Characterization of heterotypic interaction effects in vitro to deconvolute global gene expression profiles in cancer

Martin Buess*, Dimitry SA Nuyten§, Trevor Hastie†, Torsten Nielsen¶, Robert Pesich* and Patrick O Brown*‡

Abstract

Background: Perturbations in cell-cell interactions are a key feature of cancer. However, little is known about the systematic effects of cell-cell interaction on global gene expression in cancer.

Results: We used an ex vivo model to simulate tumor-stroma interaction by systematically co-cultivating breast cancer cells with stromal fibroblasts and determined associated gene expression changes with cDNA microarrays. In the complex picture of epithelial-mesenchymal interaction effects, a prominent characteristic was an induction of interferon-response genes (IRGs) in a subset of cancer cells. In close proximity to these cancer cells, the fibroblasts secreted type I interferons, which, in turn, induced expression of the IRGs in the tumor cells. Paralleling this model, immunohistochemical analysis of human breast cancer tissues showed that STAT1, the key transcriptional activator of the IRGs, and itself an IRG, was expressed in a subset of the cancers, with a striking pattern of elevated expression in the cancer cells in close proximity to the stroma. In vivo, expression of the IRGs was remarkably coherent, providing a basis for segregation of 295 early-stage breast cancers into two groups. Tumors with high compared to low expression levels of IRGs were associated with significantly shorter overall survival; 59% versus 80% at 10 years (log-rank \( p = 0.001 \)).

Conclusion: In an effort to deconvolute global gene expression profiles of breast cancer by systematic characterization of heterotypic interaction effects in vitro, we found that an interaction between some breast cancer cells and stromal fibroblasts can induce an interferon-response, and that this response may be associated with a greater propensity for tumor progression.

Background

Communication between different cell types is fundamental for the development and homeostasis of multi-cellular organisms. Cells of different origin communicate in a network of interactions via proteins, peptides, small molecular signals, the extracellular matrix and direct cell-cell contact. These
heterotypic interactions provide information that is necessary for the regulation of the gene expression programs in normal development [1], differentiation [2], topologic organization [3] and homeostasis [4] of complex tissue structures. Given the important physiological role of intercellular communication to maintain the delicate dynamic equilibrium of a normal tissue, it is not surprising that aberrant cell-cell interaction signals have been implicated in cancer development and progression [5-10]. Although the characteristics and roots of the heterotypic interaction effects are fundamental aspects of normal physiology and disease, they have not been systematically explored.

In cancer biology, there is increasing evidence for the importance of the interaction between the malignant epithelial cells and the surrounding stromal cells [7]. Tumors are not merely aggregates of malignant cells but are in many respects organ-like structures, which include host stromal cells, such as fibroblasts, endothelial cells and so on, with which the malignant cells themselves intermingle and interact. Inductive interactions between these different cell lineages can play not only a morphogenetic role but also an important mechanistic role in the pathogenesis and progression of malignancy. Coincubation of stromal cells with pre-malignant or malignant epithelial cells can increase tumorigenicity and the capacity to metastasize for a variety of tumor types [11,12], including breast cancer [13]. On the molecular level, results from the knockout of single genes have demonstrated the importance of specific signaling pathways in the tumor-stroma interaction. For example, conditional inactivation of the transforming growth factor (TGF)-β receptor type II in stromal cells led to development of epithelial cancer of the prostate and forestomach in mice [14]. In the mammary gland, site-specific knockout of TGF-β receptor type II in stromal fibroblasts led to defective mammary ductal development and increased carcinoma growth and metastasis [15]. Experiments exploring the interaction of tumor with stromal cells in vitro have revealed changes in expression of several genes involved in cancer [16-18]. These effects reveal the significance of one specific signaling mechanism, but a more complete overview of the molecular systems that mediate these cell-cell interaction effects remains to be revealed.

Biopsy samples of human carcinoma frequently contain both malignant cells and stromal cells. Since gene expression profiles of human cancer are generally derived from these mixed cell populations of grossly dissected tissues, the effects of heterotypic interactions among the cells in the tumor tissue are expected to leave their traces in the global gene expression profiles. Datasets representing expression profiles of thousands of genes in collections of benign and malignant tissues from hundreds of patients have steadily grown in recent years and might be a rich latent source of insights into heterotypic interaction effects on global gene expression. The superposition of the cell specific profiles, however, results in complex gene expression patterns that are difficult to interpret. In breast cancer, Allinen et al. [19] attempted to resolve this complexity by fractionating the tissue using cell-surface markers to separate different cell types. This led to the identification of cell type specific gene expression profiles. As a result of this analysis they suggested that a myofibroblast expression of CXCL14 and CXCL12, which can bind to the respective receptor CXCR4 on the epithelial cells, is a specific tumor promoting mechanism leading to enhanced proliferation, invasion and metastasis. In a different approach to search for the relevance of stromal signals in cancer data, West et al. [20] identified stromal-cell specific gene expression signatures in breast cancer using gene expression data from fibroblastic tumors as in vivo models of homogenous populations of malignant mesenchymal cells. Based on stromal-cell specific signatures they were able to segregate breast cancer samples into two subgroups with distinct clinical outcome.

A further layer of complexity, in addition to the simple additive effects of the involved cells, might arise from the effects on gene expression profiles induced by heterotypic cell-cell interactions. The deconvolution of these intercellular signaling effects poses an even greater challenge, since they result in supra-additive non-linear behaviors, which are hard to disentangle and distinguish from the cell-intrinsic regulatory processes. These cell interaction effects might account for a significant proportion of the unrevealed information in the gene expression data from tissue specimens. Given the evidence that interactions between cells can play critical roles in tumor progression, such data might be even more meaningful than prominent expression patterns that are driven by the proportional representation of a given cell type in a tissue [21].

The primary aim of this work was to survey and characterize the effects of cell-cell interaction in an attempt to disentangle the complex network of intercellular signaling in a multi-cellular tissue and specifically in breast cancer. To extract the information about tumor-stroma interaction from global gene expression profiles of cancer tissue, we applied an approach based on in vitro modeling combined with subsequent testing of the in vitro findings in published cancer datasets. Observation of fundamental biological processes in vitro, such as the cell cycle [22] and the reaction of fibroblasts to serum [23,24], or observation of the common response of different cell types to hypoxic conditions [25] has proven to be a worthwhile approach to better understand complex biological mechanisms underlying global gene expression profiles in human cancer. Using a simple ex vivo co-culture system allowed us to address a few basic questions about heterotypic cell-cell interactions. First, is global gene expression in a co-culture setting different from the expression in mono-culture and, if so, in which respect is it different? Second, how do the responses to co-culture differ among different cell combinations? Third, are the in vitro observations transferable in vivo using published gene expression datasets from
human tissue specimens? We analyzed heterotypic interaction effects in stromal fibroblasts and a diverse set of benign and malignant breast epithelial cells in a mixed co-culture setting by measuring changes in global gene expression using DNA microarrays. The global view of the gene expression responses facilitated the identification of specific changes and pathways underlying these effects. Gene expression signatures paralleling a response to heterotypic interaction in this ex vivo model were shared by clinically distinct subgroups of breast cancer.

**Results**

**Identification of heterotypic interaction effects**

As a model for investigating the gene expression program in response to heterotypic cell-cell interaction in normal breast and in breast cancer, we examined cells representing the benign and malignant epithelial cell compartment and the mesenchymal cell compartment in an in vitro mixed co-culture setting. The cells were co-cultivated for 48 h in low fetal bovine serum medium (0.2% FBS) to allow reciprocal signal exchange with minimal background from the influence of undefined molecular signals inherent in FBS. We examined the effects of co-cultivation for each cell pair in at least two independent biological replicates. The gene expression profiles of the co-cultures were compared to the expression profiles of the corresponding cells kept in monoculture using cDNA microarrays containing approximately 40,700 elements, representing 24,472 unique Unigene clusters (build number 173, released on 28 July 2004). To establish the experimental approach, we first focused our experiments on the breast cancer cell line MDA-MB231, the primary fibroblast CCL-171 and the co-culture of these two cell types. The data were organized using unsupervised hierarchical clustering of the replicate experiments to provide an overview of the effects on global gene expression (Figure 1a). In the co-culture, most genes displayed intermediate expression levels, which closely approximated the proportionally weighted average of their expression levels in the two cell types in monoculture. However, one set of genes showed a consistent, significant increase in transcript abundance in the co-culture compared to either monoculture, suggesting that induction of these genes was an effect of co-cultivation. Most of these induced genes were known to be interferon regulated (Figure 1b). They included those encoding the myxovirus resistance proteins 1 and 2 (MX1 and MX2), 2′,5′-oligoadenylate synthetase 1 and 2 and 3 (OAS1, OAS2, OAS3) and interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), phospholipid scramblase 1 (PLSCR1), eukaryotic translation initiation factor 2-alpha kinase (EIF2AK2) and the signal transducer and activator of transcription (STAT1). One of these genes, EPSTI1, had previously been reported to be induced by co-cultivation of MDA-MB231 and a fibroblast [17]. Our results suggest that the interferon response pathway mediates this induction. Although several of the genes induced in this co-culture model have not previously been linked to interferon induction (for example, zinc finger protein 187 (ZNF187), Homo sapiens peroxisomal proliferator-activated receptor A interacting complex 285 (PRIC285), heet domain and RLD 6 (HERC6)), we have confirmed that they are induced in MDA-MB231 cells by treatment with a type I interferon. As a more explicit approach to identify genes with consistent changes in expression in response to co-culture we used significance analysis of microarrays (SAM) [26]. A set of 42 genes represented by 49 image clones were identified with a false discovery rate (FDR) of 0 (Additional data file 1).

To further validate the results obtained by cDNA microarray analysis OAS2 transcript levels were measured by quantitative real time PCR (Figure A in Additional data file 2). Moreover, for STAT1 the increase in transcript levels in co-culture (2.8-fold) was paralleled by an increase in STAT1 protein as detected by fluorescence assisted cell sorting (FACS) analysis (Figure B in Additional data file 2).

Since breast cancer is a clinically and molecularly heterogeneous disease, we selected a broad spectrum of different breast cancer cell lines to sample this heterogeneity and explored the effects of heterotypic culture looking for subtype-specific and shared response patterns. We focused on epithelial-mesenchymal interactions co-cultivating fibroblasts of different origins (HTB125 (breast stromal fibroblast), HDF (fibroblast from breast skin) and CCL-171 (embryonic lung fibroblast)), in combination with normal breast epithelial cells (human mammary epithelial cells (HMECs)) and seven widely used breast cancer cell lines.

The changes in gene expression due to heterotypic interaction were subtle compared to the large intrinsic variation in expression patterns among the involved cell types, as Figure 1a illustrates for the cell pair MDA-MB231 and CCL-171. To identify the gene expression changes resulting from cell-cell interaction, we needed to control for the simple additive effects that reflect the proportional contribution of the two cell types to the total population of each gene’s transcript in the co-culture. Eliminating these proportionally weighted additive contributions would allow us to isolate supra-additive interaction effects. The fact that transcript levels of most genes did not change in response to co-culture allowed a linear regression model based on the transcript profiles of each monoculture to be fitted to the co-culture data for normalization. An example of such an analysis is shown in Figure 2a. For each gene, the ratio of the measured transcript level and the level estimated by the linear model provides a measure of the heterotypic interaction effect. This is illustrated in Figure 2b, which shows the distribution of the gene expression changes of the CCL-171/MDA-MB231 co-culture. The genes identified by SAM as differentially expressed in co-culture compared to monoculture are highlighted to illustrate the performance of this approach. Interaction effects, represented as gene-expression changes, are converted to
Figure 1
Effect of heterotypic interaction between breast cancer cell line MDA-MB231 and CCL-171 fibroblasts. (a) Biologically independent replicates of the monocultured fibroblast CCL-171, the breast cancer cell line MDA-MB231 and the mixed co-culture of CCL-171 and MDA-MB231 were grown for 48 h at low serum conditions and characterized by DNA microarray hybridization. Hierarchical clustering of a total of 4,333 elements that display a greater than 3-fold variance in expression in more than 3 different experimental samples. Data from individual elements or genes are represented as single rows, and different experiments are shown as columns. Red and green denote expression levels of the samples. The intensity of the color reflects the magnitude of the deviation from baseline. Unsupervised hierarchical clustering of the experiments grouped the biological replicates together. Gene expression varied considerably between fibroblast and MDA-MB231 cultures, as expected for cells of mesenchymal or epithelial origin, respectively. The co-culture profile showed mainly intermediate expression levels. However, the vertical black bar marks a cluster of genes induced in all co-cultures compared to both monocultures, indicating that they are induced by heterotypic interaction. (b) Zooming in on the genes up-regulated in co-culture compared to monocultures reveals that they are associated with the response to interferon.

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There was obvious heterogeneity in the responses of different pairs of cells to co-cultivation. The patterns of gene expression changes due to co-cultivation were mainly determined by the type of the epithelial cell involved whereas the origin of the fibroblasts had a minor influence. Importantly, CCL-171, a lung fibroblast, and HTB125, a fibroblast derived from the breast of a cancer patient, induced distinct but very similar interferon responses in co-cultivation with different epithelial cells (Figure 2c). To highlight consistent features of the responses of distinct normal or malignant epithelial cells, representing the distinct types of breast cancer, to co-cultivation with fibroblasts, we collapsed our data into eight groups, one group for each epithelial cell co-cultured with three different types of fibroblasts. There were 3,000 genes that showed a significant reproducible change (FDR < 1%) in transcript levels in response to co-culture in at least one of the groups. Clustering the averaged values of culture-induced changes for each group revealed specific and shared effects (Figure 2d). For several cell combinations, co-cultures led to an induction of smooth muscle actin (ACTA2), myosin regulatory light chain interacting protein (MYLIP), myosin, light polypeptide kinase (MYL), myosin regulatory light chain 2, smooth muscle isoform (MYL9), calponin 2 (CNN2) and fibronectin (FN1). Induction of these genes has previously been described to be associated with the acquisition of a myofibroblast phenotype [27]. The ability of the tumor cells to induce this ‘myofibroblast’ expression program varied among the breast cancer cell lines; the strongest effect was seen with MCF7 cells. In a previous study, conditioned medium of MCF7 cells was shown to induce a myofibroblast phenotype [28]. Targets of the TGF-β pathway, such as the gene encoding latent transforming growth factor beta binding protein (LTBP2) and transforming growth factor induced gene (TGFBI), were induced in parallel with the ‘myofibroblast response’. In fact, TGF-β has previously been shown to induce a ‘myofibroblast’ phenotype [29], suggesting that the response observed in these co-cultures might be mediated by the TGF-β pathway.

The most consistent coordinated response, however, was an induction of interferon-associated genes by cultivation of fibroblasts with four of the seven breast cancer cell lines. This response was seen in the co-cultures involving the estrogen-receptor negative breast cancer cell lines MDA-MB231, MDA-MB436, HS578T and BT549, but neither in HMECs nor in the estrogen-receptor positive breast cancer cells MCF7, T47D and SKBR-3. Although the gene expression profiles of these epithelial cells grown as monocultures reflected their molecular differences, including some consistent differences between the estrogen-receptor negative and estrogen-receptor positive breast cancer cell lines, there were no consistent
differences between these groups in baseline expression of
the interferon-induced genes in the monocultures. The cell-
type specificity is a strong hint that the interferon-response
activation is a specific effect of heterotypic interaction. Since
we compared the gene-expression responses in the co-cul-
tures with the responses in the corresponding monocultures
kept under the same conditions, we can exclude responses to
serum stimulation or withdrawal as sources of the interferon
response observed in these experiments. The response does
not represent an effect of crowding, which is a known inducer
of an interferon response [30], since the cell density in our
experiments was maintained below the threshold at which the
interferon response genes were turned on (data not shown).
Furthermore, we were unable to identify any infective
agent in any of the cultures despite extensive testing for
mycoplasma, reverse transcriptase activity and viral tran-
scripts, using microarrays that provide a broad survey of
human viruses [31] (data not shown). The consistent cell-type
specific, coordinated response suggests that it depends on a
specific physiological feature shared among the estrogen-
receptor negative human breast cancers, which is retained in
long-term culture, enabling them to activate this specific
response upon contact with stromal cells.

Localizing expression of interferon-response genes to
breast cancer cell lines
We investigated in which cell the interferon-response genes
were induced in response to heterotypic interaction by differ-
entially labeling the epithelial cells and the fibroblasts with
distinct fluorescent dyes prior to co-culture, then sorting
them after co-culture using FACS. Comparing gene expres-
sion patterns of cells in monoculture with those of the same
cell type after co-cultivation showed that in the CCL-171
fibroblasts, the interferon-response genes were induced on
average by a factor of only 2.7 whereas in the MDA-MB231
breast cancer cell line these genes were induced 11-fold (Fig-
ure 3a). This result of a predominant induction in the tumor
cell is in line with immunohistochemical evidence that in vivo
the interferon- response genes STAT1, EPSTI1 [17] and
EIF2AK2 [32] are expressed in the malignant epithelial cells
and to a much lesser extent in the stroma. To test whether a
soluble factor is sufficient to induce the interferon response
genes or whether direct cell-cell contact is needed for their
induction, we let the cells interact in transwell co-cultures at
low serum conditions. In this setting, neither the MDA-
MB231 breast cancer cell line nor the CCL-171 fibroblasts
showed induction of interferon response genes, indicating
that close cell-cell contact is necessary for interaction. If the
induction of interferon-response genes depended on short-
range epithelial-mesenchymal interactions, we would expect
to find the expression of interferon-response genes mainly
at the tumor-stromal interface. To test this hypothesis we
stained normal breast and breast cancer sections using anti-
odies specific for STAT1, the key transcriptional activator of
the interferon-response genes, and itself a protein over-
expressed in response to interferon stimulation (Figure 3b).

No staining was evident in normal breast samples. In tumor
tissue sections consisting of a homogenous tumor island
surrounded by stroma, we typically observed a distinctive
pattern of STAT1 expression concentrated at the periphery
of the tumor islands, near the tumor-stroma boundary, sup-
porting the idea that the interferon-response genes are induced
preferentially in the tumor cells in closest proximity to the
stromal cells. The gradient in the response further suggests
involvement of a soluble factor acting over a short range.

Induction of interferon in co-culture
To investigate the possible roles of soluble factors or direct
cell-cell contact in triggering the observed interferon
response, we tested the ability of conditioned medium from
selected cultures to induce the response in a monoculture of
MDA-MB231 cells. Conditioned medium from monocultures
of either CCL-171 or MDA-MB231 cells did not induce inter-
feron-response genes. However, conditioned media from an
MDA-MB231/CCL-171 co-culture did induce the interferon
response genes in MDA-MB231 cells. Thus, interferon-
response genes are induced by a soluble factor, the induction
depending upon direct contact between the tumor cells and
fibroblasts. In contrast to the MDA-MB231/CCL-171 co-cul-
ture supernatant, the conditioned medium of the T47D/CCL-
171 co-culture did not induce the interferon response genes
when applied onto MDA-MB231 cells (Figure 4a). Con-
versely, when T47D cells were exposed to MDA-MB231/CCL-
171 co-culture supernatant, the interferon-response was
induced (Figure 4b). However, the response of the T47D cells
to the co-culture supernatant was weaker than that of the
MDA-MB231 cells. This implies that while the interferon-
response genes can be induced in either tumor cell line, only
the interaction of MDA-MB231 with fibroblasts released a
soluble factor into the medium capable of inducing an inter-
feron response. We speculated that the factor released by
the fibroblasts might be a type I interferon. To confirm and
localize the expression of type I interferon we used quantita-
tive RT-PCR to analyze sorted cells after co-cultivation. We
found over-expression of IFNβ in CCL-171 in response to
interaction with MDA-MB231 but not in response to T47D
(Figure 4c). Expression analysis of IFNα gave us the same
result (data not shown), indicating that the expression of type
I interferon genes by co-cultured fibroblasts might underlie
the observed interferon response.

Taken together, these results demonstrate that heterotypic
interaction between fibroblasts and a specific subset of breast
cancer cells can induce the fibroblasts to express type I inter-
ferons, resulting, in turn, in induction of interferon-response
genes in the tumor cells and to a lesser extent in the fibro-
blasts (Figure 5). In our in vitro system, both estrogen-recep-
tor positive and estrogen-receptor negative tumor cells are
responsive to type I interferons, but the ability to induce
expression of interferons in co-cultivated fibroblasts was spe-
cific to the estrogen-receptor negative breast cancer cells.
Figure 2 (see legend on next page)
Genomic analysis of epithelial-mesenchymal interaction effects in human cancers

Interactions between cancer cells and non-malignant cells in the surrounding microenvironment are important determinants of cancer development and progression [11,14,33,34]. We reasoned that identifying and characterizing gene expression programs characteristically induced by interaction between specific pairs of cells in culture might enable us to recognize and interpret specific features in the expression profiles of human cancer that reflect similar interactions between tumor and stromal cells in vivo. The most consistent response to ex vivo co-cultivation of breast cancer and stromal cells was the induction of the interferon-response genes. We therefore looked for this response in the expression patterns in published data from 295 early stage (stage I and II) breast cancer samples from the Netherlands Cancer Institute (NKI) [35] (Figures 6a,b and 7). The interferon-response genes showed a strikingly coherent variation in expression among these cancers, enabling these cancers to be divided into two groups, one with relatively high expression and the other with relatively low expression of the interferon-response genes. Clustering the breast carcinomas based only on expression of the interferon response genes directed them into two main clusters, one with high-level expression of most of the interferon genes and the other with lower expression of these genes (Figure 6a). The same coordinated behavior and segregation of tumors could be observed in a different set of advanced breast cancer samples [36,37], suggesting that variation in this interferon-response program is a general feature in breast cancer (Additional data file 3).

As a first assessment of its potential biological relevance, we compared distant metastasis-free survival and overall-specific survival between the two groups distinguished by the interferon-response genes (Figure 6b). We found that tumors with high expression levels of interferon-response genes had a significantly shorter metastasis-free survival ($p = 0.0014$; 58% at 10 years) and overall survival ($p = 0.001; 59% at 10$ years) than tumors with low expression levels (metastasis free survival, 74% at 10 years; overall survival, 80% at 10 years).

The same trend toward unfavorable outcome in patients with cancers showing high levels of interferon-response gene transcripts ($p = 0.067$) could be seen in an analysis of published data from advanced-stage breast cancers [36,37]. As a metric that can be compared to known prognostic parameters and applied to other prospectively collected samples, we defined an 'interferon-response score' by averaging the gene expression levels for the 42 genes of the interferon-response gene list. The interferon response did not significantly correlate
with clinical parameters such as age of the patient, tumor size, nodal stage or angio-invasion. It was, however, very significantly correlated with tumor grade and estrogen receptor status ($p < 10^{-6}$; Additional data file 4), paralleling our in vitro findings that cell lines representing estrogen-receptor negative tumors preferentially induce the interferon-response genes in co-culture.

We also investigated the relationship between the interferon-response gene signature and three previously identified gene-expression signatures, which were useful prognosticators in this dataset. The first signature is a set of 70 genes [38], which was identified in a supervised analysis of a subset of the NKI early stage breast cancer dataset [35], to predict freedom from metastasis at 5 years. The second signature was identified in vitro by exposing fibroblasts to serum to mimic a wound response, and has been shown to predict risk of progression [39]. The third signature, the response to hypoxia in vitro [25], is also associated with a poor prognosis. The interferon signature was only very weakly correlated with either the wound signature or the hypoxia signature, and moderately correlated with the 70-gene prognostic profile, whereas the wound signature and the 70-gene score were more strongly correlated to one another (Figure 7). Thus, the interferon response appears to be a distinct feature of breast cancer biology, identifying a subgroup of cancers with a higher propensity for progression.

**STAT1 protein expression in a second independent breast cancer dataset**

As an independent test of the prognostic significance of interferon-response gene expression in primary early stage breast cancer we performed immunohistochemical staining for STAT1 in a tissue collection derived from a case series of women who underwent surgery for primary breast cancer at the Vancouver General Hospital between 1974 and 1995 [40]. Consistent with the variation we found in interferon-response gene expression in breast tumors, we found a large variability in the expression of STAT1 protein, the principal transcriptional regulator of the interferon response genes, in these tumors. Of the 353 primary tumors with interpretable results, 102 displayed high (28.9%), 184 low (52.2%) and 67 absent (18.9%) STAT1 expression. Paralleling the results from the NKI dataset, patients from Vancouver with tumors displaying high STAT1 expression levels had a higher risk of death due to breast cancer (33% dead from breast cancer at 10 years) than patients with tumors showing low or absent STAT1 expression (25% dead at 10 years) ($p = 0.056$; Figure 8).

**Discussion**

The main objective of this study was to examine and characterize the effects of heterotypic cellular interaction, to gain insight into the underlying biology of these effects in normal mammary tissue and breast cancer. To isolate specific, direct interactions from more complex interactions involving multiple cell types in a whole tissue or organism we used a simple ex vivo co-culture model. Since some important heterotypic interactions can require direct cell-cell contact, we focused on a co-culture model where the two cell types were mixed. A challenge in the analysis of a mixed co-culture model is the separation of the interaction effects induced by signal exchange between the two cell types from the simple additive combination of their intrinsic gene expression patterns in the overall gene expression profile of the co-culture. Our strategy of normalizing for the simple additive effects based on a linear regression model proved to be advantageous, since it does not depend on prior knowledge of the exact proportional contribution of the different cell types to the superposed gene expression pattern. A similar approach has been described to define the proportional contribution of different cell cycle states in a mix of cells, although without taking into account interaction effects [41]. This strategy was effective in isolating the cell-cell interaction effects on gene expression.

We examined the effects on global gene expression of the molecular crosstalk between stromal fibroblasts and each of a diverse set of breast cancer cell lines or normal breast epithelial cells as they interact in vitro. Not unexpectedly, the picture of heterotypic interaction effects that emerged from combinatorial co-cultivation of multiple different cell types was complex, reflecting the different abilities of normal and malignant cells to send and to respond to extrinsic signals. The overall pattern of gene expression changes were dominated by the type of epithelial cells. Against our expectations, which were based on the knowledge that fibroblasts from different parts of the body show distinct gene expression patterns [42] leading to different physiological properties that

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**Figure 4 (see following page)**

Induction of interferon response in two types of breast cancer cell lines. (a) MDA-MB231 cells were incubated in conditioned media from CCL-171 monoculture, MDA-MB231 monoculture, T47D monoculture, CCL-171/MDA-MB231 co-culture and CCL-171/T47D co-culture. OAS2 gene expression was determined by quantitative RT-PCR. The gene expression level of GAPDH was used for normalization between the samples. A strong induction of OAS2 by the supernatant from the CCL-171/MDA-MB231 co-culture can be seen in MDA-MB231. (b) Incubation of T47D cells with conditioned media from CCL-171 monoculture, MDA-MB231 monoculture, T47D monoculture, CCL-171/MDA-MB231 co-culture and CCL-171/T47D co-culture showed that only the CCL-171/MDA-MB231 co-culture supernatant induced OAS2 gene expression, although to a much lesser extent than in MDA-MB231 cells. (c) Gene expression levels of IFNβ were determined by quantitative RT-PCR. CCL-171 cells show much higher IFNβ expression levels when isolated by FACs after co-culture with MDA-MB231 than with T47D cells. Expression levels in tumor cells are shown as controls. The error bars show the standard deviation from the normalized mean.
Figure 4 (see legend on previous page)
malignancies, including leukemias [43], ovarian cancer [44], gastric cancer [45], lung cancer [46] and breast cancer [30,43]. In breast cancer, in an attempt to validate the previously described intrinsic gene signatures [36,37], Hu et al. [47] assigned a small group of tumors with very high gene expression levels known to be induced by interferon as the ‘interferon subtype’ with a poor clinical outcome. Despite its common occurrence, the origin and the consequences of this phenomenon are unknown. Some reports have proposed that this program might reflect a viral infection or invasion of inflammatory cells in response to the tumor [43]. Our data suggest that the interferon response is not necessarily dependent on immune cells since our in vitro co-culture system comprises only fibroblasts and epithelial cells and no immune cells. Despite considerable effort to identify infective agents, we could not find any evidence for an infection in our cell culture causing the interferon response. Without excluding these possibilities, we propose that in a subset of breast cancer, the interferon response arises as an effect of the interaction of the malignant epithelial cells with the stroma.

At a first glance, the proposed link between interferon signaling and tumor-stroma interaction is surprising. However, interferons are pleiotropic cytokines, and while best known for their function as a viral defense mechanism they are also involved in other biological processes [48], such as the induction of cell cycle arrest, apoptosis, cell differentiation, immune stimulation and regulation of bone metabolism [49]. The induction of interferons at the interface between tumor cells and the surrounding stroma may have profound biological significance. In response to viral infection, induction of the interferon response genes, such as EIF2AK2, can lead to a global arrest of translation and subsequent apoptosis [50]. Interferon treatment has an anti-proliferative effect in some cultured cancer cells, and some human cancers shrink in response to interferon [51], leading to the speculation that an interferon response might be linked to a better prognosis [43]. In fact, our results show the opposite effect; patients with breast cancers displaying high interferon-response gene expression were 1.7 times more likely (95% confidence interval 1.1-2.6; \( p = 0.018 \)) to develop metastasis and 1.8 times more likely to die of the disease (95% confidence interval 1.2-2.7; \( p = 0.006 \)) than patients with tumors showing low expression levels of the interferon-response genes. Similar results have been reported by others. For example, an increase in EIF2AK2 expression and activity during tumor progression had been described in melanoma and colorectal cancer [52]. In breast cancer cells EIF2AK2 was elevated compared to normal breast epithelial cells [53]. Also, IFI 27, known to be inducible by IFNα, is frequently over-expressed in breast cancer [54]. IFITM1 over-expression in gastric cancer cells was reported to enhance migration and invasion in vitro [55]. These findings along with the observation that interferon response gene expression in cancer is highly coordinated, suggests the possibility that the interferon response program can promote cancer progression.
The role of STAT1, the main regulator of the interferon response genes, is controversial too. Our finding of a worse outcome for patients with tumors with high levels of STAT1 protein expression support our results of STAT1 mRNA expression levels and is in accordance with the expression levels of the full set of interferon response genes. The mechanism for the negative association between interferon response gene induction and patient outcome is not yet understood. Several mechanisms are possible. Up-regulation of STAT1 was found to be associated with resistance to radiotherapy [56]. IFITM1 was reported in another model to be involved in IFNα induced radioresistance [57]. For patients treated with radiotherapy, high expression of interferon response genes leading to radioresistance of the tumors could contribute to an unfavorable outcome compared to the more radiosensitive tumors. Since all patients receiving breast conserving therapy from the NKI dataset underwent adjuvant radiotherapy, this hypothesis cannot be further substantiated.

Figure 6
Interferon response gene expression in early stage breast cancer (a) The expression values of genes in the ‘interferon response gene set’ were extracted from a published expression study of 295 early stage breast cancers from the Netherlands Cancer Institute [35]. Genes and samples are organized by hierarchical clustering. The tumors segregated into two groups defined by high (red) or low (blue) expression levels of 29 genes matching the ‘interferon response gene set’. (b) Correlation of interferon response with distant metastasis free and overall survival. Kaplan-Meier curves for the clinical outcomes of indicated tumors exhibiting high (red curve) and low (blue curve) interferon responses are shown.
from our data because of the lack of an appropriate control group. Another possible mechanism, independent from an effect on therapeutic efficacy, could be mediated by an effect on invasiveness of the tumor. Up-regulation of STAT1 has been reported in breast cancer micrometastasis in the bone marrow [58], suggesting a more metastasis-prone phenotype.

The finding that an interferon response can be induced in response to tumor-stroma interaction raises questions for further inquiry. First, our results do not allow us to distinguish whether the interferon response has any role in contributing to tumor progression or is merely an incidental feature of certain cancers that tend to be more aggressive. If it

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**Figure 7**
Correlation of the 70 genes signature [38], the wound signature [24], the hypoxia signature [25] and the interferon response score in the NKI dataset. Pairwise scatterplot-matrix of four gene signatures. Pearson correlations are shown in the lower part of each plot.
Genome Biology provide a valuable interpretive framework for analyzing the processes and features of the tumor microenvironment. Our experience suggests that orchestrate the process of cancer progression and metastasis. Interactions among the multiple molecules operating in these cells to understand the mechanisms underlying tumor growth and spread can provide a window to explore complex interactions in vivo. The approach used in this work, employing an in vivo model to develop gene expression signatures as an experimentally tractable window on the more complex interactions in vivo can be deliberately extended to other cell types, such as endothelial, inflammatory and immune cells. This technique may allow us to explore complex interactions among the multiple molecules operating in these cells to orchestrate the process of cancer progression and metastasis. Our experience suggests that in vitro modeling of specific processes and features of the tumor microenvironment can provide a valuable interpretive framework for analyzing the gene expression patterns in more complex heterogeneous in vivo samples and identify effects of heterotypic cellular interactions.

Materials and methods

Cell culture

HMECs (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) were expanded in mammary epithelial basal medium supplemented with bovine pituitary extract, human epithelial growth factor, insulin and antibiotics (Clonetics, Cambrex Bio Science Walkersville, Inc.). MCF-7, T47D, MDA-MB231, MDA-MB436, SKBR-3, Hs578T, BT549, CCL-171, HTB-125 (ATCC) and HDF (Cambrex Bio Science Walkersville, Inc.) were propagated in DMEM supplemented with 10% FBS (HyClone, Logan, UT, USA), glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO, Grand Island, NY, USA). For co-culture experiments the cells were cultivated for 48 h at 50,000 cells/cm² in endothelial basal media (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) with 0.2% FBS, which was a good universal medium for all cells involved. Separated co-cultures were kept in Transwell chambers with a 0.4 μm pore size (Costar, Corning Inc., Corning, NY, USA). The cells for analysis were always harvested from the bottom well and reciprocal interactions were tested. Cells negatively tested for mycoplasma infection using MycoAlert™ (Cambrex Bio Science Rockland, Inc.) and VenorGem® (Sigma, Saint Louis, MO) mycoplasma detection kits used according to the manufacturer’s instructions.

Flowcytometry

Cells were fixed and stained using the Cytofix/Cytoperm™ Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions using 20 μg/ml STAT1a mAB (Abcam, Cambridge, MA, USA) and a fluorescein-5-isothiocyanate labeled goat anti-mouse IgG (Sigma-Aldrich, St Louis, MO, USA) for detection. Goat serum 1:200 was used for blocking. Analytical flow cytometry was done on a modified dual laser LSRScan (BD Immunocytometry Systems, San Diego, CA, USA) in the Shared FACS Facility, Center for Molecular and Genetic Medicine at Stanford, using FlowJo software (TreeStar, Ashland, OR, USA) for data analysis.

For FACS sorting, cells were stained with the lipophilic carbo-cyanine dye DiO (Vybrant® DiO cell-labeling solution, Molecular Probes™ Invitrogen, Eugene, OR, USA) in serum-free DMEM medium for 20 minutes and washed three times in calcium- and magnesium-free PBS according to the manufacturer’s instructions before co-culturing. After 48 h, the cells were detached by incubation in Trypsin/EDTA (GIBCO, Grand Island, NY, USA) for 3 minutes and washed in ice-cold PBS and then immediately put on ice. Cell sorting was done on a MoFlow cell sorter (Becton Dickinson, Mountain View, CA, USA) in the Shared FACS Facility, Center for Molecular and Genetic Medicine at Stanford. The sorted cells were harvested in TRIZOL® LS Reagent (Invitrogen, Carlsbad, CA, USA).
USA). FlowJo software (TreeSTAR) was used for data analysis.

**RNA isolation and amplification**

After discarding the culture medium and washing the cell layer once with PBS, total RNA was isolated by lysing the cells in the culture dish with RLT buffer (Qiagen, Valencia, CA, USA) and extraction with the RNeasy® Mini Kit (Qiagen). Total RNA (500 ng) was amplified using the MessageAmp™ II aRNA Kit (Ambion, Austin, TX, USA). The amplification product was checked for integrity by electrophoresis in a 1% agarose gel in MOPS buffer.

**cDNA microarrays and hybridization**

We used human cDNA microarrays containing 40,700 elements that represent 24,472 unique genes based on Unique Clusters. Arrays were produced at the Stanford Functional Genomic Facility. Complete details regarding the clones on the arrays may be found at Stanford: functional genomics facility [60]. cDNA produced from 6 µg amplified RNA were hybridized to the array in a two-color comparative format, with the experimental samples labeled with one fluorophore (Cy5) and a reference pool of mRNA (Universal human reference, Stratagene, La Jolla, CA, USA) labeled with a second fluorophore (Cy3). Fluorescent dyes were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Hybridizations were carried out using the standard protocol described previously [61].

**Data analysis and clustering**

Array images were scanned using an Axon Scanner 4000B (Axon Instruments, Union City, CA, USA), and image analysis was performed using GenePix Pro version 5.0 3.0.6.89 (Axon Instruments). The raw data files were stored in the Stanford Microarray Database [62]; the data used for the paper are available at the accompanying website [63]. Data were expressed as the log₂ ratio of fluorescence intensities of the sample and the reference, for each element on the array.

The (Cy5/Cy3) ratio is defined in the Stanford Microarray Database as the normalized ratio of the background-corrected intensities. Spots with aberrant measurements due to obvious array artifacts or poor technical quality were manually flagged and removed from further analysis. A filter was applied to omit measurements where the fluorescent signal from the DNA spot was less than 50% above the measured background fluorescence surrounding the printed DNA spot in either the Cy3 or Cy5 channel. Genes that did not meet these criteria for at least 80% of the measurements across the experimental samples were excluded from further analysis. Valid data were filtered to exclude elements that did not have at least a three-fold deviation in the mean in at least three samples. Data were evaluated by unsupervised hierarchical clustering [64] and SAM [26] and displayed using Treeview [65].

**Determination of the heterotypic interaction effect on gene expression**

To facilitate the identification of heterotypic interaction effects on global gene expression in a mixed co-culture experiment, we normalized the gene expression data based on the proportional contribution of each cell type to transcript abundance. Given that the average gene does not change due to heterotypic interaction and that there are simple additive effects to account for, we used a linear regression fit for normalization. To determine the contribution of each cell type to the combined gene expression pattern in the linear regression model, the expression levels of the monocultures are the predictors and the expression levels of the co-culture, the response.

Specifically, a set of equations (1-n) is established (one per gene), as illustrated in the additional data file 5 in which the expression level of gene n (en, co-culture) in the mixed co-culture equals the fraction a of mRNA from cell type 1 minus the relative expression level of gene n in type 1 mono-cultured cells plus the fraction (1-a) of mRNA from cell type 2 times the relative expression level of gene n in type 2 mono-cultured cells multiplied by the interaction coefficient \( I_n \). We assume that the average gene is not influenced by heterotypic interaction in the mixed co-culture represented as I = 1. Since the dataset over \( e_{1-n} \) is skewed, we empirically identified a linear regression fit based on Gamma errors and identity link as a good model to calculate a. Then the equations 1-n can then be solved for \( I_{1-n} \), which results in a profile of interaction effects for the genes \( e_{1-n} \). These interaction effects can be analyzed in much the same way as conventional gene expression measurements.

**Real time quantitative PCR**

Total RNA (500 ng) was mixed with dT16 primer in a volume of 11 µl, incubated at 65°C for 10 minutes and immediately put on ice. Following addition of 100 units Superscript II reverse transcriptase (GIBCO, Carlsbad, CA, USA), reverse transcription was performed for 2 h at 42°C in 1× RT reaction buffer (GIBCO), 10 µM dithiothreitol, 500 µM dNTP (Amersham Biosciences, Pittsburgh, PA, USA) with 2.5 µM dT16 primer in a volume of 20 µl. PCR reactions were performed in a final volume of 20 µl with cDNA prepared from 20 ng RNA and a final concentration of 1× SYBR® Green PCR Master Mix (ABI, Foster City, CA, USA) and 200 nM of each primer (sequences: GAPDH, forward GAGGTGAAGGTCGGAGTTC, reverse GAAGATGGTATGAGAGGAGTC, OAS2, forward GGATACCTGAAGCCCTAGA, reverse CCTGCAGACGT-CACAGATGGT; IFNα, forward CCTCGCCCTTTGCTT-TACTG, reverse GCCCAGAGCAGCTTGACT; IFNβ, forward ACCTCGAAAGATCAGACTTCA, reverse TGCT-TGGTTGAAGATGCTTG). The reaction was run in an ABI 7700 Sequence Detection System with the following cycling conditions: 50°C for 2 minutes, 94°C for 10 minutes, then 40 cycles of 94°C for 15 s and 60°C for 60 s. For each gene a...
standard curve was prepared and triplicate measurements were performed for each sample.

**Immunohistochemistry**

Large biopsy or tissue microarray sections were cut from paraffin blocks, deparaffinized in xylene, and hydrated in a graded series of alcohol. The slides were pretreated with citrate buffer and a microwave step. Immunostaining was performed using the DAKO Envision+ System, Peroxidase DAB, (DAKO, Cambridgeshire, United Kingdom) for STAT1a monoclonal antibody (1:100 dilution; Abcam).

We stained 1,024 tissue cores from 521 donor blocks. Immunohistochemistry images were acquired with the BLISS Microscope System (Bacus Laboratories, Lombard, IL, USA). Staining results were assessed using a three-point scoring system, where 0 = invasive tumor cells present in the tissue core and no staining seen, 1 = invasive tumor cells present with weak staining intensity and/or < 20% of the cells stained, and 2 = invasive tumor cells present with strong staining in > 20% of the cells. Tissue cores that failed to adhere to the glass slide, did not contain invasive carcinoma or were otherwise uninterpretable were excluded. Scoring of the arrays was analyzed using the Deconvoluter software as previously described [66], with each sample receiving the higher of the scores for two replicate cores.

**Human breast cancer dataset**

The dataset for breast cancer contained 295 tumors analyzed on a 25,000 spot oligonucleotide array as described [35]. In brief, patients were diagnosed and treated at the NKI for early stage breast cancer (stage I and II) between 1984 and 1995. The clinical data were updated in January 2005. The median follow-up for patients still alive is now 12.3 years.

The interferon response gene list consists of 42 genes represented by 49 image clones on the cDNA Stanford array. Clones having the same Unigene locus were removed. The gene sequences were mapped to spots on the NKI array using Unigene build number 184 (released on 9 June 2005) to give 29 unique spots. In order to overcome possible overweighting of clones from Unigene clusters that were matched to more than one probe on the NKI array, expression values derived from probes that were not matched to the same Unigene cluster were averaged. Expression measurements for each gene were mean centered. The resulting dataset was subjected to hierarchical clustering with average linkage clustering [64] and displayed with Treeview [65].

Distant metastasis was analyzed as first event only (distant metastasis-free probability). If a patient developed a local recurrence, axillary recurrence, contralateral breast cancer or a second primary cancer (except for non-melanoma skin cancer), she was censored at that time and subsequent distant metastases were not analyzed. This is based on the theoretical possibility that the locally recurrent or second primary cancer could be a source for distant metastases. An ipsilateral supra-clavicular recurrence was soon followed by a distant metastasis in all but one patient. An ipsilateral supra-clavicular recurrence was thus considered the first clinical evidence for metastatic disease for this analysis and patients were not censored at the time of ipsilateral supra-clavicular recurrence. Overall survival was analyzed based on death from any cause and patients were censored at last follow up. Kaplan-Meier survival curves were compared by the Cox/Mantel log/ rank test using Winstat for Microsoft Excel (RFitch Software, Staufen, Germany). Multivariate analysis by the Cox proportional hazard method was performed using the software package SPSS R 11.5 (SPSS, Inc., Chicago, IL, USA).

A dataset of gene expression patterns from advanced breast cancers was described by Sorlie et al. [36,37]. Expression data from 19 image clones representing the interferon response gene list were included in this dataset. Genes and samples were organized by hierarchical clustering. Relapse-free and overall survival were calculated as described above.

The independent breast cancer tissue microarray validation series is as described [40]; immunohistochemical scores for STAT1 were related to breast cancer-specific survival by Kaplan-Meier analysis with log-rank test.

**Abbreviations**

DMEM, Dulbecco’s modified Eagle’s medium; EIF2AK2, eukaryotic translation initiation factor 2-alpha kinase; FACS, fluorescent assisted cell sorting; FBS, fetal bovine serum; FDR, false discovery rate; HMEC, human mammary epithelial cell; IFIT, interferon-induced protein with tetra-tricopeptide repeats; IFN, interferon; OAS, 2',5'-oligoadenylate synthetase; NKI, Netherlands Cancer Institute; PBS, phosphate-buffered saline; SAM, significance analysis of microarray data; STAT, signal transducer and activator of transcription; TGF, transforming growth factor.

**Authors’ contributions**

MB and POB designed the research; MB and RP performed the research; MB, TH and TN contributed new reagents/analytical tools; MB, DSAN, TH and POB analyzed the data; and MB and POB wrote the paper. The authors declare no conflict of interest.

**Additional data files**

The following additional data are available with the online version of this paper. Additional data file 1 is a table listing the interferon response genes. Additional data file 2 shows the expression of OAS2 measured by RT-PCR in the co-culture CCL171/MDA-MB-231 and the expression of STAT1 measured by immunofluorescent staining and FACS analysis. Additional data file 3 shows the analysis of the interferon
response signature in advanced human breast cancers. Additional data file 4 shows box and scatter plots illustrating the correlation of the interferon score to clinical parameters with known prognostic significance. Additional data file 5 is an illustration of the linear regression model used to normalize for additive effects in the mixed co-culture gene expression data.

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References

1. Cunha GR, Hom YK: Role of mesenchymal-epithelial interactions in mammary gland development. J Mammary Gland Biol Neoplasia 1996, 1:21-35.

2. Levine JF, Strockdale FE: Cell-cell interactions promote mammary epithelial cell differentiation. J Cell Biol 1985, 100:1415-1422.

3. Weaver M, Batts L, Hogan BL: The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. Epithelial Cell Biol 1993, 2:79-89.

4. Howlett AR, Bissell MJ: Signalling pathways implicated in early mammary gland morphogenesis and breast cancer. PLoS Genet 2006, 2:e112.

5. Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW: The organizing principle: microenvironmental influences in the normal and malignant breast. Differentiation 2002, 70:537-546.

6. Bissell MJ, Radisky D: Putting tumours in context. Nat Rev Cancer 2001, 1:46-54.

7. Hanahan D, Weinberg RA: The hallmarks of cancer. Cell 2000, 100:57-70.

8. Arias AM: Epithelial mesenchymal interactions in cancer and development. Cell 2001, 105:425-431.

9. Sneddon JB, Zhen HH, Montgomery K, van de Rijn M, Tward AD, West R, Gladstone H, Chang HY, Morganroth GS, Oro AE, Brown PO: Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated fibroblasts and can promote tumor cell proliferation. Proc Natl Acad Sci USA 2006, 103:14842-14847.

10. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR: Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 1999, 59:5002-5011.

11. Camps JL, Chang SM, Hsu TC, Freeman MR, Hong SJ, Zhou HE, von Eschenbach AC, Chung LW: Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. Proc Natl Acad Sci USA 1990, 87:75-79.

12. Kuperwasser C, Chavarria T, Wu M, Magram G, Gray JW, Carey L, Richardson A, Weinberg RA: Reconstruction of functionally normal and malignant human breast tissues in mice [see comment]. Proc Natl Acad Sci USA 1993, 90:999-1003.

13. Huang H, Cai T, Wang Z, Yang L, Richardson A, et al: Molecular characterization of the tumor microenvironment in breast cancer [see comment]. Cancer Cell 2004, 6:17-32.

14. Khodarev NN, Yu J, Labay D, Tlsty TD, Cunha GR, Arteaga CL, Neilson EG, Hayward SW, Moses HL: Beta type II receptor in fibroblasts promotes mammary carcinogenesis. J Cell Biol 2006, 175:49-60.
Naem R, Carey VJ, Richardson AL, Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1 and CXCL12 secretion. Cell 2005, 121:335-348.

35. van de Vijver MJ, He YD, van’t Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, et al.: A gene-expression signature as a predictor of survival in breast cancer. New Engl J Med 2002, 347:1999-2009.

36. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, et al.: Gene expression patterns of breast cancers distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001, 98:10869-10874.

37. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Peschl R, Geisler S, et al.: Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA 2003, 100:8418-8423.

38. van’t Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Rijn M, Florence S, Rust ME, et al.: Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002, 415:530-536.

39. Chang HY, Nuyten DS, Sood DN, Hsiang T, Hester K, Dai H, He YD, van’t Veer LJ, Barrette T, Tan K, et al.: Robustness, scalability, and interpretation of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 2005, 102:3738-3743.

40. Makretsov N, Huntsman DG, Nielsen TO, Yorid C, Peacock M, Reynolds E, Dressler L, Pacyna-Gengelbach M, van de Rijn M, Rosen GD, Perou CM, Whyte RB, et al.: Oncomine: comprehensive web-based analysis of microarray data. Genes Cancer 2006, 1:161-176.

41. Kita S, Sugaya S, Zhai L, Wu YP, Wano C, Chigira S, Nomura J, Takahashi S, Ichinose M, Suzuki N: Involvement of LEU13 in interferon-induced refractoriness of human RSa cells to cell killing by X rays. Radiat Res 2003, 160:302-308.

42. Woeille U, Cloos J, Sauter G, Riedthof L, Janicke F, van Diest P, Brakenhoff R, Pantel K: Molecular signature associated with bone marrow micrometastasis in human breast cancer. Cancer Res 2003, 63:5679-5684.

43. Ohashi K, Burkart V, Flohe S, Kolb H: Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol 2000, 164:5356.

44. Stanford: Functional Genomics Facility [http://www.microarray-pubs.stanford.edu/]

45. Ericsson A, Cappola S, Fujita J, Zhang W, Ahn K, Liu W, Relich S, Chheda R, Geiss SR, Woon H, et al.: Robustness, scalability, and interpretation of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 2005, 102:3738-3743.

46. Makretsov N, Huntsman DG, Nielsen TO, Yorid C, Peacock M, Reynolds E, Dressler L, Pacyna-Gengelbach M, van de Rijn M, Rosen GD, Perou CM, Whyte RB, et al.: Oncomine: comprehensive web-based analysis of microarray data. Genes Cancer 2006, 1:161-176.

47. Stanford: Functional Genomics Facility [http://www.microarray-pubs.stanford.edu/]

48. Ericsson A, Cappola S, Fujita J, Zhang W, Ahn K, Liu W, Relich S, Chheda R, Geiss SR, Woon H, et al.: Robustness, scalability, and interpretation of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 2005, 102:3738-3743.

49. Ohashi K, Burkart V, Flohe S, Kolb H: Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol 2000, 164:5356.

50. Stanford: Functional Genomics Facility [http://www.microarray-pubs.stanford.edu/]

51. Ericsson A, Cappola S, Fujita J, Zhang W, Ahn K, Liu W, Relich S, Chheda R, Geiss SR, Woon H, et al.: Robustness, scalability, and interpretation of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 2005, 102:3738-3743.

52. Stanford: Functional Genomics Facility [http://www.microarray-pubs.stanford.edu/]

53. Ericsson A, Cappola S, Fujita J, Zhang W, Ahn K, Liu W, Relich S, Chheda R, Geiss SR, Woon H, et al.: Robustness, scalability, and interpretation of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 2005, 102:3738-3743.

54. Rasmussen UB, Wolf C, Mattei MG, Chenard MP, Belloco JP, Cham- bon P, Rio MC, Basnet P: Identification of a new interferon-alpha-inducible gene (p27) on human chromosome 14q32 and its expression in breast carcinoma. Cancer Res 1993, 53:4096-4101.

55. Yang Y, Lee HJ, Kim KY, Song HK, Kim KJ, Yoon SR, Cho D, Song KS, Lee YH, Choi J: The interferon-inducible 9-27 gene modulates the susceptibility to natural killer cells and the invasiveness of gastric cancer cells. Cancer Lett 2005, 221:191-200.

56. Khodarev NN, Beckert M, Labay E, Darga T, Roizman B, Weichselbaum RR: STAT1 is overexpressed in tumors selected for androgen independence and confers protection from radiation in transduced sensitive cells. Proc Natl Acad Sci USA 2004, 101:1714-1719.

57. Kita S, Sugaya S, Zhai L, Wu YP, Wano C, Chigira S, Nomura J, Takahashi S, Ichinose M, Suzuki N: Involvement of LEU13 in interferon-induced refractoriness of human RSa cells to cell killing by X rays. Radiat Res 2003, 160:302-308.

58. Woeille U, Cloos J, Sauter G, Riedthof L, Janicke F, van Diest P, Brakenhoff R, Pantel K: Molecular signature associated with bone marrow micrometastasis in human breast cancer. Cancer Res 2003, 63:5679-5684.

59. Ohashi K, Burkart V, Flohe S, Kolb H: Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol 2000, 164:5356.

60. Stanford: Functional Genomics Facility [http://www.microarray-pubs.stanford.edu/]

61. Ericsson A, Cappola S, Fujita J, Zhang W, Ahn K, Liu W, Relich S, Chheda R, Geiss SR, Woon H, et al.: Robustness, scalability, and interpretation of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 2005, 102:3738-3743.

62. Stanford: Functional Genomics Facility [http://www.microarray-pubs.stanford.edu/]

63. Ericsson A, Cappola S, Fujita J, Zhang W, Ahn K, Liu W, Relich S, Chheda R, Geiss SR, Woon H, et al.: Robustness, scalability, and interpretation of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 2005, 102:3738-3743.