Stromal genes discriminate preinvasive from invasive disease, predict outcome, and highlight inflammatory pathways in digestive cancers

Amel Saadi[a], Nicholas B. Shannon[a], Pierre Lao-Sirieix[a], Maria O'Donovan[a,b], Elaine Walker[a], Nicholas J. Clemons[a], James S. Hardwick[c], Chunsheng Zhang[c], Madhumita Das[a], Vicki Save[b], Marco Novelli[d], Frances Balkwill[e], and Rebecca C. Fitzgerald[a,1]

[a]Medical Research Council Cancer Cell Unit, Hutchison Medical Research Council Research Centre, Cambridge CB22 0XZ, United Kingdom; [b]Histopathology Department, Addenbrooke's Hospital, Cambridge CB2 0QQ, United Kingdom; [c]Merck Laboratories, West Point, PA 19486; [d]Royal Free and University College Medical School, University College London, London WC1E 6BT, United Kingdom; and [e]Centre for Cancer and Inflammation, Institute of Cancer and the Cancer Research United Kingdom Clinical Centre, Barts and The London School of Medicine and Dentistry, London EC1M 6BQ, United Kingdom

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The stromal compartment is increasingly recognized to play a role in cancer. However, its role in the transition from preinvasive to invasive disease is unknown. Most gastrointestinal tumors have clearly defined premalignant stages, and Barrett's esophagus (BE) is an ideal research model. Supervised clustering of gene expression profiles from microdissected stroma identified a gene signature that could distinguish between BE metaplasia, dysplasia, and esophageal adenocarcinoma (EAC). EAC patients overexpressing any of the five genes (*TMEPAI*, *JMY*, *TSP1*, *FAPα*, and *BCL6*) identified from this stromal signature had a significantly poorer outcome. Gene ontology analysis identified a strong inflammatory component in BE disease progression, and key pathways included cytokine–-cytokine receptor interactions and TGF-β. Increased protein levels of inflammatory-related genes significantly up-regulated in EAC compared with preinvasive stages were confirmed in the stroma of independent samples, and in vitro assays confirmed functional relevance of these genes. Gene set enrichment analysis of external datasets demonstrated that the stromal signature was also relevant in the preinvasive to invasive transition of the stomach, colon, and pancreas. These data implicate inflammatory pathways in the genesis of gastrointestinal tract cancers, which can affect prognosis.

Barrett's esophagus | gene set enrichment | intraepithelial neoplasia | inflammation | microarray

Normal epithelial cells depend on stroma, consisting of extracellular matrix and mesenchymal and nerve cells, to sustain their survival and proliferation. Alterations in the stroma are central to carcinogenesis (1). Most of these cell lineages in the stroma have been identified as "coconspirators" either hindering the elimination (2) or helping the formation and development (3) of cancer.

The development of an epithelial malignancy is a multistep process in which a preinvasive lesion gradually acquires the capacity to invade (4). However, the stage at which the stromal compartment contributes to the de novo development of cancer is unknown. Precursor lesions exist for most gastrointestinal tumors. Esophageal adenocarcinoma (EAC) develops through a premalignant, metaplastic lesion called Barrett's esophagus (BE). BE is an ideal tool for the study of cancer progression because it is visible endoscopically and highly accessible, there is a field effect that can be spatially mapped, and patients are monitored over time, giving rise to longitudinal data (5). Furthermore, gastroesophageal reflux is the key risk factor for this rapidly increasing disease (6, 7); BE could therefore be regarded as a response to chronic injury, and in this context the stroma might be predicted to play an important role.

We hypothesized that the altered expression of stromal genes influences the progression from preinvasive to invasive disease. An unbiased expression-array analysis in microdissected stroma from different stages of EAC progression was performed, and stromal genes affecting prognosis were identified. Expression of genes of interest was validated on independent human samples to establish the cellular source of expression and their functional relevance. The significance of our esophageal stromal signature was assessed in other gastrointestinal cancers with a well-characterized preinvasive stage.

Results

Distinct Stromal Signatures Separate Different Stages of BE Progression. The stroma from metaplasia, dysplasia, and EAC was microdissected, and gene expression was analyzed using an Agilent 44k array. Pair-wise comparison of gene expression in the stroma alone was sufficient to differentiate between the stages of progression (Fig. 1; $P < 10^{-4}$ in each case).

Identification of Key Genes. The most consistently dysregulated genes from the metaplasia–dysplasia–EAC sequence were identified using strict filtering and short-listing criteria (*SI Materials and Methods* and Fig. S1). The 12 short-listed genes encompass a number of functions: inflammatory mediators [*interleukin 6* (*IL-6*), *cyclooxygenase 2* (*Cox-2*), and *CCAAT element binding protein beta* (*C/EBPβ*)]; TGF-β–related genes [*thrombospondin 1* (*TSP1*), *periostin* (*POSTN*), and *transmembrane prostate androgen induced* (*TMEPAI*)]; potential modulators of invasion [*matrix metalloproteinase 1* (*MMP1*) and *junction-mediating and regulatory protein* (*JMY*) (8)]; transcription factors [*B cell lymphoma-6* (*BCL6*)]; metabolizing enzyme for toxicants and carcinogens [*UDP glucuronosyltransferase 1 family, polypeptide A4* (*UGT1A4*)]; and unsurprisingly, stromal activation markers [*fibroblast activation protein alpha* (*Fap-α*) and *alpha smooth muscle actin* (*α-SMA*)].

Their stromal expression was confirmed at the protein level by immunohistochemistry (IHC) for all up-regulated targets for which specific antibodies were available, to enable cell localization to be determined. The predominant stromal cell lineages expressing the targets of interest were fibroblasts, inflammatory

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus database (accession nos. GSE19632 and GSE19529).

[1]To whom correspondence should be addressed. E-mail: rcf@hutchison-mrc.cam.ac.uk.

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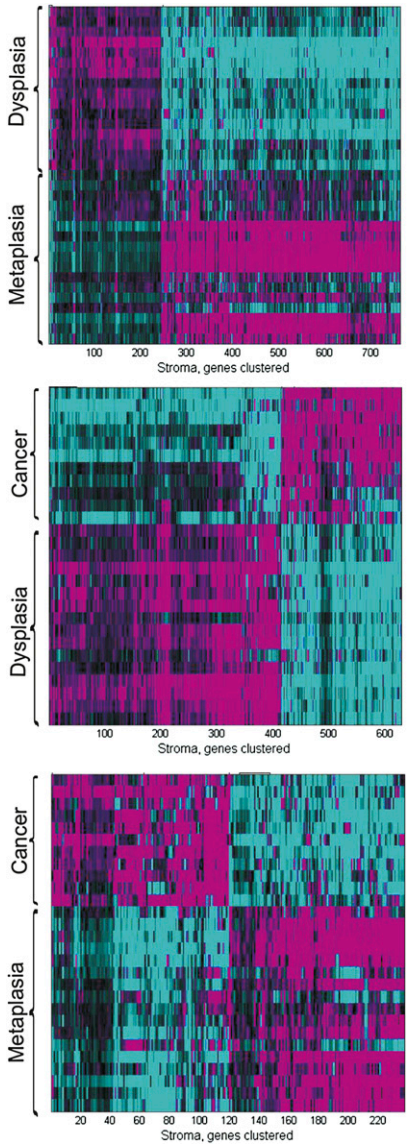

Fig. 1. Hierarchical clustering showing discrimination between the different stages of progression ($P < 10^{-4}$ for all comparisons).

cells, and endothelial cells (Fig. S2). Specific antibodies were not available for C/EBPβ, JMY, and IL-6. However, IL-6 is known to be expressed by fibroblasts and inflammatory cells (9). C/EBPβ is central to IL-17 stimulation of MMP1 in fibroblasts (11) and was shown to stimulate COX-2 and IL-8 expression (10), implying a stromal localization. UGT1A4 was the only significantly down-regulated target short-listed (Fig. S1), and because no antibody was available this was validated by in situ hybridization, which showed both epithelial and stromal localization (Fig. S2).

Expression of Stromal Genes Predicts Poor Outcome in EAC. Functional relevance of this stromal signature was determined by linking the expression level of the 12 targets to outcome for 63 chemo-naïve patients with invasive EAC. Up-regulation of *TMEPAI* and *JMY* was significantly associated with a worse prognosis ($P < 0.005$ for both; Fig. S3), and up-regulation of *TSP1*, *FAPα*, and *BCL6* had a trend for significantly worse outcome. Patients with up-regulation of any one or more of these five targets had a worse prognosis than patients with no increase in expression of any of these stromal genes ($P = 0.022$; Fig. 2).

Inflammation Ontologies Are Up-Regulated in the Progression from Metaplasia to EAC. Key processes altered along the disease sequence were determined by ontology analysis by comparing metaplasia vs. dysplasia and dysplasia vs. EAC. Ontologies conserved along the metaplasia–dysplasia–EAC sequence contained up-regulation of inflammation-related genes (three ontologies: mediation of immune response, inflammatory response, and negative regulation of immune response) and cell–cell communication related genes (two ontologies: cell–cell signaling and cell surface receptor linked signal transduction) (Table 1). Furthermore, analysis of signaling pathways deposited in a public database [Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways] highlighted the statistically significant up-regulation of cytokine–cytokine receptor interaction in the metaplasia vs. dysplasia comparison and of the TGF-β pathway in the dysplasia vs. EAC comparison (Table 1). These data are particularly striking because, in keeping with the supervised clustering analyses, the most dysregulated genes are inflammation-related genes (*IL-6*, *C/EBPβ*, and *Cox-2*) and are TGF-β–related genes (*TMEPAI*, *POSTN*, and *TSP1*) (Fig. S1).

Fibroblasts Express Similar Inflammation-Related Ontologies. Because seven of eight (with suitable antibodies) of the most dysregulated stromal genes were expressed by fibroblasts (*TMEPAI*, *BCL6*, *MMP1*, *TSP1*, *POSTN*, *αSMA*, and *FAPα*), we expression profiled 13 clonally derived primary stromal fibroblasts (origin confirmed; Fig. S4), generated from metaplasia, dysplasia, and EAC specimens (*SI Materials and Methods*). Three of 12 key genes, *MMP1*, *IL-6*, and *COX-2*, were dysregulated in the fibroblast signature. In addition, the inflammation-related ontologies and KEGG pathways identified for the microdissected stromal biopsy signature were all statistically up-regulated in the fibroblast signature (Table 1). Furthermore, three ontologies related to an invasive phenotype (chemotaxis, cell adhesion, and regulation of angiogenesis) differentiated cancer-associated fibroblasts from BE fibroblasts.

Functional Role of Selected Stromal Genes. *BCL6*, *TSP1*, and *POSTN* were selected for further study to investigate their role in progression to cancer because they were validated at the protein level across the metaplasia–dysplasia–EAC sequence (Fig. S2) and had relevance to inflammation and TFG-β and a little-known role in BE.

In keeping with previous studies (12–14), the up-regulated IL-6 levels were positively correlated with transcript levels for *BCL6* ($P < 0.05$) and inversely correlated with *TP53* ($P = 0.01$) and *PDCD4* ($P < 0.0001$) (Fig. S5). In vitro stimulation of the esophageal epithelial cancer cell line FLO-1 with recombinant IL-6 led to a significant and time-dependent increase in *BCL6* mRNA expression (10 h vs. 4 and 0 h; $P < 0.01$), with a con-

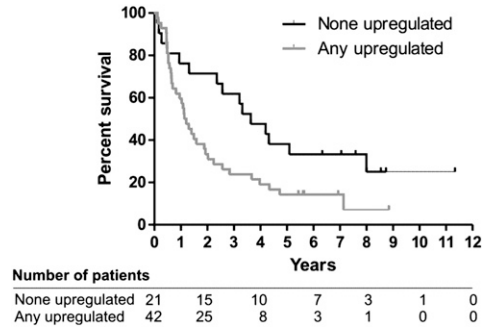

Fig. 2. Kaplan-Meier curve comparing survival in patients with (gray) and without (black) up-regulation of the five-gene signature (*TMEPAI*, *JMY*, *TSP1*, *FAPα*, or *BCL6*).

Table 1. Ontology analysis

Parameter	Stromal (MET vs. DYS)	Stromal (DYS vs. EAC)	Fibroblast (MET vs. EAC)
Conserved ontologies in the progression from metaplasia to dysplasia to cancer			
Up-regulated			
Inflammatory response	0.00137	0.03085	3.36×10^{-10}
Immune response	0.00298	0.00932	6.40×10^{-08}
Cell–cell signaling	0.01318	0.01719	5.59×10^{-16}
Cell surface receptor linked signal transduction	0.01770	0.01301	NS
Proteolysis	0.03944	0.02702	0.00279
Negative regulation of immune response	0.03765	0.04098	NS
Negative regulation of blood vessel endothelial cell migration	0.04091	0.04709	NS
Down-regulated			
Oxidation reduction	0.0162682	0.0278318	3.85×10^{-07}
KEGG pathway analysis			
Cytokine–cytokine receptor interaction	0.00008	NS	2.09×10^{-10}
Cell adhesion molecules	0.00163	NS	NS
Notch signaling pathway	0.00314	NS	NS
Complement and coagulation cascades	NS	0.00118	0.00010
TGF-β signaling pathway	NS	0.00228	0.00026
Nitrogen metabolism	NS	0.00255	NS

Table shows the ontologies conserved for each pairwise comparison for metaplasia (MET) vs. dysplasia (DYS) and dysplasia vs. EAC. All these ontologies were statistically significant in the pairwise comparisons ($P < 0.05$). All of the ontologies common to both pairwise comparisons are listed. The *P* values in the fibroblast columns indicate the ontologies also statistically different in metaplasia vs. EAC fibroblasts.

comitant reduction in *TP53* mRNA (10 h vs. 8, 4, and 0 h; $P < 0.001$) but not of *PDCD4* (Fig. 3*A*).

There was a striking increase in the intensity and density of staining for TSP1 in the metaplasia–dysplasia–EAC sequence ($P < 0.001$ for both; Fig. S2*C*). We therefore analyzed the expression of TSP1 in an independent case–control cohort of patients with BE who progressed from metaplasia to cancer over a mean of 6.5 years (range, 3–13 years) compared with patients with metaplasia who did not progressed further than low-grade dysplasia over a mean of 5.5 years (range, 3–9 years). The intensity of staining in the index biopsy was statistically higher in progressors than in controls ($P = 0.004$; Fig. 3*B*). Furthermore, patients with a maximum intensity (value of 3) were statistically more likely to progress to cancer than patients with a lower intensity; relative risk of progression to cancer of 3.8 (95% confidence interval, 1.5–9.9; $P = 0.006$).

POSTN is a TGF-β–induced secreted protein that acts as an adhesion molecule and can promote cell motility (15). POSTN expression was significantly increased in primary esophageal fibroblasts compared with epithelial cell lines (Fig. 3*C*). Addition of cancer-associated fibroblasts ($P < 0.05$) or 100 ng/mL of recombinant POSTN ($P < 0.01$), but not normal esophagus- or dysplasia-derived fibroblasts, induced in vitro invasion of an esophageal cancer cell line FLO-1 (Fig. 3*D*).

Inflammation Signature Is a General Determinant of Progression in Gastrointestinal Tumors. To determine the significance of our findings in other cancers with a well-characterized preinvasive stage, we performed gene set enrichment analysis (GSEA). GSEA determines whether a defined set of genes correlates with a particular phenotype within microarray data. Hence, we compared our stromal datasets and a primary fibroblast gene set with gene-expression datasets from the following: whole samples from which our stromal microdissection was performed; three independent esophageal sample sets (16–18); and other gastrointestinal datasets from gastric (19), colonic (20), and pancreatic (21, 22) carcinogenic stages. There was an increasing enrichment, either up or down as expected, of the stromal and fibroblast gene sets in the progression from preinvasive stages toward invasive cancer (Fig. 4). The set of genes identified from the up-regulated stromal list was the most consistently enriched gene set across the different cancer types (Table S1). The pancreatic dataset by Buchholz et al., derived from microdissection epithelium, was used as a negative control. No enrichment was seen in the ductal carcinoma in situ (DCIS) to breast cancer transition. This suggests that there is a core set of stromal genes that are dysregulated in the development and progression of preinvasive disease to invasive cancer in the gastrointestinal tract.

Because of the propensity of inflammatory-related genes observed in the ontology analysis of the in-house array data (Table 1), GSEA analysis was again applied to enriched genes involved in the inflammatory response in the nine independent datasets described above. The TGF-β, cytokine, and inflammatory pathways were consistently enriched in esophageal and colon cancer datasets compared with preinvasive disease stages (Table S2). TGFβ related genes were enriched in gastric cancer compared with intestinal metaplasia of the stomach (Table S2).

Discussion

We have identified a distinct stage-specific stromal signature in BE carcinogenesis, with predominance of inflammation- and TGF-β–related genes. Increased expression of one or more of five stromal genes was associated with a poor prognosis and had functional relevance. These inflammation-related stromal genes that distinguished between preinvasive and invasive disease in our own data were also applicable to other gastrointestinal tumors.

Only one gene expression profiling study of stroma in preinvasive disease was identified in the context of breast cancer (23). However, their data may include a field effect bias because they selected different disease stages, which were derived from cancer endpoint. In contrast to our inflammatory signature, most differentially expressed genes in this study were linked to extracellular matrix proteins, matrix metalloproteases, and to the control of cell proliferation. In BE carcinogenesis, a single publication studied the stromal contribution by subtracting an epithelial cancer cell line signature from that obtained from a whole biopsy. The investigators identified a unique set of genes that distinguished BE and EAC samples from normal tissues (16). Here, we performed laser capture microdissection (LCM) of the stroma in samples from discrete stages of preinvasive disease and cancer. Because of the subjective nature of the grading of dysplasia and the heterogeneity of the disease, we

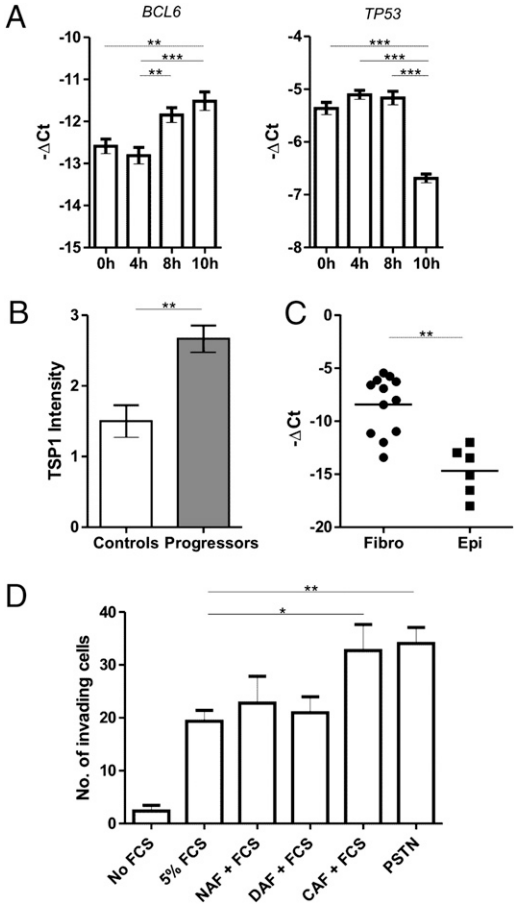

Fig. 3. Functional role of BCL6, TSP1, and PSTN. (*A*) Expression of BCL6 and TP53 in FLO-1 after stimulation with 40 ng/mL IL-6. The data are expressed as -ΔCt (ΔCt = Ct GAPDH − Ct gene of interest), and the median ± SEM is represented. $^{**}P < 0.01$; $^{***}P < 0.001$. (*B*) Intensity score of TSP1 in the index biopsy of a nested case–control study of patients with metaplasia who progressed to EAC over a mean of 6.5 years (range, 3–13 years) compared with patients with metaplasia who did not progress over a mean of 5.5 years (range, 3–9 years). $^{**}P < 0.01$. (*C*) mRNA expression measured by quantitative PCR (qPCR) of POSTN in a panel of primary esophageal fibroblasts and a panel of epithelial cells of esophageal origin (FLO-1, TE7, OE33, GihTert, Chtert, and GohTert). The data are expressed as -ΔCt (ΔCt = Ct GAPDH − Ct POSTN), and the median is represented. $^{***}P < 0.001$. (*D*) Number of cells invading through matrigel per high-power field (×400). Data are represented as mean ± SEM. Cells were treated with 5% serum (FCS), or 5% serum with 10^5 normal esophagus (NAF), dysplasia (DAF), or cancer-associated fibroblasts (CAF) were seeded in the lower chamber, or 100 ng/mL of POSTN was added to the top chamber. $^{*}P < 0.05$.

performed double-blind histopathologic diagnosis of serial sections from the same biopsies used for the expression array. Only samples homogeneous for a grade of dysplasia with a consensus diagnosis were used.

The stromally derived five-gene prognostic signature comprises *TMEPAI*, *JMY*, *TSP1*, *FAPα*, and *BCL6*. Although not all genes are prognostic on their own, the panel is informative and underlies the collaborative action of multiple genes, culminating in the development of an invasive phenotype. We also demonstrated that increased stromal expression of TSP1 was associated with a relative risk of 3.8 (95% confidence interval, 1.5–9.9%) of developing EAC compared with controls. Together with its contribution to the prognostic signature, this makes TSP1 an interesting target to study further in the establishment and development of EAC. Other key genes dysregulated in EAC compared with preinvasive BE samples included *IL-6*, *Cox-2*, *C/EBPβ*, and *POSTN*, which are in keeping with the inflammatory-related pathways ascertained from the ontology and KEGG analyses. The up-regulation of inflammation-related genes and associated ontologies fits with the idea that the inflammatory microenvironment is considered to be the seventh hallmark of cancer (2) and with previous work in our laboratory and others linking inflammation and TGF-β to BE carcinogenesis (24–27). More work is required to understand the cross-talk and combined influences of the stroma and luminal components on epithelial cell behavior in BE.

The GSEA data suggest that the genes identified in the stroma and fibroblast gene sets are following similar trends of expression in the genesis of other gastrointestinal tumors. Although we identified 12 genes relevant to Barrett's carcinogenesis, it is likely that these genes represent dysregulated processes common to other gastrointestinal tumors rather than the individual oncogenes themselves being critical. Although these datasets were from whole tissues, the stromal signature did not seem diluted by the epithelial signature because only the dataset from microdissected epithelium (22) was displaying no enrichment. However, the analysis of expression profiling from whole BE tissues from the same patients presented herein did not identify the same targets as the stromal data. Therefore microdissection of the stroma was an essential first step to identify the important pathways. Interestingly, such enrichment of esophageal stromal genes was not observed in a tested breast cancer database with normal, DCIS, invasive, and metastatic ductal samples (Table S1). Because gastrointestinal cancers (colon, stomach, and pancreas) are well known to occur in the context of inflammation (28–30), it is perhaps not surprising that there is a core set of stromal inflammatory-related genes, which are dysregulated in the progression from preinvasive disease to invasive cancer in these organs.

These data add scientific credence to the observations that anti-inflammatory drugs like aspirin may have chemopreventive potential in gastrointestinal cancers (31), and because drugs more highly targeted to specific inflammatory pathways are increasingly available, it may be possible to reverse tumor-supporting inflammation in the stroma as a new anticancer approach in the gastrointestinal tract.

Materials and Methods

A detailed description of all of the materials and methods can be found in *SI Material and Methods*.

Human Tissue Samples. Ethics committee approval was obtained for the study. All patients were consented. A breakdown of specimen types and sample sizes is in *SI Material and Methods*.

LCM, mRNA Isolation, Amplification, and Gene Expression Profiling. After microdissection of the stroma, two rounds of amplification were applied to the RNA before reverse transcription and hybridization to a 44K Agilent microarray. Transcript abundance was normalized to the Stratagene Human Universal Reference and then detrended.

Gene Expression Data Analysis. Gene expression differences between different stages of BE carcinogenesis were determined using one-way ANOVA. Only genes with more than 2-fold difference in expression between different stages were considered for further selection.

Creation of a Prognostic Panel. Junctional and esophageal tumors from 63 chemo-naïve patients were profiled, and data were normalized as described in *LCM, mRNA Isolation, Amplification, and Gene Expression Profiling* in *SI Material and Methods.* Kaplan-Meier curves were created for the 12 short-listed targets. Each target for which dysregulated expression had a trend to confer lower survival was integrated in the prognostic panel.

Ontology Analysis. Overrepresentation of gene ontology (GO) annotation terms in gene sets was determined using GeneCoDis to group related genes on the basis of GO attributes and KEGG pathway involvement.

Gene Expression Profiling of Primary Fibroblasts. RNA extracted from the fibroblast lines was processed according to Affymetrix guidelines then

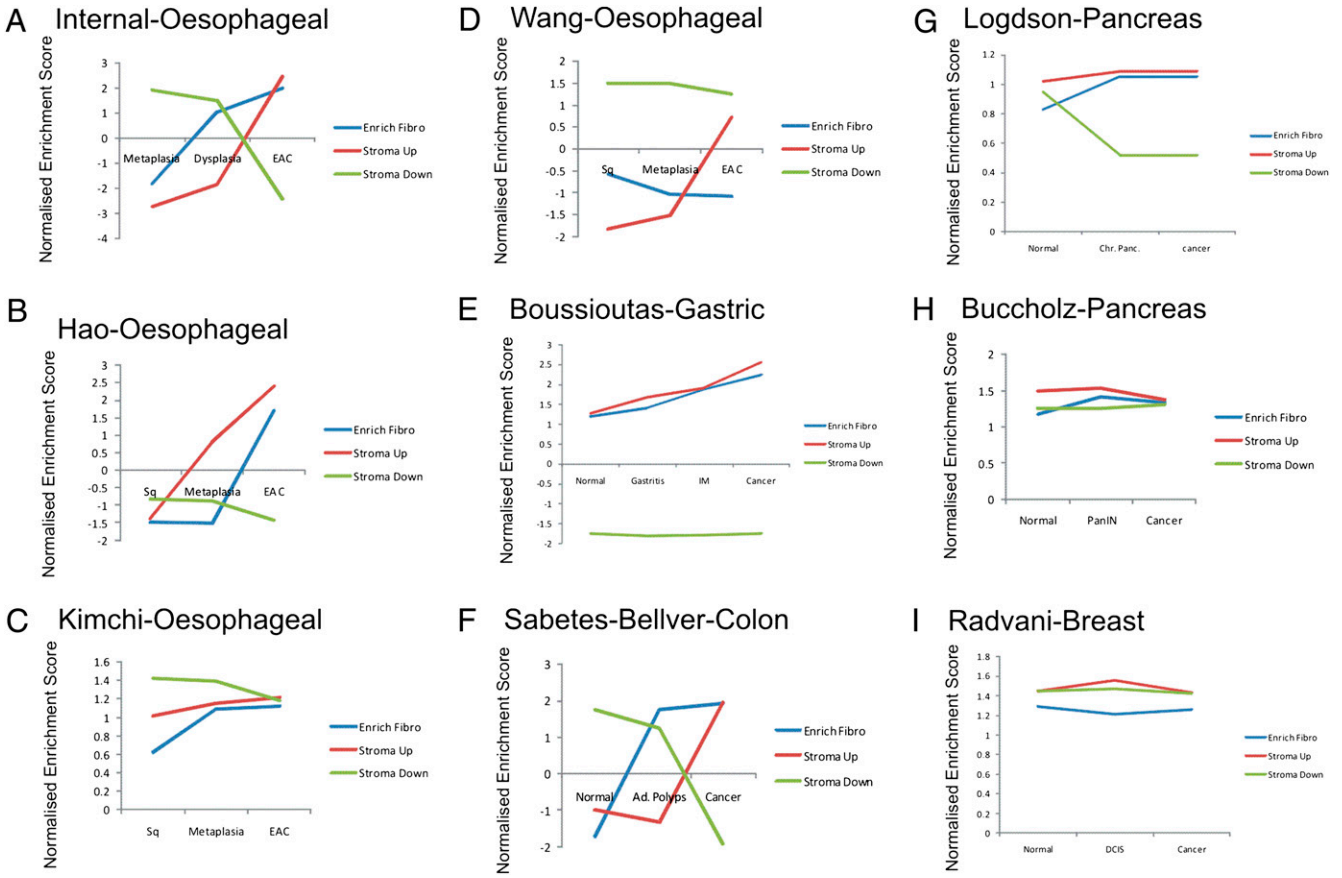

Fig. 4. Normalized enrichment score for the gene sets across progression to EAC. Normalized enrichment score for the set of up-regulated (red) and down-regulated (green) stromal genes and for the fibroblast gene set (blue) in four esophageal (*A–D*), one gastric (*E*), one colonic (*F*), two pancreatic (*G*: whole tissue; and *H*: epithelial only) and one breast (*I*).

hybridized to a GeneChip Human Gene 1.0 ST array. Data were analyzed using GeneSpringGX 9 software. Genes overexpressed in cancer-associated fibroblasts compared with BE-associated fibroblasts more than 2-fold were selected for gene enrichment analysis.

Nested Case–Control Study. Sixteen patients with BE who developed incident adenocarcinoma or high-grade dysplasia and two controls per patient were selected (cohort described in details in ref. 32). The cancer patients had at least two endoscopies before the development of high-grade dysplasia or cancer. The control cases had at least three surveillance endoscopies. The index biopsy sample was stained with TSP1.

Gene Set Enrichment Analysis. Three gene sets were generated from our microarray analyses; two from human specimens and one from primary fibroblasts. GSEA was performed using the GSEA software (http://www.broad.mit.edu/gsea/index.jsp). GSEA was performed in our in-house stromal samples and esophageal samples (from the same patients) and used to filter for genes present in both analyses at the leading edge subset. GSEA for these filtered gene-lists were run in our internal dataset as well as external datasets; esophageal (15–17), gastric (18) and colon (19), breast (33), and pancreas (20, 21).

Western Blotting, Immunostaining, Quantitative PCR, and Invasion Assays. Standards methods were used. Details can be found in *SI Material and Methods*.

Statistical Analysis of in Vitro Studies. Student's *t* test was used to identify differences between two data sets and ANOVA for multiple data sets with the Dunn's multiple comparison test as post test. $P < 0.05$ was required for significance. The Pearson *r* test was used to analyze correlation of expression of markers. Log–rank test was used to analyze the association between dysregulation of the stromal short list and survival. The Fisher exact test was used to calculate the significance of the likelihood of TSP1 intensity being higher in cases vs. controls.

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