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Activation of Host Wound Responses in Breast Cancer Microenvironment

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Abstract

Purpose: Cancer progression is mediated by processes that are also important in wound repair. As a result, cancers have been conceptualized as overhealing wounds or "wounds that do not heal," and gene expression signatures reflective of wound repair have shown value as predictors of breast cancer survival. Despite the widespread acknowledgment of commonalities between host responses to wounds and host responses to cancer, the gene expression responses of normal tissue adjacent to cancers have not been well characterized.

Experimental Design: Using RNA extracted from histologically normal breast tissue from 107 patients, including 60 reduction mammoplasty patients and 47 cancer patients, we measured whole genome expression profiles and identified a gene expression signature that is induced in response to breast cancer.

Results: This signature represents an *in vivo* "wound response" signature that is differentially expressed in the normal tissue of breast cancer patients compared with those without disease and is highly accurate (at least 92% sensitivity and 98% specificity) in distinguishing diseased and nondiseased. The *in vivo* wound response signature is highly prognostic of breast cancer survival, and there is a strong association between the groups identified by this signature and those identified using serum-treated fibroblasts and other microenvironment-derived or microenvironment-related signatures.

Conclusions: The prevalence of the wound response signature in histologically normal tissue adjacent to breast cancer suggests that microenvironment response is an important variable in breast cancer progression and may be an important target for clinical interventions. (Clin Cancer Res 2009;15(22):7020–8)

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The concept of cancers as "wounds that do not heal" arose >20 years ago based on the observation of gross cellular and molecular similarities between wounds and cancer tissue (1, 2). "Wound response" involves clotting and coagulation, tissue remodeling, cellular migration and proliferation, and angiogenesis. Many of these processes have well-recognized roles in promoting cancer. Thus, the hypothesis that tumors induce wound responses in cancer microenvironments has been the subject of recent reviews (2) and has led to suggestions that targeting the microenvironment could lead to new cancer therapies (3, 4) or chemoprevention opportunities (5). Other targets and drugs in the microenvironment have not been successful (reviewed in ref. 6], highlighting the need to better characterize of the microenvironment response to breast cancer.

The gene expression alterations present in the breast cancer microenvironment have been examined in a few previous studies. In a careful study by Allinen et al. (7), pure cell populations were isolated from two reduction mammoplasty, two ductal carcinoma *in situ*, and 10 invasive breast cancer patients. Serial

Translational Relevance

These data show that "wound responses" are activated in the gene expression profile of normal tissue adjacent to cancer and that this *in vivo* wound response signature predicts prognosis in independent tumor data sets. If normal tissue adjacent to breast cancer expresses genomic signatures with promoting characteristics, surgical interventions may need to evaluate wider margins consistent with the geographic range of these alterations. Furthermore, differential expression of wound response signatures across different tumors and tumor subtypes suggests that individualized targeting of microenvironment may be a viable treatment strategy in breast cancer. For example, significant heterogeneity in extracellular matrix or angiogenesis gene expression may suggest differential efficacy of extracellular matrix– or angiogenesis-targeted drugs. These studies provide support for the notion that the normal microenvironment is an important variable in breast cancer progression and may be an important target for future clinical interventions.

analysis of gene expression of these purified cell populations documented widespread molecular changes in all cell types of breast cancer stroma (7). These molecular changes may reflect paracrine interactions among the diverse cell types that are present in the mammary gland during carcinogenesis: progression to carcinoma is associated with increases in myofibroblasts and immune cells, as well as increased vascularization (8–13). However, recent studies comparing stroma from reduction mammoplasty patients to stroma adjacent to breast cancers failed to identify any gene expression changes associated with disease status (14). Technical differences between these two studies may account for their distinct conclusions. Further research is needed to explain differences between these two studies. Because both studies relied on relatively small numbers of patients, little is known about interindividual variation in the microenvironment response. However, previous studies on breast cancers suggest that interindividual variation is an important feature of cancer-associated wound responses. Based on the idea that fibroblasts *in vivo* are exposed to serum only during wounding, Chang et al. (15) used cultured fibroblasts from many different anatomic sites to derive a wound response signature, that is highly prognostic in breast cancer patients (15). Whether that signature is altered in the microenvironment adjacent to breast cancer has not been evaluated previously.

We hypothesized that, with larger sample sizes, a reproducible gene expression signature of activated wound response would be detectable *in vivo* in the microenvironment of breast cancer. We used whole genome microarrays to investigate gene expression in 107 patients, including 60 reduction mammoplasty patients and 47 histologically normal samples from cancer patients (referred to as cancer-adjacent normal samples). Our results show that an *in vivo* wound response signature is identifiable in the histologically normal tissue adjacent to breast cancer and that this signature has strong predictive value in distinguishing normal tissue of cancer patients from that of disease-free individuals. This signature is also prognostic, confirming the importance of wound response in breast cancer progression.

Materials and Methods

Patient samples. Patients were women undergoing elective reduction mammoplasty at Baystate Medical Center in Springfield, MA, or mastectomy at University of North Carolina Hospitals in Chapel Hill, NC. All patients enrolled voluntarily under Institutional Review Board–approved protocols. Reduction mammoplasty tissue was excluded if pathologic assessment of patient-matched, paraffin-embedded tissues suggested any abnormal malignant or premalignant findings. For histologically normal tissue adjacent to breast cancer (cancer-adjacent normal), a pathologist from the Tissue Procurement Facility at University of North Carolina at Chapel Hill confirmed histologically normal tissues. Only patients receiving no neoadjuvant therapy before surgery were included. All tissues were handled by snap freezing immediately after surgery, and RNA was isolated using a single protocol as described by Hu et al. (16).

Microarrays. All microarrays were done at the University of North Carolina at Chapel Hill. Two-color Agilent human arrays were used. Cy3-labeled reference was produced from total RNA from Stratagene Universal Human Reference (spiked with 1:1,000 with MCF-7 RNA and 1:1,000 with ME16C RNA to increase expression of breast cancer genes) following amplification with Agilent low RNA input amplification kit. The identical protocol was applied to total RNA from reduction mammoplasty or cancer-adjacent normal tissue, and all patient samples were labeled with Cy5. Some samples from reduction mammoplasty and cancer-adjacent normal groups were done on 4 × 44 k Agilent whole genome arrays, and some of each type were done on 244 k Agilent custom arrays that included all of the probes from the 4 × 44 k array. Only probes that were present on both formats were used in the final analysis and intraclass *r*'s for technical replicates assayed on the same platform (range, 0.73-0.97; median, 0.89; $n = 8$) did not show meaningful differences from those conducted on different platforms (range, 0.708-0.938; median, 0.90; $n = 12$). Principal component analysis suggested no substantial difference between the two platforms. Duplicate microarrays corresponding to the same patient sample were combined by averaging. All data are publicly available through the Gene Expression Omnibus (GSE16113).

Data normalization and age adjustment. Data were lowess normalized, and only genes with signal intensity >10 in both channels were included in analyses. Because the reduction mammoplasty patients tended to be younger than the cancer-adjacent normal patients, we selectively adjusted for confounding by age using the following algorithm. First, we estimated the effect of tissue type (reduction mammoplasty versus cancer-adjacent normal) in a linear regression model that included tissue type as a main effect and age as a covariate (full model). We compared the main effect estimate from this full model to that of a reduced model that included only the main effect (no age parameter).We concluded that age was a confounder if inclusion of age in the model induced a change in the main effect parameter >10% and that change in effect was in the top 80th percentile. To ensure that the change-in-effect estimate was stable, we did 100 bootstrap samplings and confirmed that genes were similarly affected in the original analysis and in the bootstrap analysis. That is, in most cases, those genes that were unadjusted in the original data remained unadjusted in the bootstrap. We used the parameter estimates from the original analysis to select genes for age adjustment, and as a result, we adjusted for age in 19% of genes. This is concordant with previous literature from lymphoblastoid cell lines suggesting that at least 10% of genes were significantly associated with donor age (17). Identical normalization parameters and age-adjustment parameters and algorithms applied to the training set were used to adjust for age in the test set.

Prediction analysis. The age-adjusted training data set was analyzed using Distance Weighted Discrimination (DWD) to identify

genes that distinguished reduction mammoplasty and cancer-adjacent normal samples (18). The top 200 DWD loadings were selected for the predictive signature based upon the observation that this was the smallest gene set that provided maximum predictive accuracy in the training set (by 10-fold cross-validation). Average predictive accuracy in 10-fold cross-validation was 95.4% (95% confidence interval, 95.1-96.3%) in the training data set, with 91.9% sensitivity (95% confidence interval, 91.2-94.1%) and 97.98% specificity (95% confidence interval, 97.92-97.92%), and the same set of genes was used to assign class membership in the test set.

To evaluate the Chang et al. (15) serum response as a predictor of cancer-adjacent normal versus reduction mammoplasty status, all patients in the training set were classified according to the Pearson correlation of its expression levels of the core serum response genes to the serum activated fibroblast centroid. Patients with correlation >-0.15 were classified as "activated," and patients with correlation ≤-0.15 were classified as "quiescent", as described in the original paper.

Prediction of breast cancer survival. To evaluate the prognostic value of our signature, we used published gene expression data for breast tumors from 295 patients, derived by researchers from Netherlands Cancer Institute and Rosetta Inpharmatics-Merck using Agilent oligonucleotide microarrays (15, 19, 20). To evaluate the prognostic value of our *in vivo* wound response signature, we mapped our 200 probes, representing 155 genes, to 112 genes on the Netherlands Cancer Institute data set. We then used principal component analysis to define three groups of equal size. These groups were then evaluated in univariate Kaplan-Meier analyses using WinSTAT. We compared our prognostic signature to the wound response signature published for these tumors by Chang et al. (15). For that comparison, we used the group definitions from the original paper (two groups, activated and quiescent). In addition, we compared our signature to the Stroma-Derived Prognostic Predictor of Finak et al. (21) and the 66-gene desmoids-type fibromatosis signature (22), both of which were used to define three groups of equal size by principal component analysis.

Immunohistochemistry. A series of cancer-adjacent normal ($n = 7$) and reduction mammoplasty ($n = 8$) samples that had been analyzed by microarray were also analyzed by immunohistochemistry. Five-micrometer sections cut from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized and rehydrated following standard protocol. Sections were incubated with antisera against COX-2/PTGS2 (Cayman Chemical) and CYR61 (H-78, Santa Cruz), following manufacturers' instructions. Antigen-antibody complexes were visualized using horseradish peroxidase–labeled polymer kit following standard protocol (DAKO). Sections were counterstained in hematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted in Permount.

Results

Activated wound responses in normal tissue adjacent to breast cancer. Using data from 48 reduction mammoplasty samples and 34 histologically normal samples adjacent to cancer, we did Distance Weighted Discrimination to select the top 200 probes that were differentially expressed between reduction mammoplasty and cancer-adjacent normal patients. In 10-fold cross-validation, these 200 probes, representing 155 genes, had 98% accuracy in predicting reduction mammoplasty versus cancer-adjacent normal status. Figure 1 shows a hierarchical cluster of these 82 patients using this gene list (complete gene list and values for all 200 probes shown in Supplementary Table S1).

The cancer-adjacent normal signature seems to be enriched for many genes previously shown to have an important role in wound healing and inflammation (Fig. 1; Supplementary Table S2). For example, extracellular matrix (ECM) deposition is an important process in repairing of wounds and ECM genes are differentially expressed between reduction mammoplasty and cancer-adjacent normal samples. Figure 1C shows a group of genes more highly expressed in reduction mammoplasty tissue than in cancer-adjacent normal tissue that includes genes in the Gene Ontology (GO) categories for ECM, such as *EGFL6*, *BMPER*, *ELA2*, and *TFPI2*. *TFPI2* is the negative regulator of F3, and consistent with lower expression of its inhibitor, the transcript levels of F3 are higher in cancer-adjacent normal samples (Fig. 1D), along with a number of other genes involved in ECM formation, such as *ADAMTS4*, *OGN*, *PDGFRL*, *FBLN1*, *LUM*, and *EFEMP1*. Thus, clusters 1C and D show dysregulation of ECM in the microenvironment of cancer. Functional annotation clustering with DAVID[8] shows ECM genes are significantly overrepresented in our *in vivo* signature for response to cancer (Supplementary Table S2). Three functional annotation clusters, including ECM genes, were identified with GO terms such as extracellular region (Benjamini $P = 1.0 \times 10^{-11}$), extracellular region part (2.7×10^{-8}), and ECM (3.7×10^{-4}) statistically overrepresented. Supplementary Table S2 shows DAVID functional annotation clustering results.

Angiogenesis is an important process in wound healing. Consistent with a role for angiogenesis in the cancer microenvironment, Fig. 1E includes important immediate early angiogenesis genes such as *PTGS2/COX-2*, *CYR61*, and *CTGF* (23–26). Furthermore, well-documented binding partners for CYR61 and CTGF (including THBS1 and BMPER) are upregulated in cancer-adjacent normals. Thus, these pathways and the CCN family of genes may be important in regulating the host response to cancer. *PTGS2* and *CTGF* are immediate early genes that are in the GO category "response to wounding," which was enriched in our data ($P = 1.4 \times 10^{-5}$), and the role of CYR61 in wound response is well established in recent literature (27). PTGS2 has been previously shown to be elevated in the microenvironment of breast cancer (28). Figure 1E therefore represents an important wound response angiogenic signature that is activated in the breast cancer microenvironment.

Genes from the GO "cellular adhesion" category were represented in Fig. 1F, including *LAMA3* and *LAMC2*. The cluster in Fig. 1F also included a number of chemotaxis genes such as *IL1B*, *CXCL1*, and *CXCL14*, the latter of which has previously been shown to be upregulated in the histologically normal tissue of breast cancer patients (7). Figure 1G includes a wide range of genes involved in re-epithelialization after wounding, including keratins 6A, 6B, 6C and 14, 14p, and 16 (29–31). Re-epithelialization is one of the major processes necessary for successful wound repair (32). Keratins are overrepresented in our functional clusters ($P = 5.2 \times 10^{-5}$), in a cluster that includes significant enrichment for GO categories epidermis development ($P = 2.8 \times 10^{-4}$), ectoderm development ($P = 2.8 \times 10^{-4}$), and cell communication ($P = 5.5 \times 10^{-6}$). Figure 1G shows overrepresentation of cellular adhesion genes, including *TP63* (33), *DST*, *COL17A1*, and *DSC3*. In sum, Fig. 1F and G show remodeling clusters that are dependent upon paracrine signaling and heterotypic interactions between cell types. Taken as a whole, Fig. 1 shows that the wound healing processes that have been well documented in the skin (32) are strongly induced in the microenvironment of normal breast tissue adjacent to breast cancers.

[8] http://david.abcc.ncifcrf.gov/

The original work hypothesizing that tumors resemble chronic wounds was referring to the tumor stroma rather than the adjacent normal tissue (1). Although we defined a wound response signature in adjacent normal tissue, we were interested to examine the relative expression of this signature in the tumors themselves. Supplementary Fig. S1 depicts the expression of the 200 probes across the 82 patients, including the paired tumor tissue for each of the cancer patients, with the groups of

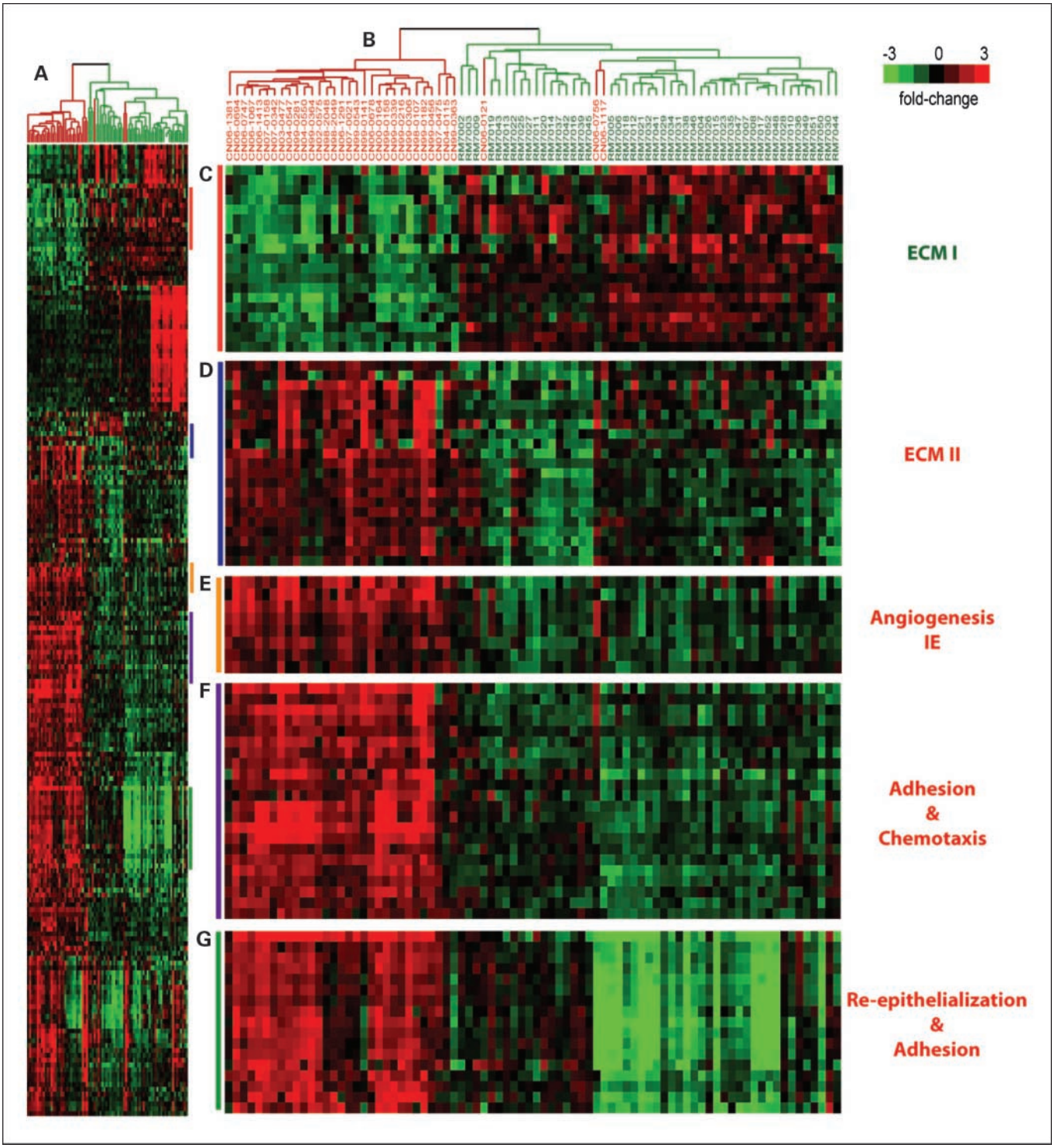

Fig. 1. Wound response signatures are differentially expressed between reduction mammoplasty patients (RM) and histologically normal tissue of cancer patients (cancer normal; CN). *A,* shows the complete dendrogram for 200 probes that distinguish reduction mammoplasty from cancer-adjacent normal tissues with 98% accuracy in predictive analyses. The dendrogram in *B* shows cancer-adjacent normal samples (*red*) and reduction mammoplasty samples (*green*) form distinct branches, with only a few cancer-adjacent normal tissues clustering with reduction mammoplasty samples. The clusters show that various biological processes involved in wound repair are differentially expressed between the two groups, including ECM alterations (*C* and *D*), immediate early (IE) genes involved in angiogenesis (*E*), re-epithelialization and cellular adhesion (*F*), and cellular adhesion and chemotaxis (*G*). Supplementary Table S1 lists $\log_2$ (red/green) values for each sample by probe ID, and Supplementary Table S2 provides DAVID analysis results for the 200-probe gene set.

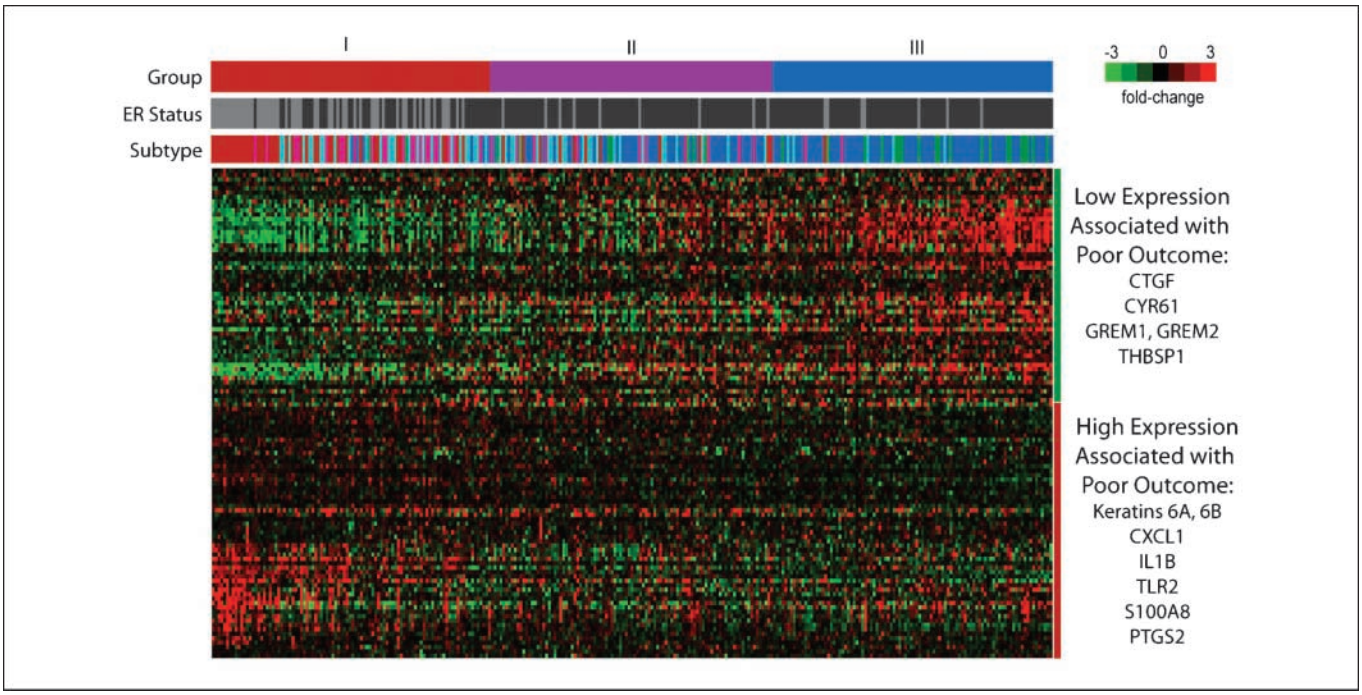

Fig. 2. Differential expression of wound response signatures in breast cancer. Expression of our *in vivo* wound response signature (Fig. 1) was examined in 295 tumors from the Netherlands Cancer Institute (15). Principal Component Analysis was used to divide the samples into three groups of equal size (groups I, II, and III), and the samples are ordered according to their assigned group. Gray, estrogen receptor (ER) negative; black, ER positive. For breast cancer subtype, red is basal-like; magenta, HER2 enriched; light blue, luminal B; dark blue, luminal A; green, normal-like. Two gene clusters were identified: one wherein low expression predicted poor outcome and one wherein high expression predicted poor outcome.

genes labeled according to the corresponding clusters from Fig. 1. This figure shows that, for some clusters (e.g., Fig. 1C) wherein cancer-adjacent normal has lower expression than reduction mammoplasty, the decreased expression is even more pronounced in the tumors. Likewise, many of the genes in Fig. 1F that were upregulated in cancer-adjacent normal are more strongly upregulated in tumors. However, there are groups of genes from Fig. 1D to F that are homogeneously expressed in cancer-adjacent normal tissue and heterogeneously expressed in tumors, and there are genes (e.g., Fig. 1G) that are upregulated in cancer-adjacent normal and expressed at lower levels in tumors. In the case of the cluster in Fig. 1G, many of these genes are immune response/chemotaxis genes, and therefore, downregulation in the immunosuppressive tumor microenvironment, but not the adjacent normal, may be expected. In sum, our gene list has a unique expression profile in each of the tissue types (cancer-adjacent normal versus reduction mammoplasty versus tumor), consistent with some of the known biological features of each tissue type, and our signature reliably segregates normal breast samples from patients with disease from those without disease (Fig. 1B; dendrogram in Supplementary Fig. S1).

Localization of PTGS2/COX-2 and CYR61 by immunohistochemistry. To confirm the changes that we observed by microarray and to identify the cell types responsible for altered expression, we did immunohistochemistry for two markers from the Angiogenesis/IE cluster in Fig. 1E. Representative images are shown in Supplementary Fig. S2. COX-2 was more highly expressed in the luminal epithelium of cancer-adjacent normal patients than reduction mammoplasty patients (two-tailed *t* test with unequal variance; $P = 0.0005$), consistent with previous observations (28). Less than half of the reduction mammoplasty samples (5 of 13) were positive for COX-2 staining, with an average score of 0.7 (range, 1-2). However, 16 (89%) of 18 of cancer-adjacent normal samples were positive for COX-2, with an average score of 2.1 (range, 1-3). CYR61 was moderately or strongly expressed in the luminal epithelium of cancer-adjacent normal and reduction mammoplasty patients, and we also observed CYR61 expression in fibroblasts, endothelial cells, plasma cells, and lymphocytes. Thus, the 2- to 4-fold change in CYR61 RNA levels in cancer-adjacent normal relative to reduction mammoplasty may reflect changes in expression levels of specific cellular populations or may reflect changes in the distribution of cell types present in the tissue.

Predictive accuracy of the wound response signature. Our *in vivo* wound response signature distinguished cancer-adjacent normal samples from reduction mammoplasty samples with 98% accuracy in the training set, 94% sensitivity, and 100% specificity. This accuracy and the striking expression differences between cancer-adjacent normal and reduction mammoplasty shown in Fig. 1 suggest that this signature can be used to predict the disease status of histologically normal samples in an independent test set. To our knowledge, there are no comparable, publicly available data set of grossly dissected human breast tissue, and therefore, we collected a separate test set of 25 additional arrays (12 reduction mammoplasty and 13 cancer-adjacent normal). In this independent test set, we correctly predicted cancer-adjacent normal versus reduction mammoplasty status with 96% accuracy (92% sensitivity and 100% specificity), with only 1 of 13 cancer-adjacent normal samples incorrectly classified as a reduction mammoplasty.

The population undergoing reduction mammoplasty surgery may be systematically different from general population.

Whereas some women undergo unilateral reduction mammoplasty for cosmetic reasons, others undergo bilateral reduction mammoplasty to address ergonomic problems. This latter group may have more fatty tissue and larger breasts, and therefore, it is possible that some of these patients have biology that differs from normal breast biology in the general population. To evaluate generalizability of our study, we obtained a second test set comprised of 10 additional samples of normal breast tissue, none of which was from reduction mammoplasty patients: three normal breast samples from women with benign diagnoses (one benign cyst and two fibroadenoma) and seven normal breast tissue samples adjacent to ductal carcinoma *in situ*. In this independent test set, we applied the same normalization, data preprocessing algorithms, and parameters as were applied to the training set. Of the 200 probes that comprised our classifier in the training set, 149 were present with >80% good data in this second independent test set. All three of the normal tissues adjacent to benign lesions were classified as reduction mammoplasty–like, and four of seven normal breast tissue samples adjacent to ductal carcinoma *in situ* samples were identified as cancer-adjacent normal. These results suggest that the normal breast signature that we observed in reduction mammoplasty is not peculiar to women who elect reduction surgery, and furthermore, documents that the cancer-adjacent normal–like signature is also present adjacent to ductal carcinoma *in situ* in some patients, even before the disease becomes invasive.

Because Chang et al. (15) had previously published a serum treatment/wound response signature, we were interested in whether their *in vitro* signature, which had little overlap in terms of exact gene content but substantial overlap in terms of the processes represented, would also predict cancer-adjacent normal versus reduction mammoplasty status. We classified each of our samples as activated or quiescent using their signature and classification parameters, expecting that perhaps activated would be more common in the cancer-adjacent normal samples. However, ~37% percent of reduction mammoplasty samples were classified as activated and 35% of cancer-adjacent normal samples were classified as activated, showing that the *in vitro* serum response signature does not offer predictive value in distinguishing reduction mammoplasty and cancer-adjacent normal samples (χ^2 test; 1 *df*; $P = 0.8$). This suggests that, although many of the processes involved in wound response may be common to different experimental and observational systems, the specific signatures of wound response may be tissue and/or cell-type dependent. Our wound response signature, being breast tissue derived, seems to offer advantages over the *in vitro* serum-derived signatures in identifying abnormal breast tissues.

In vivo *wound response and patient survival*. Because the aforementioned signature derived from serum-treated fibroblasts, commonly referred to as the wound response signature, was highly prognostic for breast cancer patient outcomes, we

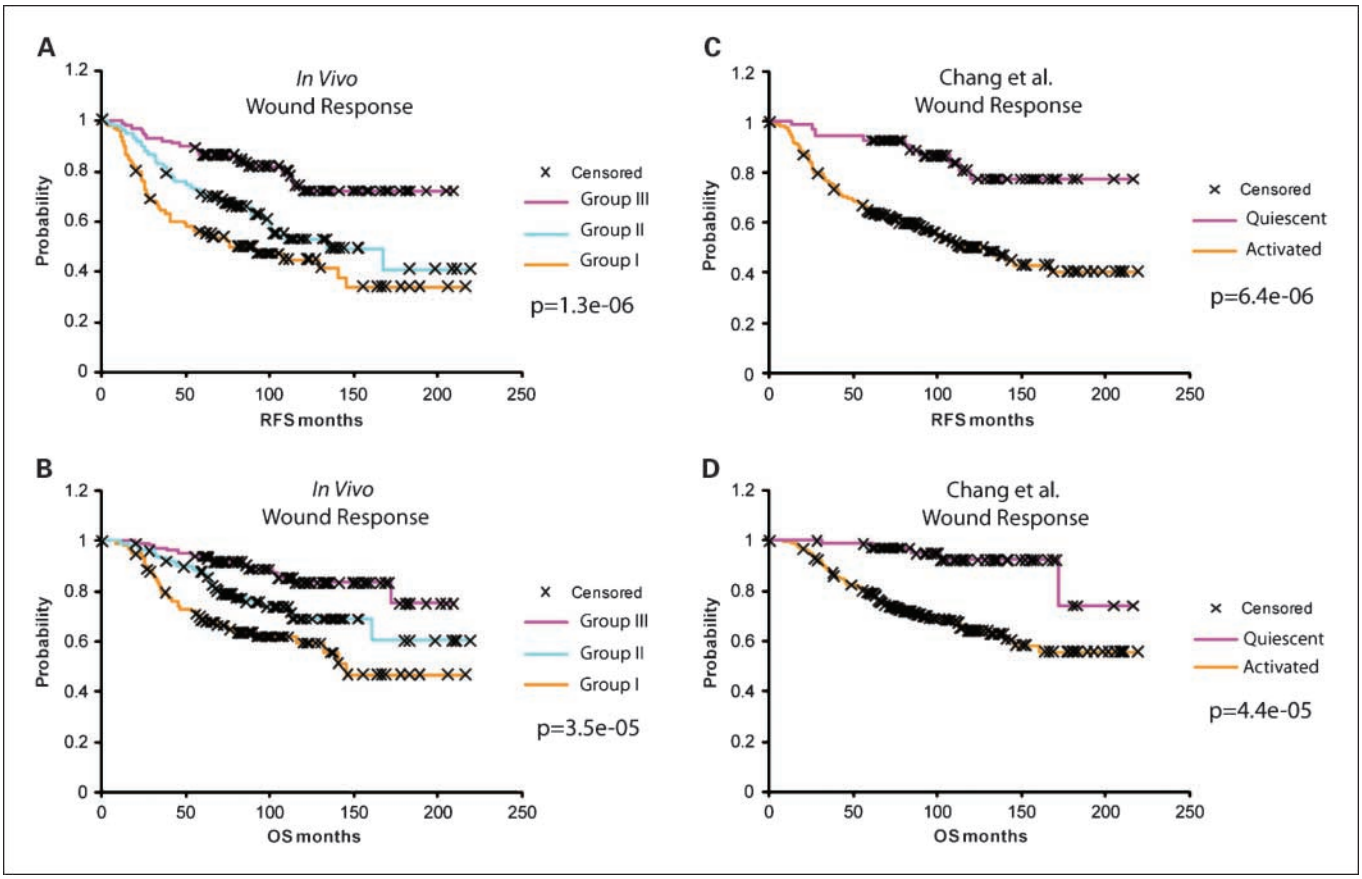

Fig. 3. *In vivo* wound response signature and previously published serum response signature show similar prognostic value. Kaplan-Meier analyses were conducted using the principal component analysis–assigned group I, II, or III (*A* and *B*) and using the Chang et al. (15) core serum response assignments (*C* and *D*). Both classification schemes are associated with overall and relapse-free survival.

Table 1. Concordance in classifications between the *in vivo* wound response signature and previous serum response or microenvironment-related prognostic signatures

***In vivo* wound response**		**Core serum response (15)**		**SDPP (19)**		**DTF signature (20)**		**Skin injury (40)**		**Involution (41)**	
Class	**No. of patients**	**Class**	**No. of patients**	**Class**	**No. of patients**	**Class**	**No. of patients**	**Class**	**No. of patients**	**Class**	**No. of patients**
Group III	98	Quiescent	50	Good	67	Good	50	Good	56	Good	70
		Activated	48	Mixed	25	Mixed	19	Mixed	36	Mixed	23
				Poor	6	Poor	29	Poor	6	Poor	5
Group II	99	Activated	88	Mixed	51	Mixed	30	Mixed	39	Mixed	43
		Quiescent	11	Good	28	Good	39	Good	34	Good	23
				Poor	20	Poor	30	Poor	26	Poor	33
Group I	98	Activated	92	Poor	72	Poor	40	Poor	66	Poor	60
		Quiescent	6	Mixed	23	Mixed	49	Mixed	24	Mixed	33
				Good	3	Good	9	Good	8	Good	5
		$\chi^2 = 67.7$; 2 *df*; $P < 0.001$		$\chi^2 = 152.9$; 4 *df*; $P < 0.001$		$\chi^2 = 44.0$; 4 *df*; $P < 0.0001$		$\chi^2 = 96.6$; 4 *df*; $P < 0.0001$		$\chi^2 = 121.7$; 4 *df*; $P < 0.0001$	

NOTE: The rows are arranged so that the number of group I, II, or III samples that are represented in the analogous category from each of the other predictors is visible by looking across the gray bar.
Abbreviations: SDPP, Stroma-Derived Prognostic Predictor; DTF, desmoid-type fibromatosis.

hypothesized that our *in vivo*, 200-probe, breast tissue–derived wound response signature might also have prognostic value. Thus we evaluated the prognostic value of our signature on a test set of 295 patients from the Netherlands Cancer Institute (15, 34). These 295 patients were divided into three groups based on the rank order of the first principal component of our *in vivo* wound response signature across their tumor expression profiles (gene expression shown in Fig. 2), and we conducted Kaplan-Meier analysis to evaluate the association between these groups and overall and relapse-free survival (Fig. 3).

In Fig. 2, the poor prognosis group (group I) had low expression of immediate early genes *CTGF* and *CYR61* and low expression of *GREM1* and *GREM2*. GREM1 is an important developmental signaling molecule that is part of a fibroblast growth factor/GREM1 inhibitory loop that halts tissue outgrowth during development and regeneration (35). These tumors also have low expression of angiogenesis inhibitor THBSP1 (36). In contrast, the good prognosis group III tumors had high expression of these same genes. The group I poor prognosis tumors had high expression of a number of chemotaxis and immunoregulatory genes, including *IL1B*, *TLR2*, *CCL2*, and indoleamine 2,3-dioxygenase, a targetable enzyme that modulates the response of T cells to tumors (37). These tumors have high expression of keratin 6 and 14, the former of which contains an IL-1–responsive DNA element (38). Keratin 6 is expressed in the skin in hyperproliferative disorders and in response to stressful stimuli such as wounding (29). Keratin 14 is a basal keratinocyte marker that is differentially expressed in wound repair (30). We observed that basal-like tumors were more likely to have the group I wound response signature, whereas luminal tumors were more likely to be group III (Fig. 2). The correlations between subtype and some of the gene expression changes were consistent with those in previous studies. For example, *PTGS2* has been shown to be highly expressed in basal-like tumors (39) previously, and we also observed this here.

Based on the three groups of equal size identified in Fig. 2, we then evaluated associations with survival. The three groups had different relapse-free (Fig. 3A) and overall survival (Fig. 3B), and the predictive value of these signatures compare favorably with other prognostic signatures that have used microenvironment biology to predict outcome. For example, our signatures show prognostic value similar to that provided by the Chang et al. (15) wound response signature in predicting relapse-free (Fig. 3C) and overall (Fig. 3D) survival.

To further evaluate concordance of our *in vivo* wound response with previous wound response or microenvironment-related signatures, we applied methods described in Fan et al. (34). Concordance between predictors was evaluated by classifying each patient and then conducting a χ^2 test to identify associations between classifications made by each predictor. Table 1 shows that there was high concordance between those classified as activated by Chang et al. (15) and our group I and II tumors. Likewise, our predictor showed significant concordance with the three-class Stroma-Derived Prognostic Predictor (21) and desmoids-type fibromatosis signatures (22), both of which were more likely to classify group III tumors as good prognosis and group I tumors as poor prognosis.

Although our signature represents many of the processes observed to occur in healing skin (32), we sought to strengthen the claim that our signature reflects wound repair using results from previous gene expression studies on healing skin and wound response in breast. Using the same concordance evaluation methods (34) that we applied for Stroma-Derived Prognostic Predictor and desmoid-type fibromatosis signatures, we found a significant association between our signature and a previously published gene expression signature for the early response of skin to injury (40). This signature was measured only 30 or 60 minutes after skin injury and does not reflect chronic injury, and it is also derived from the skin, not the breast. Thus, we also sought comparisons with breast tissue injury. A breast injury signature has not been reported, but mammary gland involution has established features of wound repair

(41). Therefore, we examined concordance with the mammary gland involution signature and found a strong and significant correlation. The results are presented in Table 1. Taken together, these findings show reproducible concordance between our signature and wound repair– and microenvironment-based signatures.

Discussion

It has been argued that solid tumors are composed of two distinct but interdependent compartments: the malignant parenchyma and its supporting microenvironment (42). In this article, we have studied how the normal microenvironment responds to the presence of the tumor. Our results show that a wound response signature is activated in the histologically normal tissue of cancer patients. Wound healing includes numerous overlapping processes such as blood clotting, inflammation, ECM alterations, angiogenesis, and tissue remodeling (reviewed in refs. 2, 32). Many of these processes are expressed differentially in normal tissue adjacent to breast cancer and in tumors but not in the normal tissue of women undergoing reduction mammoplasty.

Previous microarray studies have examined molecular similarities between wound repair and cancer using *in vitro* models. First, Iyer et al. (43) studied the response of fibroblasts to serum based on the idea that serum has a very specific meaning *in vivo*: cells encounter serum only in the event of local injury. In response, tissues mount a rapid concerted multicellular response to preserve tissue integrity. Serum promotes growth and survival of normal and cancer cells in culture, and in parallel, serum causes rapid cell proliferation in tissue-level wound repair responses. Given these parallels, Iyer et al. (43) were able to document a serum response of foreskin fibroblasts that included genes involved in clotting and coagulation during remodeling, including tissue factor 3, genes promoting migration and proliferation of fibroblasts (e.g., *CTGF*), and genes involved in angiogenesis (e.g. *PTGS2* and *IL1B*). Many of these same genes were modified in our *in vivo* wound response signature. To extend the observations of Iyer et al. (43), Chang et al. (15) hypothesized that a canonical gene expression signature might be identified for serum response of fibroblasts from different anatomic sites. Using gene expression profiles of 50 samples from ten sites, Chang et al. (15) recapitulated a wound response signature originally reported by Iyer et al. (43) and documented that this core serum response was evident in a subset of breast tumors. Breast tumors that expressed the serum response signature had a poorer prognosis than tumors with a quiescent serum response phenotype. Although the *in vitro* wound response signatures of Iyer et al. (43) and Chang et al. (15) did not have predictive accuracy in distinguishing reduction mammoplasty and cancer-adjacent normal tissues in our data set, we did observe that the same biological processes are activated in association with breast cancer *in vivo*, outside the boundaries of the tumor itself.

The association between microenvironment characteristics, survival, and breast cancer subtype has been reported in previous articles. For example, in a tissue microarray study on 479 invasive breast carcinomas using 28 different markers, basal-like breast cancers were more likely to highly express genes (such as laminin) that are involved in ECM remodeling (44). In a separate study, the ECM composition of 28 primary breast carcinomas were examined using microarrays, and a gene expression profile of 278 ECM-related genes was used to divide the tumors into four main groups that correlated with outcome and basal-like subtype (45). The current results confirm that many ECM genes are enriched in basal-like cancers and that these genes have prognostic value but suggest that the enrichment may be part of a broader chronic response of the host as it attempts to repair the tissue injury caused by cancer.

Strengths of our investigation include the large number of samples relative to previous studies on histologically normal tissue, the application of methods to control for confounding by age, and the systematic comparison with previous microenvironment-derived signatures. In addition, we documented the generalizability of our findings to women with premalignant or benign breast disease and showed the validity of our predictor in two independent test sets. Our results show that the use of whole tissue, which includes epithelial cells, as well as stromal cells, does not hamper our ability to detect important microenvironment signatures. Many of the genes we detected as differentially expressed are reported to be expressed by fibroblasts (e.g., *CTGF*) and neutrophils (e.g. *IL1B*), for example, whereas others are epithelial contributors (keratins 6 and 14). This reiterates the findings of Perou et al. (46) showing that signatures from distinct cellular compartments can be identified in microarray data from mixed cell populations.

Our results suggest some important questions for future investigation related to the spatial and temporal parameters of the wound response to cancer. Although our tissue procurement protocols ensured that the histologically normal tissue was collected from regions outside of tumor margins, the distance to the margin was not measured precisely, so we are unable to document the geographic zones of gene expression alterations. Methylation changes have been reported as far as 4 cm from the site of the primary tumor (47); however, the geographic zones for gene expression alterations have not been characterized. Based on the notion that wound response is a local phenomenon, it might be expected that the wound response would not be present in contralateral breast or that it may dissipate as the distance from the primary tumor increases, but this has not been investigated. The geographic limits of the wound response to cancer may have translational implications. For example, most tumor recurrences occur at the site of previous resection even when margins are clear (48). If gene expression alterations with promoting characteristics have a defined range of action outside the primary tumor margins, future guidelines for surgical intervention may need to consider wider margins consistent with the geographic range of microenvironment alterations. It is also not known how early in development of breast cancer gene expression alterations are activated in adjacent normal tissue. Leukocyte infiltration occurs early in carcinogenesis (49), suggesting that aberrations in gene expression may already be present during ductal carcinoma *in situ*, but this has not been investigated. What is clear from our results is that wound response signatures are strongly activated in histologically normal tissue adjacent to invasive tumors.

A role for wound response in breast carcinogenesis is increasingly well documented. Differential expression of wound response signatures in different tumors suggests that individualized targeting of microenvironment may be a viable treatment strategy in breast cancer. For example, there is significant heterogeneity in ECM across tumors (44, 45), suggesting the differential

efficacy of ECM-targeting drugs. The strong angiogenesis signature observed in group I tumors, which are enriched for basal-like breast cancers, supports the application of chemotherapy combinations that target VEGF and angiogenesis in triple-negative breast cancers (50). By better characterizing microenvironment heterogeneity, improved strategies for application of novel and existing therapies may be possible. The similarities between cancer and wound response are compelling, and continued investigation of host responses to cancer may lead to new biological insights and translational opportunities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986;315:1650–9.
2. Schafer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. Nat Rev Mol Cell Biol 2008;9:628–38.
3. Overall CM, Kleifeld O. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nat Rev Cancer 2006;6:227–39.
4. Yamaguchi Y. Microenvironmental regulation of estrogen signals in breast cancer. Breast Cancer 2007;14:175–81.
5. Albini A, Sporn MB. The tumour microenvironment as a target for chemoprevention. Nat Rev Cancer 2007;7:139–47.
6. Kenny PA, Lee GY, Bissell MJ. Targeting the tumor microenvironment. Front Biosci 2007;12:3468–74.
7. Allinen M, Beroukhim R, Cai L, et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 2004;6:17–32.
8. Bissell MJ, Radisky D. Putting tumours in context. Nat Rev Cancer 2001;1:46–54.
9. Gusterson BA, Warburton MJ, Mitchell D, et al. Distribution of myoepithelial cells and basement membrane proteins in the normal breast and in benign and malignant breast diseases. Cancer Res 1982;42:4763–70.
10. Ronnov-Jessen L, Petersen OW, Bissell MJ. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev 1996;76:69–125.
11. Shekhar MP, Pauley R, Heppner G. Host microenvironment in breast cancer development: extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. Breast Cancer Res 2003;5:130–5.
12. Gudjonsson T, Adriance MC, Sternlicht MD, Petersen OW, Bissell MJ. Myoepithelial cells: their origin and function in breast morphogenesis and neoplasia. J Mammary Gland Biol Neoplasia 2005;10:261–72.
13. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer Res 2006;66:605–12.
14. Finak G, Sadekova S, Pepin F, et al. Gene expression signatures of morphologically normal breast tissue identify basal-like tumors. Breast Cancer Res 2006;8:R58.
15. Chang HY, Sneddon JB, Alizadeh AA, et al. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. PLoS Biol 2004;2:E7.
16. Hu Z, Fan C, Oh DS, et al. The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 2006;7:96.
17. Tan Q, Zhao J, Li S, et al. Differential and correlation analyses of microarray gene expression data in the CEPH Utah families. Genomics 2008;92:94–100.
18. Benito M, Parker J, Du Q, et al. Adjustment of systematic microarray data biases. Bioinformatics 2004;20:105–14.
19. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999–2009.
20. van 't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530–6.
21. Finak G, Bertos N, Pepin F, et al. Stromal gene expression predicts clinical outcome in breast cancer. Nat Med 2008;14:518–27.
22. Beck AH, Espinosa I, Gilks CB, van de Rijn M, West RB. The fibromatosis signature defines a robust stromal response in breast carcinoma. Lab Invest 2008;88:591–601.
23. Arun B, Goss P. The role of COX-2 inhibition in breast cancer treatment and prevention. Semin Oncol 2004;31:22–9.
24. Babic AM, Kireeva ML, Kolesnikova TV, Lau LF. CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. Proc Natl Acad Sci U S A 1998;95:6355–60.
25. Babic AM, Chen CC, Lau LF. Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin αvβ3, promotes endothelial cell survival, and induces angiogenesis *in vivo*. Mol Cell Biol 1999;19:2958–66.
26. Shimo T, Nakanishi T, Nishida T, et al. Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells *in vitro*, and angiogenesis *in vivo*. J Biochem 1999;126:137–45.
27. Chen CC, Mo FE, Lau LF. The angiogenic factor Cyr61 activates a genetic program for wound healing in human skin fibroblasts. J Biol Chem 2001;276:47329–37.
28. Gauthier ML, Pickering CR, Miller CJ, et al. p38 regulates cyclooxygenase-2 in human mammary epithelial cells and is activated in premalignant tissue. Cancer Res 2005;65:1792–9.
29. Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M. Keratins and the keratinocyte activation cycle. J Invest Dermatol 2001;116:633–40.
30. Hosoya A, Lee JM, Cho SW, et al. Morphological evidence of basal keratinocyte migration during the re-epithelialization process. Histochem Cell Biol 2008;130:1165–75.
31. Bernot KM, Coulombe PA, McGowan KM. Keratin 16 expression defines a subset of epithelial cells during skin morphogenesis and the hair cycle. J Invest Dermatol 2002;119:1137–49.
32. Martin P. Wound healing-aiming for perfect skin regeneration. Science 1997;276:75–81.
33. Hu M, Yao J, Carroll DK, et al. Regulation of *in situ* to invasive breast carcinoma transition. Cancer Cell 2008;13:394–406.
34. Fan C, Oh DS, Wessels L, et al. Concordance among gene-expression-based predictors for breast cancer. N Engl J Med 2006;355:560–9.
35. Verheyden JM, Sun X. An Fgf/Gremlin inhibitory feedback loop triggers termination of limb bud outgrowth. Nature 2008;454:638–41.
36. Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 1994;265:1582–4.
37. Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med 2005;11:312–9.
38. Komine M, Rao LS, Freedberg IM, et al. Interleukin-1 induces transcription of keratin K6 in human epidermal keratinocytes. J Invest Dermatol 2001;116:330–8.
39. Gauthier ML, Berman HK, Miller C, et al. Abrogated response to cellular stress identifies DCIS associated with subsequent tumor events and defines basal-like breast tumors. Cancer Cell 2007;12:479–91.
40. Cole J, Tsou R, Wallace K, Gibran N, Isik F. Early gene expression profile of human skin to injury using high-density cDNA microarrays. Wound Repair Regen 2001;9:360–70.
41. Stein T, Morris JS, Davies CR, et al. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. Breast Cancer Res 2004;6:R75–91.
42. Dvorak HF. Rous-Whipple Award Lecture. How tumors make bad blood vessels and stroma. Am J Pathol 2003;162:1747–57.
43. Iyer VR, Eisen MB, Ross DT, et al. The transcriptional program in the response of human fibroblasts to serum. Science 1999;283:83–7, see comments.
44. Sarrio D, Rodriguez-Pinilla SM, Hardisson D, et al. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 2008;68:989–97.
45. Bergamaschi A, Tagliabue E, Sorlie T, et al. Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome. J Pathol 2008;214:357–67.
46. Perou CM, Brown PO, Botstein D. Tumor classification using gene expression patterns from DNA microarrays. New technologies for life sciences: a trends guide 2000:67–76.
47. Yan PS, Venkataramu C, Ibrahim A, et al. Mapping geographic zones of cancer risk with epigenetic biomarkers in normal breast tissue. Clin Cancer Res 2006;12:6626–36.
48. Dalberg K, Mattsson A, Sandelin K, Rutqvist LE. Outcome of treatment for ipsilateral breast tumor recurrence in early-stage breast cancer. Breast Cancer Res Treat 1998;49:69–78.
49. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006;6:24–37.
50. Anders C, Carey LA. Understanding and treating triple-negative breast cancer. Oncology (Williston Park) 2008;22:1233–9; discussion 1239-40, 1243.