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PRINCIPAL INVESTIGATOR: Izhak Haviv, Ph.D.

CONTRACTING ORGANIZATION: University of Melbourne
Victoria 3010, Australia

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Izhak Haviv, Ph.D.

E-mail: izhak.haviv@petermac.org

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14. ABSTRACT

The focus: Despite compelling cell biological studies and histopathological observations incriminating stromal cells in tumorigenesis, our knowledge of the genes that mediate changes in the tumor microenvironment and interactions among various cell types in epithelial cancer and their role in tumorigenesis is limited. Here, we describe a comprehensive molecular characterization of stromal-epithelial cell interactions, using microarray analysis of co-cultured cell pairs. We further show that these gene expression changes indeed are common feature of in vivo profiles of invasive epithelial cancers. Using In situ hybridization we confirmed previous assignment of which cells express which genes in response to co-culture.

We provide proof of principle evidence that the in vitro system recapitulates some aspects of cancer cell biology and may contribute new markers to the invasive events. We directly demonstrate that events in the interaction of epithelial cells and their neighboring fibroblasts play initial role in recruiting inflammatory cells and provoking immune surveillance.

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Report (according to Statement of Work) of: "the Regulation of leukocyte infiltration into ovarian cancer by tumour-stroma interactions; a microarray view of cancer microenvironment".

Introduction:

In normal tissues, a basement membrane separates epithelia and mesenchymal elements (collectively termed stroma), which support the survival of epithelial cells and maintain tissue architecture and cellular boundaries (Jacks and Weinberg, 2002). Invasion of stroma by transformed epithelial cells, a hallmark of cancer, transgresses the normal boundary that separates these cell lineages and results in abnormal heterotypic cell-cell interactions [1, 2]. The neighbors of the epithelial cancer cells, blood vessels [3-5], infiltrating immune cells [6, 7], and fibroblasts [3, 8], all exert distinguishable responses to this process (angiogenesis, inflammation, fibrosis). These responses affect cancer progression in a several ways. Some critical invasion-promoting enzymes that remodel the extracellular matrix (ECM) are secreted by stromal elements [9-11]. The fibroblasts that reside in the cancer microenvironment are phenotypically different than normal fibroblasts, as has been shown for breast and prostate cancer. They lack the ability to attenuate the growth of neighboring transformed epithelial cells [12], and have gained the ability to accelerate cancer progression [13, 14]. The mechanisms responsible for this phenotypic change may include an inherent genomic predisposition [15, 16], somatic genomic aberrations in the fibroblasts [17-19], telomere attrition [20], methylation status of some genes [21], extracellular matrix density [22] and morphological changes such as desmoplasia [23]. While the incidence of visible changes in the stroma, such as desmoplasia are well recorded [23], the incidence of the above described phenotypic and genotypic changes in fibroblasts and how they contribute to carcinoma pathology are harder to assess and discern from one another. Concurrently definitive changes in the gene expression patterns of the carcinoma fibroblasts have been observed, but the underlying mechanism is unknown [3, 24-26].

DNA microarrays are widely used to study changes in gene expression in tumors. Whilst it is clear that different cell populations differ in their gene expression [27], it is well appreciated that the changes observed in RNA isolated from whole tumors represents the summation of expression of all cellular subtypes [28]. Hypothetically, genomic predisposition, somatic

mutations, telomere attrition, and methylations changes in the fibroblast should result in unique gene expression signatures. Therefore, the presence of these unique signatures in tumour expression profiles [3, 24-26] could be used to infer the underlying fibroblast biology. The ability of the cancer fibroblast to promote or to attenuate cancer growth should also be associated with a distinct gene expression signature. If the genes unique to each of these changes were identified, they could allow us to assess the frequency of the associated biology in human cancers.

This DoD idea development project involves expression profiling of interactions between ovarian cancer epithelial cells and fibroblasts with the specific aim of identifying the underlying mechanisms of tumor-stroma interaction. Through comparing the expression response to co-culture, to those observed in hundreds of human cancer specimens, we validate the physiological relevance of our observations to human ovarian cancers. We defined an epithelial-fibroblast interaction signature, supporting previous observations [29-31], and show that this signature is the product of reciprocal exchange of soluble factors. Fibroblast-produced IL8 and EGF induce epithelial cells to produce TNFα, inducing the fibroblasts to mount an inflammatory reaction, through chemokines, tissue remodeling, and the extracellular matrix. This interaction may prove to be the primary trigger of the innate immune response (parallel to TLR-mediated responses) and the underlying mechanism of the chronic wound healing responses in tumor tissues [32]. In this report we show in situ hybridization stains of tissue sections with cancer specimens that demonstrate the same genes that fibroblasts express in response to co-culture *in vitro*, are expressed by tumor-juxtaposed fibroblasts *in vivo*. To address the physiological effect of infiltrating immune cells on ovarian cancer progression we introduced human ovarian cancer infiltrating immune cells into nude mice in xenograft assays of ovarian cancer growth. So far, these experiments have failed, due to graft versus host reaction. We are in the process of depleting mouse antigen reactive-cells from the tumor infiltrating immune cells.

Task 1. To Extend an analysis of co-culture regulated genes in other appropriate cell combinations, including cells derived from different ovarian tumor stroma and epithelium (Months 1-6):

No new developments in the fresh co-cultures, no new genes were discovered in 6 more fresh preps and co-culture assays. The assays on ovarian epithelial and fibroblastic cells from patients have continued on affymetrix U133+2 arrays, as described in previous report. However, no novel co-culture responsive genes or candidate novel signaling pathways have been suggested by the new results.

To do in the last year:

a. In order to specifically look for more interaction signaling pathways than the TNFα and the TGFβ that we found so far, and those associated with them in our system (such as IL8 and EGF), we will continue to collect viable cells from ovarian cancer tissue, but will only perform the co-culture experiment if the frozen tissue from this patient lacks the expression profile we found is correlated with our signaling in the in vivo expression profiles. The assumption is that the absence of the correlated genes indicates the absence of this interaction of the tumor cell with the stroma. It is still possible that other signaling pathways do operate in these patients, which may agree with previous co-cultured pairs we found, where the response we characterized so far was missing. Another source for novel signaling pathways between the cancer cell and the stroma, we will obtain viable cells from the peritoneum and omentum, to test whether the metastatic environment involves a different response.

b. Express ectopic CDH1 in OvCa lines with mesenchymal morphology (Hey and A2780), or pSUPER-based siRNA knock down vectors for this gene in epithelial-like OvCa lines (such as OVCAR-3). Use parental and derived cells in co-culture experiments. Score for gene expression changes by RT-PCR (Months 2-6).

c. Similar to Task 1b, explore defined cellular changes to the ovarian epithelial cell line including DNA damage and anoikis, hormone responses, etc. (Months 6-9).

d. Identify potential new candidate soluble molecules as mediators of the stroma inflammation, in case the expression of their genes are affected by the EMT. Explore those factors as in *Task 2*.

e. Microarray profile the gene expression of cultures of non-fibroblast stromal cells, when grown in serum free medium versus conditioned media conditioned by epithelial or fibroblast cultures, as well as co-cultures thereof. Tested cells will be endothelial (HUVEC), Tumor Infiltrated Lymphocytes (TIL), macrophages, Theca albea, and Granulosa cells (Months 6-12). In case the soluble molecules appear labile, we will use tissue culture inserts (as in figure 5) rather than conditioned media.

Task 2. **To identify the molecule(s) that mediate the co-culture response.**

a. Assign responder-secretor role to the cells in the co-culture. For example in point a.2.7 epithelial cells is the secretor of TNFα. The first interaction we will

focus on is the response of the macrophages to a fibroblast-derived factor, that potentiates the macrophage to recruit T cells. Here the responder is the macrophage and the secretor is the inflamed fibroblast.

In task 1 we identified responses of leukocytes to the co-culture, and identified the factors induced by the binary co-culture with the fibroblasts, mainly chemokines, that would induce a similar response in the macrophage and natural killer cells, as the co-culture conditioned media would. Interestingly, the chemokines and their corresponding response genes in the leukocytes are correlated in ovarian cancer expression profiles. We are now characterizing antibodies, antagonists, and mainly interfering RNA constructs, in attempt to abrogate the leukocyte response to co-culture. This part of the work is done in collaboration with Shaun McCall in Adelaide University, a world expert in chemokines signaling, and a lab loaded with chemokines reagents. The combinations tested are mainly focused on chemokines/cell pairs that lead to migrational effects. We are currently troubleshooting the difficulty to attain good quality array results from the leukocyte population (with and without co-culture or in response to commercial chemokines/cytokine), as our responding genes were mainly observed with real time PCR on candidates, chosen from the ovarian cancer expression profiles and the literature, such as CCL18 for macrophages.

b. Construct siRNA vectors (pRETROSUPER) for knockdown of candidate molecules (if ovarian epithelial cell is the "responder" in the co-culture, siRNA of the receptor for candidate molecule will be targeted) and transfect them into the ovarian epithelial cells, clone plasmid-carrying derivative cells. Validate that these vectors mediate expression change of their specific genes in stable ovarian epithelial cell clones (Months 7-10).

We are in the process of obtaining and managing a clone set (similar to cDNA arrays) of the Elledge/Hannon genome-wide shRNA lentivirus library[33]. This will spare us the need to clone and construct the reagents against the target gene of interest. We so far have obtained three siRNA vectors that modify the co-culture reaction in a mnner consistent with the equivalent siRNA molecules from Ambion®. Cells modified by these constructs are currently introduced into mice xenografts, with the aim of identifying the part of the co-culture response that affects cancer growth, and thus deserves further targeting.

c. Repeat this analysis for epithelial response to fibroblast that appears like an interferon-γ response (fibroblast is secretor and ovarian epithelial cell is responder), as in Task 2a-e (second year).

Yet to do during the third year. The stumble block is, that in spite of the response looking like Interferon γ, in terms of the genes induced, the commercial factor won't provoke this response. We are exploring the possibility that alternative interferons, such as interferon kappa, or combinations of the factors we have identified to accumulate in the co-culture media, such as TNFα, IL8 and IL6,

mimic an interferon response. Particularly confusing is the result that JAK1 kinase inhibitor, and siRNA knocking down of uPAR, lead to complete attenuation of the signal in the epithelial cell. We are exploring this possibility now.

d. If Task 1a-e identify new co-culture responses that are robust and hint at clinically relevant processes in the AOCS data, repeat this analysis for this new response, as in Task 2a-e (year 3).

Analysis of the time course of co-culture found TNFα is induced in 8 hours of the co-culture. We therefore tested which cytokines induce TNFα expression in epithelial cells. We found EGF+IL8 or EGF+LIF induce a 200 fold increase in TNFα in the epithelial cells. Indeed, siRNA against IL8RA attenuates the co-culture (not shown). This observation is consistent with IL8 being expressed by the fibroblasts. We are testing siRNA modified fibroblasts and epithelial cells in ovarian cancer xenograft, to see if (1) the co-culture signature is modified, or (2) cancer growth is modified, if TNFα signaling is impeded in the fibroblast, or if IL8 signaling is impeded in the epithelial cells.

Task 3. **To compare the expression pattern of key molecules that come up in co-culture in surgical specimens from CDRP project OC000109, with the aim of assessing how general our observations are, and in order to relate our observations with *in vivo* clinical features such as lymphocyte infiltration in tumors and patient survival (parallel to Task 2, after each candidate is identified).**

a. Establish as many as possible Tissue arrays with needle punch sections arrayed on glass slides.

240 of AOCS ovarian cancers and parallel tissue banks have been generated in Peter MacCallum Cancer Centre for this project.

b. Obtain antibodies against candidate molecules that are reactive and specific in Immunohistochemistry.

A drawback in the task is that many of the interesting genes are not reactive on formalin fixed material and require fresh frozen tissue. This means we stain individual specimen, rather than the high throughput of the tissue array. As an alternative we resort to Task 3c, which means ISH and we have the assay pipelined and validated many of the CRGs *in vivo* (see below).

c. Stain for candidate molecules by both in situ hybridization for RNA expression, as well as immunohistochemistry to assess correlation of expression with biological processes that are the focus of this study, such as TIL (CD3+ cells in the tumor sections), angiogenesis, inflammatory reactions, invasion, and apoptosis.

AOCS tissue arrays were stained with CRG-specific antisense probes for in situ hybridization. Sense probe showed no staining. Shown below are genes that our previous analysis assigned as fibroblast response genes. Indeed *in vivo* sections show that the genes are expressed in fibroblasts immediately juxtaposed to cancer cells.

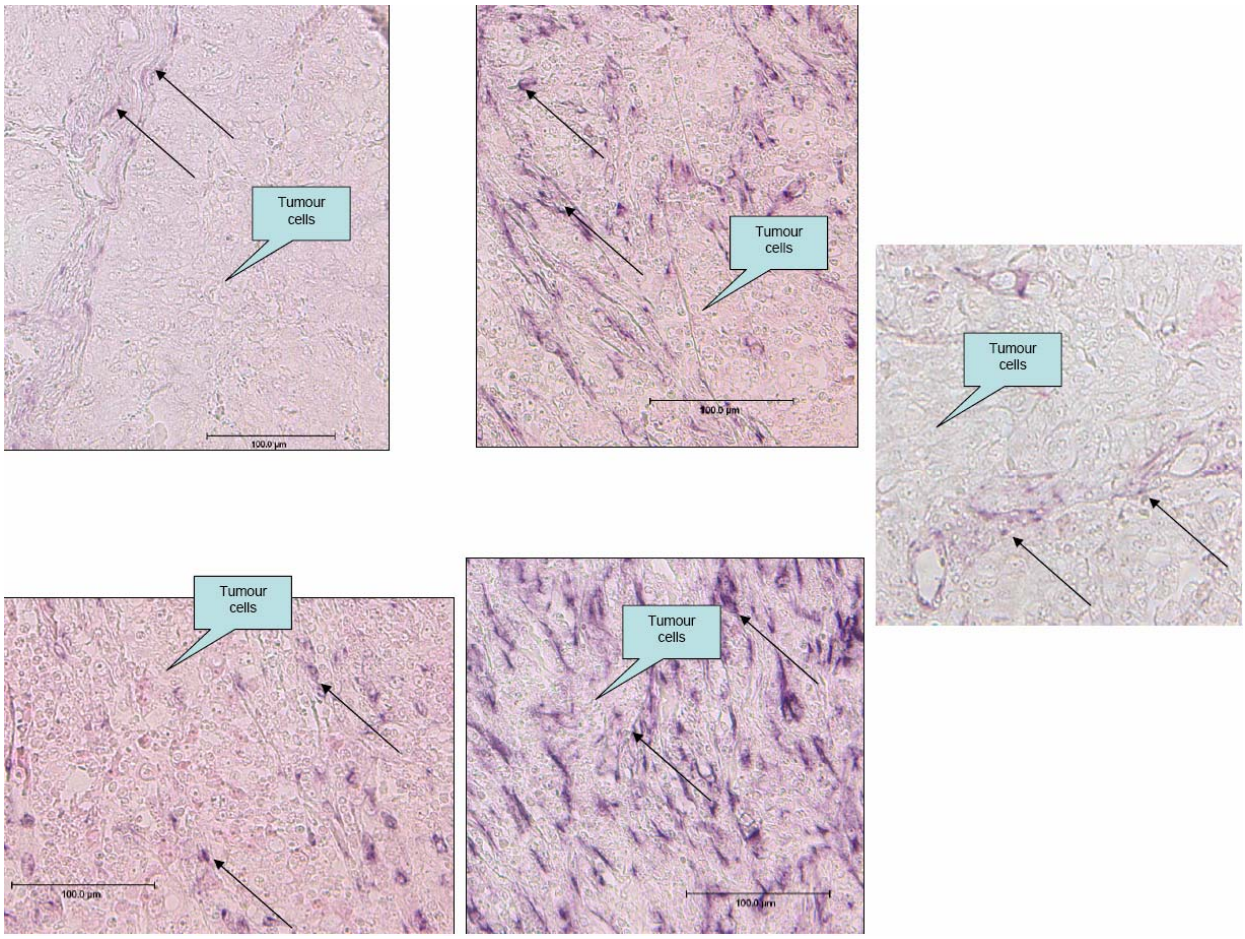

Figure 1: In situ hybridization of CRGs on cancer specimen sections, shown in clockwise sequence from top left; MMP3, TNFAIP6, INHBA, SPARC and PDGFRB. Similar results were obtained from TNC, SERPINE1 (PAI1), IL8 and OAS1. In all cases, the staining cells are almost exclusively the fibroblasts, immediately juxtaposed to cancer cells.

d. Assess our hypotheses in light of the expression associations with the known clinical data of those specimens.

To do in the last year.

***Task 4.* To characterize the biological significance of the gene expression changes observed in co-cultures and their effects on cancer progression and immune surveillance of cancers *in vitro* and *in vivo*.**

a. Use flow cytometry kits like APO-BrdU™ in CytoPerm-Cytofix ™ buffers, for the analysis of cell cycle and cell death process in cells growing in monocultures versus co-cultures. Individual cell populations are analyzed in the mixed cell populations by gating the results with CK7 as marker for epithelial cells.

Nothing new to report.

b. Construct Ectopic expression vectors for molecules that are found in Task 2 to confer the co-culture responses. Use a modified version of pBABE vectors, carrying ovarian 3 specific promoter.

Open Biosystem ® TRC and Alledge/Hannon constructs of miRNA in lentiviruses, directed against IL8RA, JNK, STAT3, IκKγ and TNFR1 are all stably transfected into fibroblasts and ovarian cancer cells.

These modified cells will be tested for the steady state mRNA level of the target genes, and knocked down colonies will be introduced into xenograft cancer model, to test the degree of synergy with the fibroblasts, in conditions, which abrogate the interaction we characterized *in vitro*. Growth curves will be performed as described in the previous report.

i. For Lymphocyte recruitment, ovarian epithelial cancer cells will be used from our own tissue bank collection. These cells have corresponding peripheral blood lymphocytes, which avoids allograft rejection reactions in the xenograft. Obtain these lymphocytes, co-inject them with the cancer cells, fibroblasts, and macrophages, all from the same patient (so far have such cell cultures from two patients).

Most xenografts performed this year focused on mice carrying human leukocytes. We found that in the absence of immune compromising drugs, the mice all die very quickly (within ten days). We are currently trying to rectify this problem by depleting mouse reactive cells from our leukocyte and TIL populations. If these immune cells become stable in the mice, we will proceed with this proposed experiment. Otherwise, we will resort to attenuating the immune response with drugs that prolong graft survival, which would be unfortunate to the aim of the experiment.

j. Follow mice for survival, tumor infiltration with lymphocytes, inflammatory reaction, cancer histology, tumor burden time course, immunohistochemistry with antibodies against co-culture induced gene products (such as CXCL9 and MMP2).

Key Research Accomplishments:

1) In situ hybridization confirmation of the co-culture response.

Reportable Outcomes:

Enclosed please find a final version of paper, in final stages of preparation before submission. This is where most of the effort is going currently, as this work is long due, and DoD deserves acknowledgement for its support.

Conclusions:

No new conclusions to share.

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Gene expression analysis of interacting fibroblast and epithelial ovarian tumor cells recapitulates a cell inflammatory signature common in cancer

Haviv, I.[1, 2], Kowalczyk, A.[3], Boussioutas, A.[1, 4], Alsop, K.[1], Tinker, A.V.[1], Tothill, R.[1], Holloway, A.[1, 2], Khavar, Y.[1], Korakis, N., Cullinane, C.[1], Robbie, M.J.[5], Thorne, N.[6], Speed, T.P.[7], Bowtell, D.D.L.[1, 2, 8]

Abstract: Gene expression analysis of a whole tumor sums the mRNA contributions of cell lineage, cell-autonomous genomic changes, and non-cell autonomous cell-matrix and cell-cell interactions. Extrapolating the latter from the reaction of cells to co-culture was used to derive intercellular signals that contribute to cross-talk between ovarian epithelial cells and fibroblasts. A reciprocal interaction, where fibroblast-derived IL8 and EGF provoke the epithelial cells to express TNFα, thus driving inflammatory gene products in the fibroblasts, was found to be a highly consistent feature of human cancer expression profiles. This work offers a compendium of experimental growth conditions and their consequential gene expression changes which may be suitable for future attempts to modulate the cancer microenvironment, by focusing on the signals that are most robust and consistent in a large fraction of cancer patients of a variety of carcinomas.

Running Title: Reciprocal interactions between fibroblastic and epithelial cells drive cancer specific expression profiles.

[1]Trescowthick Research Laboratories and the Ian Potter Centre for Cancer Genomics and Predictive Medicine, Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, VIC Australia 3002.
[2]University of Melbourne, Department of Biochemistry and Molecular Biology, Parkville, Melbourne.

[3] National Information and Computing Technology Australia Institute, Australian National University, Canberra
[4] University of Melbourne, Department of Medicine, Parkville, Melbourne.
[5] New South Wales Pathology Laboratory,
[6] Cambridge, UK
[7] Walter and Eliza Hall Institute, Parkville, Vic 3052, Australia
[8] Corresponding author, d.bowtell@petermac.org

NOTE: total article < 55,000 characters

Introduction

In normal tissues, a basement membrane separates epithelial and mesenchymal elements (collectively termed stroma), which support the survival of epithelial cells and maintain tissue architecture and cellular boundaries (Jacks and Weinberg, 2002). Invasion of stroma by transformed epithelial cells, a hallmark of cancer, transgresses the normal boundary that separates these cell lineages and results in abnormal heterotypic cell-cell interactions (Liotta et al., 1980; Yang et al., 2002). The neighbors of the epithelial cancer cells, blood vessels (Allinen et al., 2004; Bamberger and Perrett, 2002; Zurita et al., 2003), infiltrating immune cells (Haskill et al., 1982; Zhang et al., 2003), and fibroblasts (Allinen et al., 2004; Kohno et al., 1982), all exert distinguishable responses to this process (angiogenesis, inflammation, fibrosis). These responses affect cancer progression in several ways, importantly, critical invasion-promoting enzymes that remodel the extracellular matrix (ECM) are secreted by stromal elements (Hiratsuka et al., 2002; Lynch and Matrisian, 2002; Sugiyama et al., 2001). The fibroblasts that reside in the cancer microenvironment are phenotypically different than normal fibroblasts, as has been shown for breast and prostate cancer. They lack the ability to attenuate the growth of neighboring transformed epithelial cells (Sadlonova et al., 2005), and have gained the ability to accelerate cancer progression (Olumi et al., 1999; Orimo et al., 2005). Several mechanisms may be responsible for this phenotypic change in tumor related fibroblasts, including, an inherent genomic predisposition (Deshpande et al., 2005; Morgan et al., 2005), somatic genomic aberrations in the fibroblasts (Kurose et al., 2002; Moinfar et al., 2000; Tuhkanen et al., 2004), telomere attrition (Parrinello et al., 2005), changes in the methylation status of some genes (Hu et al., 2005), changes of the extracellular matrix density (Paszek et al., 2005) and morphological alterations such as desmoplasia (Madri and Carter, 1984). While the incidence of visible changes in the stroma, such as desmoplasia, are well recorded (Madri and Carter, 1984), the incidence of the above described phenotypic behavior and genotypic changes in fibroblasts and their exact contribution to carcinoma pathology are harder to assess and discern from one another. Concurrently, definitive changes in the gene expression patterns of the carcinoma fibroblasts have been observed, but the inciting mechanism remains unknown (Allinen et al., 2004; Crnogorac-Jurcevic et al., 2001; Hu et al., 2005; Parker et al., 2005).

DNA microarrays are widely used to study changes of gene expression in tumors. Whilst it is clear that different cell populations differ in their gene expression (Perou et al., 1999), it is also well appreciated that the changes observed in RNA isolated from whole tumors represent the summation of expression of all cellular subtypes (Stuart et al., 2004). Hypothetically, genomic predisposition, somatic mutations, telomere attrition, and methylation changes in the fibroblast should each result in unique gene expression signatures. Therefore, the presence of these unique signatures in tumor expression profiles (Allinen et al., 2004; Crnogorac-Jurcevic et al., 2001; Hu et al., 2005; Parker et al., 2005) could be used to infer the underlying fibroblast biology. Therefore, the ability of the cancer fibroblast to promote or to attenuate cancer growth should also be associated with a distinct gene expression signature. If the genes unique to each of these changes were identified, they could allow us to assess the frequency of the associated fibroblast biology in human cancers.

We have expression profiled tumor-stromal interactions between cancer epithelial cells and fibroblasts with the specific aim of identifying the underlying mechanisms of tumor-stroma interaction, and have compared the profiles to those observed in hundreds of human cancer specimens. We defined an epithelial-fibroblast interaction signature, supporting previous observations (Fromigue et al., 2003; Gallagher et al., 2005; Sato et al., 2004), and show that this signature is the product of reciprocal exchange of soluble factors. Fibroblast-produced IL8 and EGF induce epithelial cells to produce TNFα, inducing the fibroblasts to mount an inflammatory reaction, through chemokines, tissue remodeling, and the extracellular matrix. This interaction may prove to be the primary trigger of the innate immune response (parallel to toll like receptor-mediated responses) and the underlying mechanism of the chronic wound healing responses in tumor tissues (Dvorak, 1986).

Results:

Co-culture of ovarian epithelial tumor cells and fibroblasts results in the induction of specific gene expression

To better understand gene expression changes that are attributable to intercellular cross-talk, we compared mono-cultured cells, with co-cultured cells, by microarray gene expression profiling (Figure 1A). Equal numbers of cells were grown either in co-culture or in mono-culture for 16-24 hours. After pooling of the monoculture cells, the RNA was isolated from both the

monoculture and co-culture cell mixtures, labeled with fluorescent nucleotides and the targets competitively hybridized with cDNA microarrays (Fig. 1A). If there was no intercellular communication in co-culture, we would expect no change in gene expression (i.e. no log ratios substantially different from zero), however, we found that the expression of a large number of genes were reproducibly altered by co-culture (Table 1, column M), particularly genes involved in ECM production and remodeling, cell signaling and inflammation. The ratio of cell types in the mixed mono-cultures and co-cultures were comparable based on FACS and cell-type expression profiles (supplement 1, Supplementary Figure 1A & B). Figure 1B shows how genes were selected for further analysis, based on their fold expression changes and the consistency of these changes across experimental repeats.

Assessing the conservation of co-culture induced genes across multiple cell pairs.

Several co-culture experiments characterizing the fibroblast-epithelial interaction by gene expression analysis have been performed (Fromigue et al., 2003; Gallagher et al., 2005). In each case one fibro-epithelial cell pair was used, assuming that the gene expression response is generalizable. We have used multiple fibro-epithelial cell pairs to assess this assumption. The co-culture experiment described above was repeated using 15 ovarian cancer cell lines and one fibroblast primary cell culture. For each cell pair, co-culture and mixed mono-cultures cDNAs were prepared and compared as in Figure 1A. We performed real time PCR using the top 125 genes that were upregulated in the original experiment, described above (OAS2 and OAS3 omitted, see Supplementary Table I). Expression level ratios of each gene between co-culture and mixed-monocultures were normalized (Vandesompele et al., 2002) and used to produce a principle component analysis, measuring the degree of similarity between the cell pairs (Figure 2A), and to generate a hierarchical cluster (Figure 2B). The overall change in gene expression is also represented by box plots (Figure 2C). The median expression change in co-culture was higher than two fold for 12 of the cell pairs (Figure 2C and Supplementary Table II). By contrast, some cell pairs exhibited little gene expression change (Figure 2A and B, blue). The 56 most robust and consistently upregulated co-culture genes are marked in bold in Table 1. The same genes were found to respond to co-culture when the experiments were repeated with fibroblasts and epithelial cells that derived from disparate organs, such as skin, breast, ovarian, gastric and lung (data not shown). Of particular interest is the observation that a robust response also

occurred when non-transformed epithelial cells were co-cultured with fibroblasts (e.g. Moody human ovarian surface epithelium, Figure 2B, and other, data not shown). This suggested that the phenomenon we are modeling in the *in vitro* co-culture is relatively conserved across different types of carcinomas, and would justify analysis of these genes in expression profiles of multiple types of human cancer specimens.

Co-culture induces genes involved in tissue remodeling, inflammation, and interferon responses.

One hundred and twenty seven genes were identified that were consistently up regulated by co-culture (Table 1, column M). The upregulated genes are strikingly enriched for molecules that mediate inflammatory responses and tissue remodeling, including 13 inflammatory mediator genes, or genes reported as TNFα-responsive, such as IL6, IL8, TNFAIP3, etc. (Tian et al., 2005), 10 immune effector response genes, and 10 γ-interferon responsive genes. Multiple mediators of tissue remodeling were also expressed, *i.e.*, 6 extracellular matrix-degrading proteases, and 4 serine protease inhibitors. Conversely, 12 extracellular matrix constituents were induced, suggesting that the cells are conditioned to reconstitute the basement membrane. Annotation of the co-culture genes (http://david.niaid.nih.gov/david/ease.htm) showed an enrichment of genes participating in particular biology. The upregulated genes, listed in order of significance, were composed of response to pathogen and external stimulus, genotoxic stress, inflammatory and innate immune defense, cell-cell interaction, and tissue remodeling (Supplementary Table II). Thirty nine genes were down-regulated in response to co-culture. The down-regulated gene list annotation was enriched for the following: cell cross-talk, signal transduction, lipid metabolism, development and morphogenesis. The inflammatory gene expression changes are remarkable, considering the absence of inflammatory B-, T-cells or macrophages in this simple binary *in vitro* culture system. As 4 of the cell pairs described above involved both epithelial and fibroblast cloned cell lines, the contribution of rare immune cells in the co-cultures is highly unlikely.

The co-culture induced gene signature is elicited predominantly by soluble factors.

To better define the signals that induced the gene expression changes in co-culture, we exposed the single cell cultures of each cell type to different experimental conditions and

expression profiled (using microarrays) the response, aiming to find conditions that best mimic the co-culture response. Gene expression profiles for each experimental condition were compared to baseline expression of pure populations of the same cells in monoculture. Genes were selected based on the approach described in Figure 1B. The co-culture expression response was largely recapitulated when fibroblasts and epithelial cells were grown on opposite sides of transwell plates (Figure 3A). These results imply that a soluble factor, secreted by epithelial cells, elicits the response in the fibroblast cells. Recapitulating the co-culture response in the transwell experiment also means that the original observation was not simply based on cell type ratio bias between the two cell mixtures (see supplement 1). By contrast, the other experimental conditions such as cell density, medium exhaustion, adherence to matrix, cell attachment growth conditions, all generated expression responses that failed to mimic the co-culture response. (Supplementary Figure 1C, Supplementary Table III). These results suggested that the cross talk is driven by soluble but not contact-mediated signals.

Identifying the cell-specific gene expression response to co-culture

The transwell experiment readily identifies which gene changes in the co-culture occur in which cell type. Eighty two of the 125 genes altered in co-culture were modulated in the same direction in fibroblast cells used in the transwell experiment (Table 1, column 2F, Supplementary Figure 1C), whereas 58 of the 125 genes were altered in epithelial cells (Table 1, columns 2E and 2008). The differential response to the co-culture is also apparent at the protein level. While MMP3 is upregulated in the fibroblast, MMP2 is upregulated in the epithelial cell (Figure 3B). The MMP2 expression illustrates the advantage of the transwell system over the cell mixture, as the fibroblast constitutive expression of the gene would quench the induction observed in the epithelial cells. Comparable expression change in the epithelial and fibroblast cell, for MMP2 and MMP3, respectively, gave different ratios of response in the initial experiment, since MMP2 is constitutively expressed in the fibroblasts, while MMP3 is not. The contribution of stroma to carcinoma invasion through MMPs is well characterized (Jessani et al., 2004). These results demonstrate that direct epithelial-fibroblast interactions, is sufficient to induce the stromal production of these enzymes in a manner reminiscent of cancer stroma.

TNFα mediates the co-culture response in fibroblasts.

A major advantage of our experimental approach stems from the potential to identify the basic inductive signal. In order to identify the factors involved in the cross-talk, we first tested the ability of different cytokines to elicit fibroblasts responses that were consistent with those seen in the transwell experiments (Figure 4). Using ELISA blot analysis of conditioned media and comparative expression profiling of cancer cell cultures between co-culture-reactive and non-reactive lines, we selected 20 secreted factors as candidate mediators of the epithelial to fibroblast signaling (Supplement 2, Supplementary Figure 2A and B). We added the candidate cytokines to fibroblast monocultures and followed their gene expression response using quantitative real-time PCR (Figure 4A, cyan treatments). The data is represented in normalized fold expression change, as compared to serum free media. As reference, we included a number of the characterized co-culture responses so that similar responses would co-cluster (Figure 4A, columns 1, 2, 6-8 black treatments). We found that the fibroblast-epithelial cell co-culture signature was most closely recapitulated when the fibroblasts were treated with epithelial conditioned medium (column 3), or recombinant IL1β or TNFα proteins (columns 4-5). Not all of the co-culture responsive genes were useful in identifying the driving factor. For example, RAB31 and some of the adjacent genes are induced promiscuously, while INFγ responsive genes (e.g. STAT1, CXCL9, MX1, IFITM1, OAS1, etc.) are almost exclusively expressed in response to co-culture. These results suggest that either IL1β or TNFα is sufficient to reproduce the majority of the fibroblast response to the co-culture.

To test whether IL1β or TNFα signaling in the fibroblast is necessary for the co-culture response, we added neutralizing antibodies against TNFα to the co-culture experiments (as in Figure 1A). Anti-TNFα antibodies specifically inhibited the co-culture response, while antibodies against TGFβ did not (supplementary Figure 1C). To further substantiate this observation, we introduced siRNA molecules to the fibroblasts, and tested these knock down fibroblasts in co-culture reactions (as in Figure 2) with ovarian cancer cell line 2008 (Figure 4B, red treatments). The genes that were promiscuous were not included in this analysis. Knock down of essential TNFα-signaling components, NIK (IκKγ, column 17) and TNFR1 (column 16), in fibroblasts resulted in a grossly attenuated co-culture responses. Knock-down of the IL1R1 (column 13), MAP3K14 (column 14), and STAT3 (column 15) attenuated the co-culture response to a lesser extent, while irrelevant siRNA molecules (IL18R, MMP26, SMAD4 and CCR1) did not affect the co-culture response (columns 2-5). Since the IL1β gene is itself co-

culture responsive, it is hard to distinguish downstream effects from a primary driving role. However, the siRNA results with IL1R1 suggest that IL1β is indeed a secondary product of the co-culture, rather than the primary driver. These results suggested that TNFα or a TNFα-like molecule secreted by the epithelial cells drives a robust inflammatory response in fibroblasts. This conclusion is further substantiated by analysis of the human cancer expression profiles (supplement 4).

Time course of the co-culture response.

TNFα protein levels were increased in co-culture conditioned media compared to the mono-culture conditioned medium (supplementary figure 2A). Since TNFα mRNA is itself not co-culture responsive at the 24 hour time point, we aimed to identify the time point at which TNFα mRNA is first induced (assuming mRNA accumulation drives the protein accumulation). We therefore profiled the time course dynamic of the co-culture response for the 2008 cell pair (Figure 5A). Genes that are upregulated or down-regulated at the 24 hour time point are marked by red and green lines, respectively. The TNFα mRNA level increased eighteen fold in the co-culture at the 8 hour time point (Figure 5A, bold line). By 24 hours, TNFα is no longer differentially expressed. TNFα mRNA accumulation occurred in the epithelial cell at 8 hours, at a time point preceding the TNFα-driven co-culture response in the fibroblast at 24 hours. This is consistent with the assumption of reciprocal dynamic interaction initiated by a signaling factor constitutively secreted by the fibroblast cell.

Identifying upstream regulators of TNFα expression.

To investigate the factor(s) responsible for inducing the the co-culture regulated gene signature in the epithelial cells, (especially MCSF, IL1β and TNFα), we profiled the response of epithelial mono-cultures to soluble factors using real time PCR (Figure 5B). Candidate soluble factors were based on genes in the published literature that mediate mesenchymal signaling to the epithelial cell, and the ELISA results with fibroblast conditioned media (Supplementary Figure 2). TNFα was upregulated 228-fold in response to combined treatment with either IL8 with EGF (column 3), or LIF with EGF (column 4). TNFα had induced its own expression (column 5), while neither IL8 (column6), LIF (column 7) or other candidates (columns 8-14)

could induce TNFα expression by themselves. The response of epithelial cells to TNF involves an inducible autocrine cascade (Janes et al., 2006). Furthermore, siRNA-mediated knock down of gp130/IL6ST (column 15), uPAR (column 16) or SMAD4 (column 17) did not reduce TNFα upregulation in the co-culture, while the knock down of STAT3 (column 18), IFNγR1 (column 19) or IL8 receptor (column 20) eliminated the TNFα response to co-culture. These results demonstrate that IL8, in the presence of EGF, is necessary and sufficient to induce TNFα in the epithelial cell.

Epithelial-Fibroblast expression signature is recapitulated in human cancer.

It is conceivable that the expression signature described above, and the signaling that leads to this coordinate gene expression change, is unique to the experimental cell culture system in which it was defined and not representative of the epithelial-fibroblast interaction response in cancer. To address the "coherence" of the genes induced in the *in vitro* experiment, in terms of their expression *in vivo* (i.e. the degree to which they are co-expressed in human cancers), we examined expression profiles of 174 carcinoma profiles (Su et al., 2001). For this analysis we defined a “query” using the 54 genes, (rationale in supplement 3) that were strongly and frequently upregulation in co-culture (Table 1, bold genes). We ranked genes according to how strongly they correlate with each gene of the query list (pearson correlation), and calculated the quartiles and mean of the ranks of the remaining 53 genes on the list. Figure 6A is a density plot that draws the quartiles and mean like topological curves along a matrix of the U95A human gene list, where each position on the X-axis defines one of the 54 query genes, according to which the rest of the U95A genes are ranked in the Y-axis. The lower the mean and quartile are along the (Y-axis) whole genome percentile, the more significant the coexpression of the overall 54-gene query list is with this gene. The 54 parallel ranks are ordered in increasing mean-rank order. By repeating this procedure for 80,000 random gene lists of equivalent size, we found that the mean at each position never exceeded that of the co-culture query list until the last gene. A typical random gene list distributes the mean between the 40-60% bars (Figure 6B). Fifty three gene from the cell-cycle module (Iyer et al., 1999) were even more tightly co-expressed than the co-culture genes (Figure 6C), only for the top 60% of the cell cycle query list.

Defining the genes that constitute the epithelial-fibroblast interaction signature in human cancer.

One of the most important uses of whole-genome expression data is the discovery of new genes with similar function to a given list of genes (the query) already known to have closely related function. Genes that show tight levels of co-expression in a large number of diverse microarray experiments are likely to function together (Eisen et al., 1998), either through direct protein-protein interactions (Rhodes et al., 2005), by directly controlling the expression of one another (Harbison et al., 2004; Long et al., 2004), or by participating in biological processes that are coordinated (Cam et al., 2004), possibly occurring in two neighboring cells types in the tissue (Becker et al., 1997). The binary co-culture *in vitro* experiment is an over-simplified model of the interactions that occur in the tumor microenvironment. The gene recommender algorithm ranks genes according to how strongly they correlate with a set of query genes. Using the same 54 gene query described above, we extended our investigations beyond the binary cross-talk and the conditions used *in vitro* to tightly linked events *in vivo*. We used the gene recommender algorithm (Owen et al., 2003), with the 54 genes query to study the expression profiles of three published datasets with a total of 712 primary human tumors (Ramaswamy et al., 2001; Su et al., 2001; Tothill et al., 2005), from 24 different organs and of different histological subtypes. Gene recommender output genes, *i.e.* the genes that most strongly correlated with the *in vitro* co-culture query list, are ranked for each of the published datasets independently (Supplementary Table II). A high degree of coherence was observed between the top ranked lists in the *in vitro* system and the *in vivo* data. This response was also conserved between independent *in vivo* datasets (colored genes). The gene recommender output was comparable when the query list was substituted with genes derived from two recently published independent epithelial-fibroblast co-culture expression profiling studies (Fromigue et al., 2003; Gallagher et al., 2005),(data not shown). Many of the top ranked genes are bone fide immune cell markers, such as CD11c, CD68, CD3, NK4, for dendritic cells, T-lymphocytes and natural killer cells, respectively. This observation suggests that the tissue where the query genes are most strongly expressed contained prevalent immune cell infiltrates. This is consistent with our *in vitro* observation that the epithelial-fibroblast interaction triggers the expression of potent chemokines which have been shown to effectively trigger immune cell infiltration (Curiel et al., 2004; Milliken et al., 2002; Zhang et al., 2003). Also prevalent in the genes correlated with the query are proteases,

suggesting that the induction of the co-culture responsive genes in human tumors is driven by disruption of normal tissue architecture and basement membrane components. We show here that the response of epithelial and fibroblast cells to simple commingling in *in vitro* co-culture is reminiscent of proteolytic-enzyme rich tissues *in vivo*. This response, in turn, induces further proteases, such as MMP1. Based on these observations, we propose the term Tissue Injury Response (TIR) to the gene signature we identified in co-culture.

Two types of wound healing in cancers.

A recent attempt to identify a common feature in human cancer expression profiles suggested that serum response of fibroblasts recapitulates a consistent aspect of cancer expression profiles and further suggested that this response also occurs during wound healing (Chang et al., 2005; Chang et al., 2004). However, although referred to as a wound healing signature, the DAVID annotation for this published fibroblast response to serum is mainly composed of cell growth and proliferation. By contrast, the TIR genes bear no resemblance to cell cycle or cell growth and proliferation signatures. We clustered expression profiles from gastric cancers focusing on gene recommender-derived genes of the two gene signatures; the published wound healing response and the TIR. Indeed, the two gene expression signatures exhibited elevated expression only in some of the patient specimens (Figure 6C). More recently, the wound healing response was linked to two genome aberrations in the tumor cells, MYC and CSN5 (Adler et al., 2006), which is in agreement with the DAVID annotation of their list, i.e. representing epithelial cell growth (MYC), rather than reactive fibroblasts. This figure illustrates that the genes derived from the serum response of fibroblasts, and the TIR genes, are both tightly coordinated expression responses, yet not related to each other and with very little cross over.

Discussion:

Genomics has the potential to revolutionize the diagnosis and management of cancer by offering an unprecedented comprehensive view of the molecular underpinnings of pathology. Computational analysis is essential to transform the masses of generated data into a mechanistic understanding of disease. We hypothesized that the introduction of microarray gene expression measurements from distinct, highly defined and controlled *in vitro* experiments will facilitate the analysis and interpretation of human tumor expression profiles, and will allow the inference of biological mechanisms from expression patterns.

Recent publications produce compelling evidence of the important contribution of cancer microenvironment to cancer pathology and phenotypes. A number of mechanisms have been proposed to explain how the inflamed stroma, without itself undergoing transformation, may contribute to the transformation process of epithelial cells. When tested in animal models, genetic perturbation of some critical signaling pathways in the stroma can lead to carcinoma (Bhowmick et al., 2004; Campisi, 2005; Greten et al., 2004; Hagemann et al., 2004; Hiratsuka et al., 2002; Pikarsky et al., 2004; Robinson et al., 2003; Weaver et al., 2002). The principle behind each of these models of stromal perturbation is based on modification of inflammation, ECM production or remodeling. For example, knock out mice of TNFα and NFκB (essential for TNFα-response) attenuate cancer formation in animal models of chronic infection (Greten et al., 2004; Pikarsky et al., 2004). Using expression profiling of both experimental models and human cancer specimens, we showed that one can define tumor-host signaling events *in vitro* and measure their frequency of occurrence in cancer patient specimens. In particular, this work focuses on the spontaneous response of epithelial and fibroblast cells to their co-culture, irrespective of the transformation status of the cells (normal cell types produce a similar response). The epithelial-fibroblast interaction *in vitro* induced the same gene groups as observed in the stromal genetic mutants studied in animal models (inflammation, cell signaling and ECM production and remodeling). In addition, siRNA-mediated knock down of critical TNFα-signaling, attenuated the gene expression provoked by the co-culture. It has been suggested that the context by which mutations in stroma lead to carcinoma is perturbation of the epithelial-stromal communication (Jacks and Weinberg, 2002; Littlepage et al., 2005). Our results provide evidence that supports this suggestion.

Although inflammatory signals are highly associated with tissue injury, the mechanisms of induction of IL1β and TNFα, master regulators of inflammation, have mainly been studied using bacterial wall-derived irritants, such as lipopolysaccharide. The high prevalence of immune recruiting chemokines in the co-culture, as well as the tight association of the co-culture signature with the leukocyte infiltrate component of tumor profiles, suggests that *in vivo* immune cells would respond to the chemokine signal by infiltrating into the corresponding tissue. This model suggests that the initiation of innate immunity and inflammatory response is governed by altering the epithelial-fibroblast interactions, ostensibly as a result of basement membrane breakdown and wounding. The dynamic reciprocal exchange of factors between the mesenchymal and ectodermal cell layers we identified leads to a transient increase in gene expression. Most of the identified genes are no longer differentially expressed by 48 hours. Matrix metalloproteinases (MMP) were amongst the genes most strongly induced by co-culture (Table, column 1). MMP enzymes have been implicated in cancer metastasis and invasion (Lynch and Matrisian, 2002; Matrisian et al., 2001; Zucker et al., 2000). We also found induction of active MMP enzymes *in vitro* (data not shown). The proteases probably induce the transient response observed in the *in vitro* co-culture by bringing the epithelial and fibroblast cells together. Considering the transient nature of the response *in vitro*, it is likely that the elevated levels of correlated genes in the cancer tissue are the result of a steady state of tissue breakdown and transient repair response, suggesting that cancer is like a chronic wound (Dvorak, 1986). Indeed the essence of cancer invasion is continuous protease mediated tissue degradation. The role of tissue architecture in cancer progression, and the role of immune surveillance (especially in ovarian cancer) are well established. We show here that the two processes are coordinated. Consequently, the role of TNFα in cancer promotion (Luo et al., 2004; Pikarsky et al., 2004), including in ovarian cancers (Lancaster et al., 2004; Pohl et al., 2005) is restricted to chronic inflammation, as the transient reaction will not lead to cancer.

Recent gene knockouts established that Amphiregulin (a ligand of the EGFR family) likely provides the epithelial-stromal signal required for normal mammary ductal morphogenesis (Sternlicht et al., 2005; Troyer and Lee, 2001) and protection from intestinal damage (Lee et al., 2004). In addition, senescent fibroblasts, which lead to carcinoma (Campisi, 2005), facilitate epithelial prostate transformation through Amphiregulin (Bavik et al., 2006). Interestingly, senescent fibroblasts express elevated levels of IL8 and MMPs (Parrinello et al., 2005),

suggesting that the transient response we observed in co-culture may be constantly firing in the senescent fibroblast irrespective of their neighboring cells. TNFα, IL8 and EGFR-signaling pathways all take part in the inflammatory response. We have show here that EGFR family signaling together with IL8 leads to atypical expression of TNFα by the epithelial cell, and consequently induces fibroblast gene expression, typical to inflammation.

Confounding the research of tumor-stroma interaction are the paradoxical effects that the stroma can have on cancer epithelium: stromal cells are reported to suppress (Kohno et al., 1982; Sugiyama et al., 2001) and enhance (Camps et al., 1990; Moore et al., 1999; Olumi et al., 1999) malignancy. Variations in human genome sequences modulate the susceptibility to cancer and other diseases. Deciphering the extent of stromal contribution to cancer progression and inferring the relative contribution of different signaling pathways involved in communication between the stroma and epithelium is limited by a lack of knowledge about the key locus of variation among individual patients' genomes. With the release of HapMap (Cheung et al., 2005), we predict that haplotype differences in the constituents that mediate the co-culture response would lead to variation of the response through hyperactive or hypoactive signaling (Chen et al., 2006; Guo et al., 2005; Kang et al., 2006; Liu et al., 2005; Seifart et al., 2005; Shih et al., 2006; Zecevic et al., 2006). Therefore, the ability to correlate the expression patterns of genes involved in the discrete aspects of tumor-stroma interaction to cancer promoting and attenuating effects may help to resolve the controversy. For example, genes like CCL18 (Leung et al., 2004) and CXCL9 (Zhang et al., 2003) are predictive of improved patient outcome, whereas CXCL12 (Oonakahara et al., 2004) and CCL22 (Curiel et al., 2004) are associated with worse outcome. The co-culture response of epithelial and fibroblastic cells displayed significant variation in the expression of these genes, across multiple isolates, in a manner similar to the expression profile variation of human cancer specimens. Specifically, CXCL9 was induced in the co-cultured epithelial cells in a frequency similar to that observed in patient specimens by Zhang et al. (53% in our assays and 58% in the published data). Identifying the upstream events that control such genes may help resolve the contention over the stromal effect on epithelial cancers. We identified soluble factors that were necessary and sufficient for the *in vitro* co-culture response, including TNFα, IL8, and EGF-like ligands, thereby making these the first candidates to test.

The explosion of information about the molecular pathways of cell transformation has led to several highly successful targeted approaches to cancer treatment, such as the recent success

of imatinib (Gleevec), a tyrosine kinase inhibitor, for treating chronic myelogenous leukemia. The “central dogma” of cancer research is that mutations in oncogenes and tumor suppressor genes converge into a downstream decision of a cell whether or not to divide. However, assuming that the levels of gene expression indicate the degree of activity of a given biological program, then the fact that many cancer tissue do not upregulate cell cycle controlled genes suggests that cell cycle is not the be all and end all of cancer. In particular as presented in Figure 6, many gastric cancer specimens do not express higher levels of cell cycle genes than non-malignant tissues. Thus, not all cancers depend on deregulated cell cycle, division and growth. Rather, some cancers upregulate the expression of the genes induced in the co-culture reaction as we described it (the TIR expression module). Co-regulated expression modules, highly resembling the TIR expression module we observed, have been described in a high throughput analysis of cancer expression profiles (Segal et al., 2005; Segal et al., 2004), as well as in ovarian, breast and gastric cancer datasets (Boussioutas *et al*, in preparation). Recent publications demonstrate that chemokines (Curiel et al., 2004; Zhang et al., 2003), COL6A3 (Sherman-Baust et al., 2003; Simojoki et al., 2003), as well as the expression module that overlaps with our TIR expression module (Spentzos et al., 2004), are all predictive of ovarian cancer survival. Thus, understanding the factors outside the cancer cell that drive it to invasion and metastasis or keep it contained could provide an entirely new avenue of therapy. An avenue that carcinoma cells may find harder to evade, and may prove relevant to a larger fraction of patients than current targeted therapy approaches, particularly for patients that do not respond to standard cytotoxic treatments (Sherman-Baust et al., 2003).

Experimental Procedures

Culturing cells from patients: For Normal Ovarian Surface Epithelial (NOSE), we used cervical brush scrapping of the ovary, and cultured them directly. For Ovarian Stroma, 0.5x0.5x1 cm Ovarian tissue was collected from consented women, in DMEM with 10% FCS from theatre in Monash Medical Centre, and sent to us promptly, minced with razor blade, washed in Trypsine-Versen, and then incubated for 2-6 hours in 37ºC in 10 ml (per 0.25 gr tissue) of RPMI with 3 mg/ml Collagenase II (Wortington®), 24 g/ml DNase I (Boehringer Manheim®). When minced pieces homogenize, the cells are washed three times in PBS with 2 % FCS, and plated in a culture flask with fresh medium (Flask precoated with BD Collagen I®). If medium contains Gentamycin, it will select for NOSE cells, if not, Stroma cells will prevail by default. This notion was confirmed by FACS staining with anti-cytokeratin and Anti Epithelial Membrane Antigen, or by microarray profile.

Basic (plastic) Co-culture: Stroma cells are plated in 50% confluence and allowed to reach 80% confluence (24-48 hours). Cells are washed twice with PBS, and overlayed with fresh Serum Free Medium (RPMI, 25mM HEPPES, supplemented with Insulin, Transferin, Albumin, and Selenium from SIGMA®). After 48 hours, epithelial cells from 80% confluence are trypsinized, washed twice in serum free medium plus Soy bean trypsin inhibitor, and plated in fresh SFM, either on top of Stroma flask, or in new plastic dish (equivalent amount of Stroma cultured are simply given fresh SFM). After varying time, for the stroma of the experiments 16 hours, cells are trypsin-harvested, washed, flash frozen, and subnjected to RNA preparation, using Triazol (Invitrogen®) followed by RNeasy (Qiagen®). Isolated Stroma and epithelial cultures are pooled prior to RNA preparation (E+S). RNA is harvested from co-cultures of Stroma and epithelium (ES).

Conditioned medium experiments:

Stromal and OSE cells were cultured to >70% confluency, washed twice with PBS, and growth was continued for 48 hours to produce the corresponding conditioned media in SFM. Conditioned media were filtered through 0.2 PVDF membrane (WHATMAN®). Fresh cells, Stromal or epithelial were plated and serum starved as above, and then fresh SFM or SFM supplemented with 5 fold dilution of their own or their counterpart cell CM, or with commercial recombinant TGF , bFGF (Sigma ®) or TNF (BD®) in 20 ng/ml concentration. Cells grow in the new medium for 24 hours, harvested and processed as above.

Contact dependent cues assays:

To identify co-culture regulated genes that are direct membrane protein interactions-dependent, we performed the co-culture experiment, using tissue culture inserts (0.4 M pores NC, Corning®) with Stromal cells first plated on upper side of the insert, and then Epithelial cells plated either on the same side (contact allowed), or on the bottom side (only soluble factors allowed). The two co-cultures are directly compared to each other in a microarray experiment.

ECM signal assays:

Collagen I, Matrigel, human normal ECM (all from BD®) were added to SFM in 50 g/ml concentration and used to overlay T175 culture flasks (Corning® filter cap) overnight. The next day, the flasks were washed with PBS and OSE cells were plated and grown in either fresh un-coated flasks, or the coated flasks. After 24 of growth, cells were harvested and processed as above.

Organotypic cultures:

Rat tail Collagen I (BD®) is mixed with 50% serum 50% RPMI medium, neutralized and allowed to gel on the bottom of a tissue culture insert (0.4μ Fibrobalsts are mixed with Rat tail Collagen I, neutralized and plated on culture dish inserts. Stromal cells plated in collagen 1 (Carter et al., 1990) proliferate and metabolise the extracellular matrix. We allowed the Stroma to grow for a week, while the gel shrinks. Epithelial

Arrays: cDNA arrays were processed as published(Tothill et al., 2005). Affymetrix® U133+2 arrays were processed according to manufacturers' instructions.

Real time PCR:

Twenty seven genes were scored by both primer sets designed in house, using SYBR-green as well as TaqMan™ primer sets from ABI®. The independent primer sets showed average correlation of R=0.85 with each other and R=0.71 with the array results.

The response to cell density was measured by comparing cultures of 25% and 90% confluence. The response to medium Exhaustion was tested by comparing cultures grown in media from 48 hour cultures, with fresh medium. The response to adherence to extra cellular matrix components was tested by comparing cells grown on plastic flasks, with cells grown on top of flasks, which were pre-coated with collagen, matrigel, and fresh extracellular matrix preparations from the fibroblasts we used for co-cultures. The response to attachment altogether was tested by growing cells in RGD peptides with 0.1mM EDTA in medium, with cells plated on the same time into normal growth conditions, and by plating cell onto agar coated plates.

Supplement 1: Methods of confirming that mixed cell population compared on expression profiling are not systematically biased towards one cell type.

Specific detection of co-culture regulated genes in the experiment depicted in Figure 1B relies on there being equivalent numbers of each cell type in the co-culture compared with the pooled monoculture. Due to the robust expression differences between epithelial and fibroblast cells, small differences in epithelial or fibroblast cell number between the co-culture and the pooled monoculture would be expected to result in lineage specific genes being incorrectly identified as co-culture regulated. To assess whether cell bias contributed to our findings, we performed FACS analysis with epithelial-specific marker, CK7. This demonstrated that equivalent numbers of each cell type were present in the samples used for RNA preparation (Supplementary Figure 1A). Furthermore, we reasoned that if the genes which are apparently induced by co-culture were an artifact of cell bias, then all co-culture genes should simply be epithelial or fibroblast specific (depending upon which cell type predominated). We therefore competitively hybridized RNA derived from epithelial and fibroblast cultures to identify cell specific gene expression (Supplementary Figure 1B) and plotted log ratio values against average log intensity (M versus A plot; (Dudoit et al., 2002)). Genes that were most differentially expressed in co-culture were highlighted (Supplementary Figure 1B, red triangles). Although many of the genes induced were normally more strongly expressed in fibroblasts they were surrounded by a cloud of fibroblast specific genes that do not change. Conversely, a number of induced genes are more strongly expressed in epithelial cells. These conclusions are supported by the recapitulation of co-culture response in the transwell experiments, which compare homogenous cell populations.

Supplement 2: Choosing candidate factors for Figures 5 and 6.

We assumed the factor that induces this response in the fibroblast would be expressed by the epithelial cell. Candidate factors were first chosen based on differential expression among the two cell types. TNFα mRNA was four fold more epithelial specific, so was TGFβ3, and InhibinA. To identify additional candidates, we harvested conditioned media from epithelial and fibroblast cells and examined proteins that accumulate in those media using ELISA assays on Chemicon® human cytokine antibody arrays (Chemiarrays™ V, VI and VII) against 120 cytokines, and growth factors. We detected IL6, IL8, MCP1 in the fibroblast cells, and EGF, and

TNFα in the 2008 ovarian epithelial cancer cells (Supplementary Figure 2A). An epithelial secreted molecule can also enzymatically activate a cytokine that is expressed by the fibroblasts. For example, MMP26 and MMP7, both known to activate TNFα are 64 and 16 fold up-regulated by the epithelial cell. Typical TNF response genes were induced in the co-culture and the transwell experiments, but not in the epithelial conditioned media treatment of fibroblasts. Conversely, the genes elicited by the transwell in epithelial cells are typical IFNγ response genes. This suggests a dynamic reciprocal interaction occurs in the co-culture and transwell experiments. We therefore analysed the factors accumulating in mono-cultures as well as co-cultures (Supplementary Figure 2A). Consistent with the expression array, OPG/TNFRSF11 and FGF4 were reduced in co-culture, while TNFα protein was only detected in co-culture media.
Lastly, to identify additional candidates, we screened for genes that are specifically expressed in cell lines that evoke a more robust co-culture reaction. We used HG_U133_plus_2 Affymetrix arrays to expression profile compare A2780, SKOV3, DOV13, OVCAR3 (none responders), with 2008 and OVHS (Supplementary Figure 2B). For example, Il18 and ICE/Casp1 (activates IL1β) were 700 fold differentially expressed among cell lines 2008, OVHS, which evoke the co-culture response, and cell lines OVCAR3, DOV13, and A2780 which do not respond to the co-culture. Responders expressed significantly higher levels of Amphiregulin, Epiregulin (EGF-like ErbB ligand), MAP4K5, NIK, and MLK3 (signaling kinases activating NFκB), than non-responder cell lines. By contrast, a co-culture induced gene that may serve to insulate the epithelial-mesenchymal interaction *in vivo* (in the basement membrane) CSPG2 was expressed 40-80 fold lower in the co-culture reactive cell lines. This could also reflect the activity of an autocrine signaling, such as WNT-pathway. Additional criterion to select candidate factors was provided by the *in vivo* cancer specimen expression profiles. Secreted factor genes that are consistently correlated with the co-culture response in the patient profiles (Supplementary TableII) were considered candidates and tested (BMP1, INHBA, CCL2, MCSF, CCL5 and uPA).

Supplement 3: Rational behind the choice of 54 genes for the gene recommender algorithm.

The expression response to co-culture, as most expression responses, included genes that were upregulated as well as genes that were down-regulated. In seeking genes that are consistent with the overall gene signature, it is complicated to include both upregulated as well as the

down-regulated genes, as these would be inversely correlated with each other. Therefore, we focused our analysis on "bait" gene list that was composed of only the genes that were upregulated, and robustly upregulated. When comparing our work with cDNA arrays, it became apparent that some genes, such as MMP3 are robust co-culture responders, yet, in the human cancer expression data, these genes are virtually only expressed in the tumors, which complicates the numerical analysis of Cy3 to Cy5 ratios. Such genes were also excluded.

Supplement 4: *In vivo* expression patterns of the co-culture responsive genes are correlated with TNFα response genes.

Recently, the physiological relevance of a transcriptional signatures, defined initially in cells *in vitro*, was tested by analysis microarray data from hundreds of tumor specimens (Lamb et al., 2003). For this analysis we used the co-culture responsive gene list described in supplement 3. A pattern of differential expression of any given gene across multiple carcinoma profiles, is used for ranking genes according to their corresponding expression correlation to one query gene, such as the one shown in Supplementary Figure 2C. To substantiate our conclusion that TNFα-like signal is responsible for the majority of the co-culture reaction, we analyzed the *in vivo* cancer specimen profiles as in Figure 3A and 3B and Supplemental Table II (Supplementary Figure 2D). To support our conclusion that a TNFα-like signal is responsible for the majority of the co-culture reaction, we analyzed the *in vivo* cancer specimen profiles in the same way as we did in the analysis leading to Figures 3A and 3B and Supplemental Table II (Supplementary Figure 2D). Our hypothesis now is that co-culture genes as a whole are much more strongly associated with TNFα-response genes than TGFβ-response genes. Proceeding as we did in our discussion above of the co-culture gene set in relation to the 226 ovarian cancer specimens, here we used the 229 tumor set (Tothill et al., 2005) and four rankings of genes on the array according to their correlation with 2 TNFα-responsive genes, TNFAIP3 and DUSP5, and with 2 TGFβ-responsive genes, TGFBI and TGFB1I4 (Supplementary Figures C and D). The selection of these representative genes was based on our own expression profiling of TNFα and TGFβ responses (Supp Fig 1C and Supp Table III) using anova between the two profiles. We then commuted the Wilcoxon rank sum statistic comparing the rankings of the co-culture gene sets with the non co-culture genes, obtaining p-values of 10^-13, 10^-6, 0.007 and 0.002 for the above respectively, for the above 4 genes. These results support our hypothesis that the co-

culture genes as a whole are much more strongly associated with TNFα-response genes than TGFβ-response genes. We scored for complex correlations across sixty three independent treatments, and listed the treatments that are most correlated with each co-culture cell pairs (Figure 2 color of treeview branches, and see Supplemental TableI on top for correlation coefficients). Twelve cell pairs were significantly correlated with treatments of fibroblast cultures with TNFα (red), or MCSF1 (yellow), while only three correlated with EGF+IL8 treatments of the epithelial cells (blue). IL1β scored highly close to TNFα and more surprisingly, TGFβ scored close to the MCSF.

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Figure Legends

Figure 1: A. Co-culture versus mono-cultures: Equal numbers of cells were grown either in co-culture or in mono-cultures for 16-24 hours. These were harvested, epithelial and fibroblast mono-cultures pooled. RNA from both mixes is compared either using microarrays, or real time PCR. **B.** Volcano plot (LOD score) of co-culture response across seven repeats of the experiment. X-axis is M value, and y-axis is the B statistic of this expression change. Each dot represents an individual gene, while dot color marks the different groups of genes, as divided by the statistical properties of the response to co-culture in seven experimental repeats. B statistics represent an empirical Bayesian statistic (Lonnstedt and Speed, 2003), T the student statistic, M the -log (ratio)), and A the log geometric mean(Dudoit et al., 2002). Using these statistical measures of significance, we chose genes that are part of the response, even though their expression fold change was less than the usual 2 fold. Included genes were double positive for either two of the M, B, and T statistics (empirical threshold as dashed line).

Figure 2: A. Co-culture versus isolated cells for 15 independent Ovarian Cancer cell lines. Experiment described in Figure 1A was repeated with fifteen ovarian cell lines listed in column bottom, and expression profiling was conducted using real time PCR with 125 primer sets (Supplementary Table I). Shown are the co-culture over the mixed monoculture fold change for each cell pair, for 75 changing genes, and nine control genes. The data was normalized relative to three control genes, 18S rRNA, CystA, and GAPDH, chosen and as in (Vandesompele et al., 2002). The tree view of the different expression profiles as organized by hierarchical clustering. Color of tree view sample branch indicates the fibroblast treatment-response that most correlated with each co-culture cell pair response (see supplementary Table I, figures 4 and 5); red is TNFα/ILβ (R=0.72), blue is InhibinA (R=0.4), yellow is TGFβ/MCSF (R=0.68). These correlations were identified via a feature of Genespring GX®. **B.** Control genes, analyzed as in A. **C.** Box plot representation of the overall response to co-culture in a logarithmic scale. Boxes represent 90% of the genes. A bar across the samples is on the level of two fold response.

Figure 3: A. An alternative approach to co-culture that enables monitoring the response to co-culture of each cell type individually. Epithelial and fibroblast cells from opposite sides of a transwell are compared to the same cells growing alone. **B.** Anti-MMP immunoblot characterization of the co-culture response. Cells grown as in Figure 3A (transwell experiment) were collected into Trizol ® for the microarray analysis. Protein fraction of the epithelial and fibroblast cell cultures were dialyzed according to manufacturer's instructions, and applied on SDS-acrylamide gel immunoblots.

Figure 4: A. Identification of signaling molecules responsible for the co-culture response in the fibroblasts, using RT-PCR based profiling. Hierarchical clustering compared three type of experiments; co-cultures (black labels, as in Figure 2, columns 1, 2, 6-8), fibroblast response to epithelial factors (column 3), and fibroblast response to candidate commercial proteins (cyan labels, columns 4, 5, 9-25). **B.** The effect of single gene knock-down on the co-culture response. Fibroblasts were treated with siRNA gene-specific reagents (red labels), and then co-cultured with 2008 cells, as in Figure 2. Hierarchical clustering compared five types of experiments; co-cultures (columns 1, 6, 9, 10, 12), fibroblast response to epithelial factors (column 11), fibroblast response to rhTNFα commercial protein (column 7) and fibroblast response to co-culture, following commercial synthetic siRNA-mediated knock down of control genes (columns 2-5), and genes that are critical for the co-culture signaling (columns 13-17).

Figure 5: A. Time course experiment of the co-culture (Iyer et al., 1999), with co-culture substituting the serum treatment, as in Figure 1A. X-axis describes culture duration until harvest. Gene expression lines are colored according to their corresponding kinetic feature. Gene depicted in bold black is TNFα. **B.** Identification of signaling molecules responsible for the co-culture-induced expression of TNFα mRNA in the 2008 epithelial cells, using RT-PCR based profiling. Hierarchical clustering compared four type of experiments; co-cultures (as in Figure 3, columns 1 and 14), co-cultures of siRNA modified epithelial cells (columns 2-4, 15 and 19), epithelial response to fibroblast factors (column 5), and epithelial response to candidate commercial proteins (columns 6-13, 16-18).

Figure 6: A. A matrix of Co-expression of the co-culture responsive. All genes were ordered (along the Y-axis) according to expression correlation with each of the 54 genes (along the X-axis). The mean (blue line) and quartile (top 25% in dashed green, 50% red, and lowest 25% in dashed purple) rank value distributions of 54 typical co-culture genes are presented. **B.** Same analysis on a typical random 54 gene list. Results vary for 80,000 random lists, but never exceed in mean correlation neither the co-culture, nor the cell cycle gene lists. **C.** The same analysis on 53 cell cycle genes, invoked in fibroblasts upon serum simulation. **D.** Hierarchical clustering of 43 premalignant and 64 cancerous Gastric specimens on 260 genes, 130 representing the co-culture correlated genes (from Supplemental table II), and 130 genes selected by serum treatment of fibroblasts.

Supplementary Figure 1: A. Flow cytometry of cell mixtures of epithelial and fibroblast cells, either following co-cultures (left), of mixed mono-cultures upon harvest (right). The epithelial fraction in those two mixtures was 37±2% and 39±2%, correspondingly, across five repeats. Such variation would not justify a cell type specific gene showing an expression difference higher than two fold (based on the differential expression described in F.). **B.** Mono-cultures of epithelial and fibroblast cells were compared by expression profiling (five repeats). Each gene is assigned an average M and A as above. Top and bottom of the graph represent epithelial and fibroblast specific genes, correspondingly. Red triangles mark the co-culture regulated genes listed in Table 1. **C.** Hierarchical clustering comparing 22 distinct treatments in attempt to identify the cellular event that best mimics the co-culture, using 1400 genes selected from the 10.5 K in house cDNA array(Boussioutas et al., 2003; Tothill et al., 2005). These genes are the total of genes changing in any of the experimental treatments.

Supplementary Figure 2: A. Factors secreted to the medium of each cell line, as indicated in the top right corners, were identified using chemiarray™ ELISA blots. **B.** Hierarchical clustering comparison of co-culture reactive ovarian cell lines (2008 and OVHS), with cell lines that are relatively non-reactive in co-culture. 189 genes were

selected from the U133+2 full genome array using ANOVA analysis. **C.** Expression pattern of a single gene, TNFAIP3, across 282 carcinoma expression profiles(Tothill et al., 2005). **D.** Correlation coefficient plot of all genes to the expression pattern of single gene across 282 carcinoma profiles (as in A). X-axis Rank order of all genes on the array. Y-axis describes the Mann Whitney score, based on expression correlation to the single gene labeled on the top left (blue S-curve). Co-culture responsive genes are marked as red circles. P-values are listed on the top right of each graph.

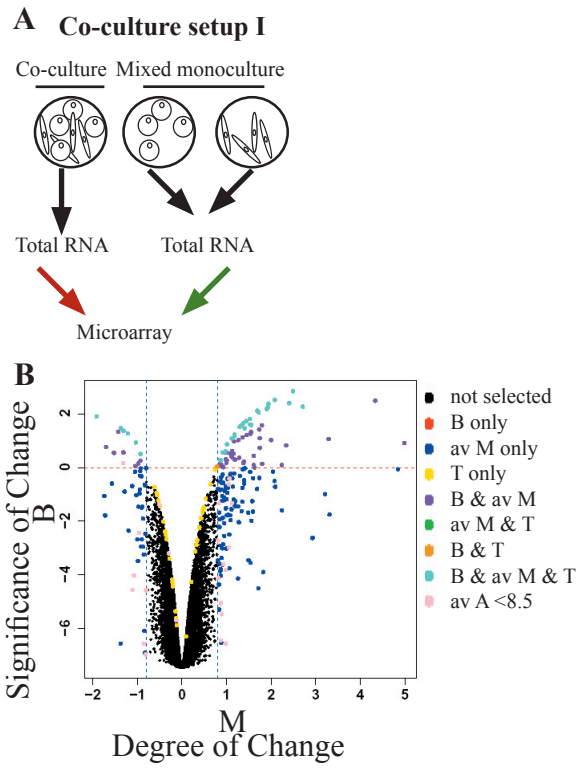
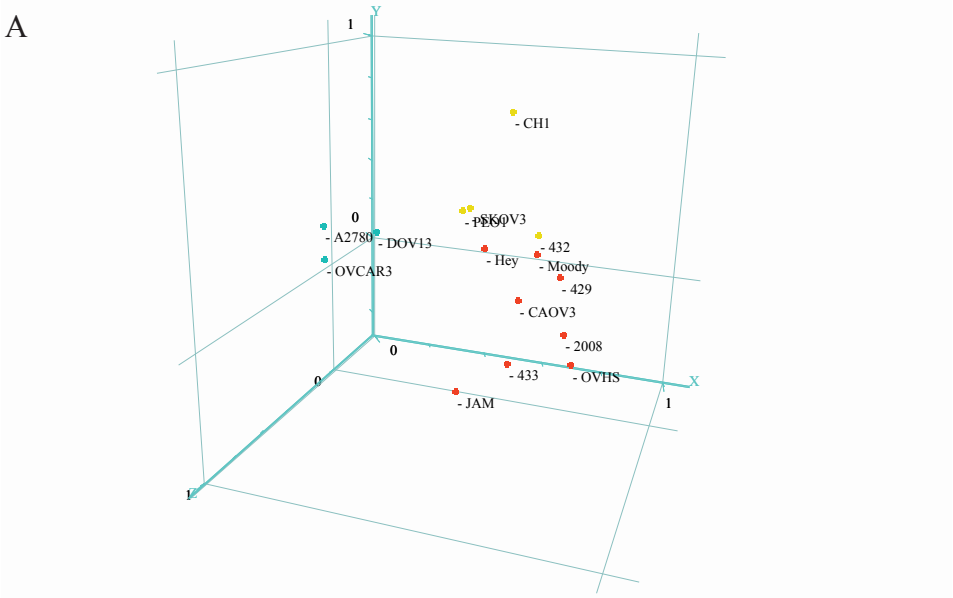

A
Co-culture setup I
Co-culture
Mixed monoculture
Total RNA
Total RNA
Microarray
B
Significance of Change
B
M
Degree of Change
not selected
B only
av M only
T only
B & av M
av M & T
B & T
B & av M & T
av A <8.5

Figure 1

A


Y
1
- CH1
0
- SKOV3
- PEO1
- A2780
- DOV13
- 432
- Hey
- Moody
- OVCAR3
- 429
- CAOV3
- 2008
0
- 433
- OVHS
X
0
- JAM
1
1
Z

B

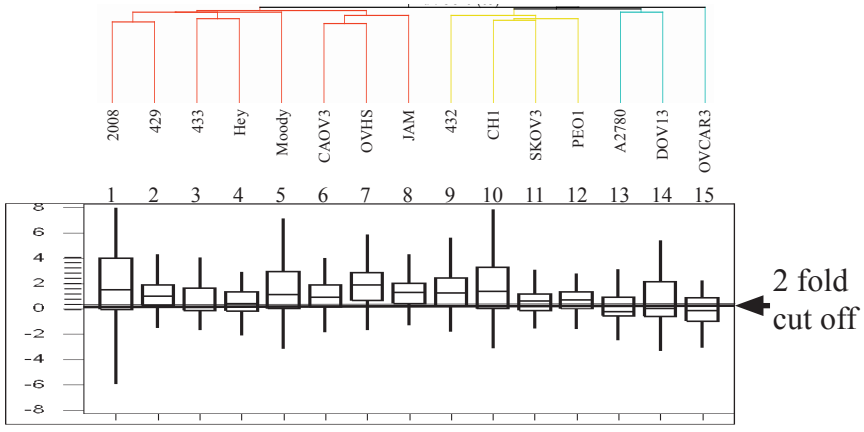

2008
429
433
Hey
Moody
CAOV3
OVHS
JAM
432
CH1
SKOV3
PEO1
A2780
DOV13
OVCAR3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
8
6
4
2
0
-2
-4
-6
-8
2 fold
cut off

Figure 2

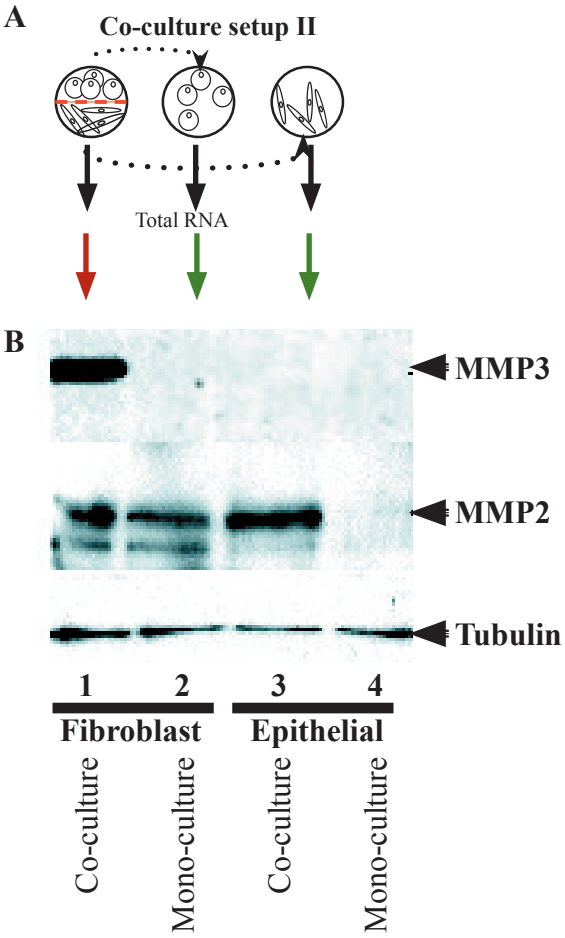

A
Co-culture setup II
Total RNA
B
MMP3
MMP2
Tubulin
1
2
3
4
Fibroblast
Epithelial
Co-culture
Mono-culture
Co-culture
Mono-culture

Figure 3

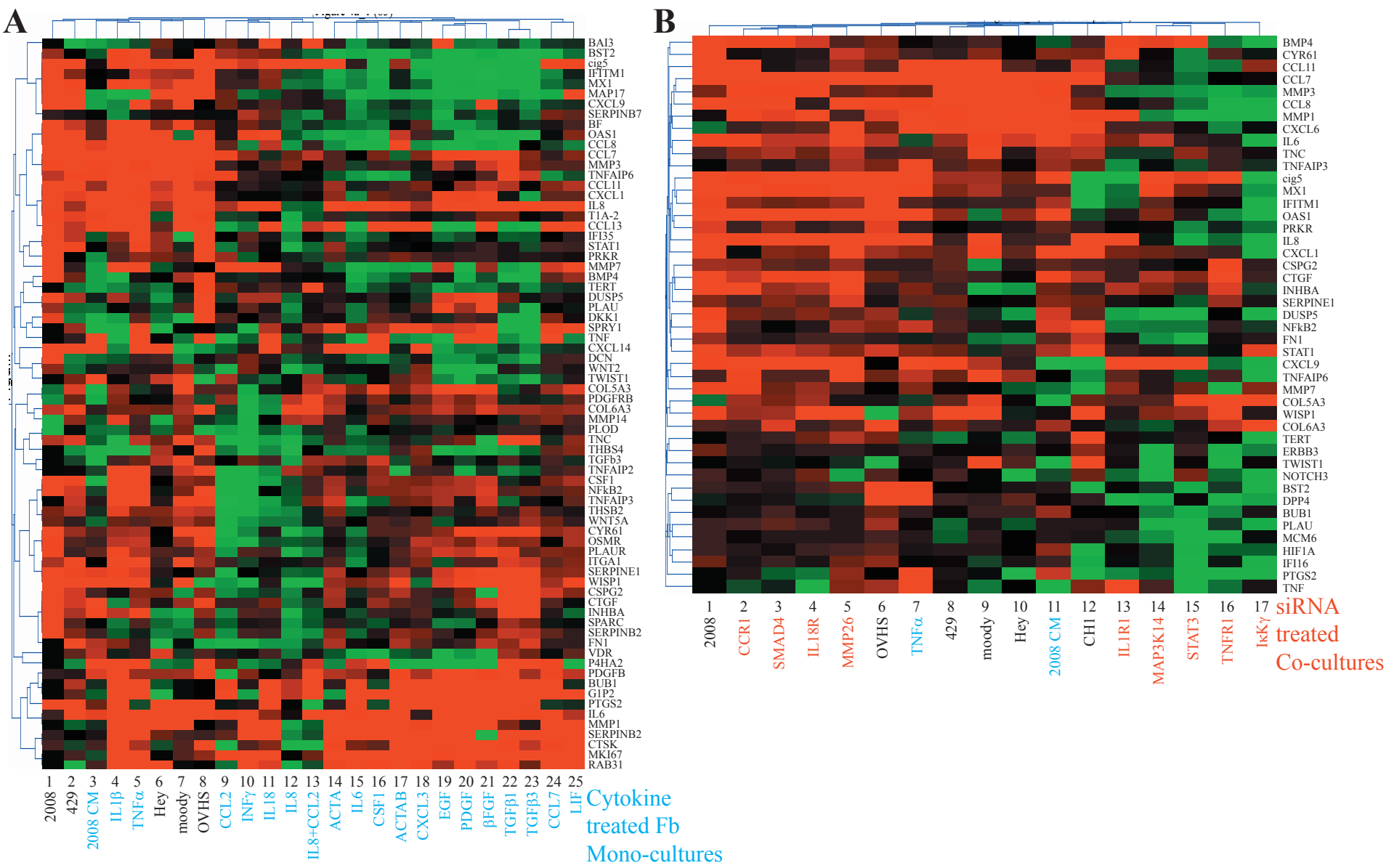

A
BAI3
BST2
cig5
IFITM1
MX1
MAP17
CXCL9
SERPINB7
BF
OAS1
CCL8
CCL7
MMP3
TNFAIP6
CCL11
CXCL1
IL8
T1A-2
CCL13
IFI35
STAT1
PRKR
MMP7
BMP4
TERT
DUSP5
PLAU
DKK1
SPRY1
TNF
CXCL14
DCN
WNT2
TWIST1
COL5A3
PDGFRB
COL6A3
MMP14
PLOD
TNC
THBS4
TGFb3
TNFAIP2
CSF1
NFkB2
TNFAIP3
THSB2
WNT5A
CYR61
OSMR
PLAUR
ITGA1
SERPINE1
WISP1
CSPG2
CTGF
INHBA
SPARC
SERPINB2
FN1
VDR
P4HA2
PDGFB
BUB1
G1P2
PTGS2
IL6
MMP1
SERPINB2
CTSK
MKI67
RAB31
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
2008 429 2008 CM IL1β TNFα Hey moody OVHS CCL2 INFγ IL18 IL8 IL8+CCL2 ACTA IL6 CSF1 ACTAB CXCL3 EGF PDGF βFGF TGFβ1 TGFβ3 CCL7 LIF
Cytokine treated Fb Mono-cultures
B
BMP4
CYR61
CCL11
CCL7
MMP3
CCL8
MMP1
CXCL6
IL6
TNC
TNFAIP3
cig5
MX1
IFITM1
OAS1
PRKR
IL8
CXCL1
CSPG2
CTGF
INHBA
SERPINE1
DUSP5
NFkB2
FN1
STAT1
CXCL9
TNFAIP6
MMP7
COL5A3
WISP1
COL6A3
TERT
ERBB3
TWIST1
NOTCH3
BST2
DPP4
BUB1
PLAU
MCM6
HIF1A
IFI16
PTGS2
TNF
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
2008 CCR1 SMAD4 IL18R MMP26 OVHS TNFα 429 moody Hey 2008 CM CH1 IL1R1 MAP3K14 STAT3 TNFR1 IκKγ
siRNA treated Co-cultures

Figure 4

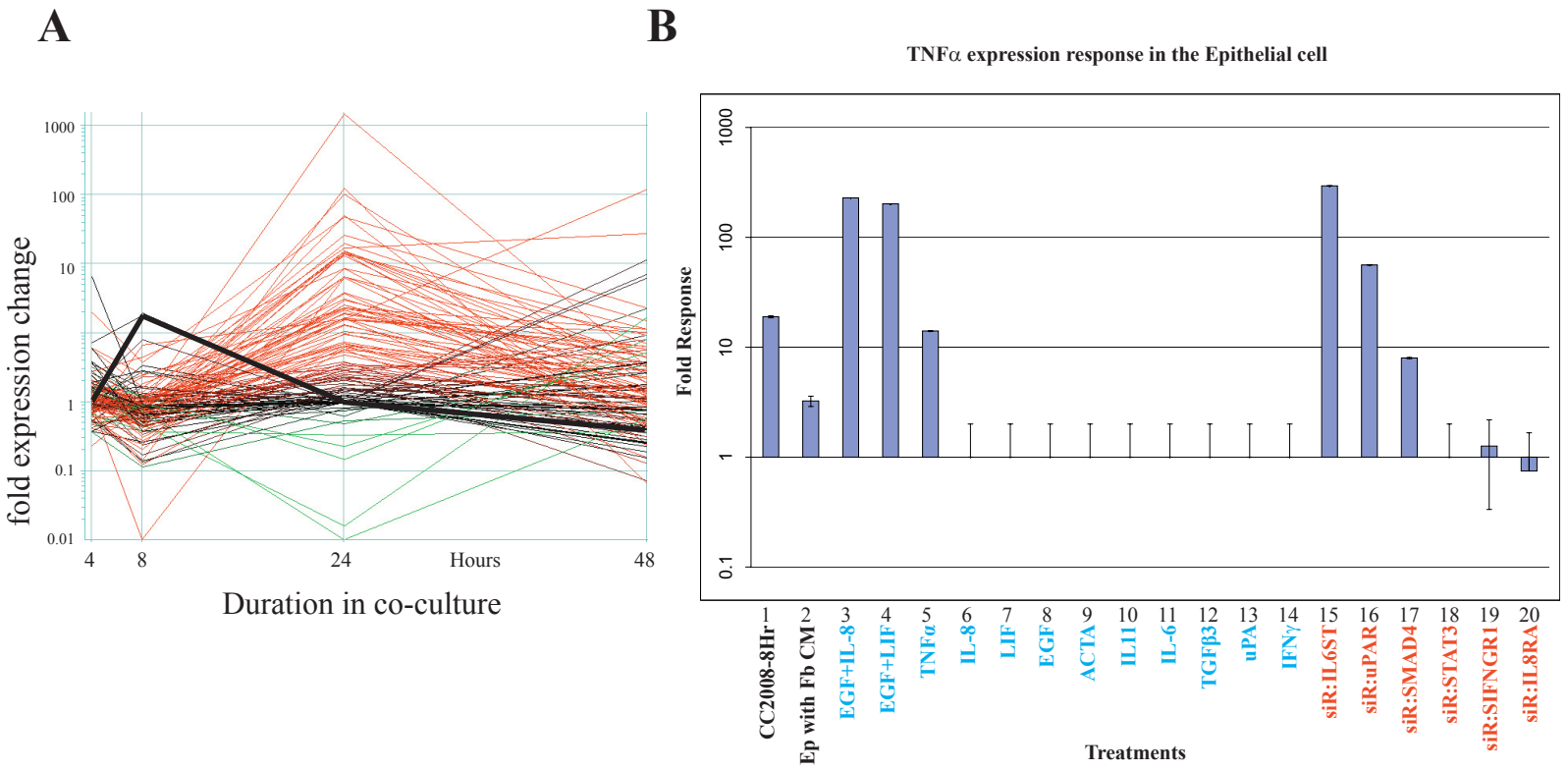
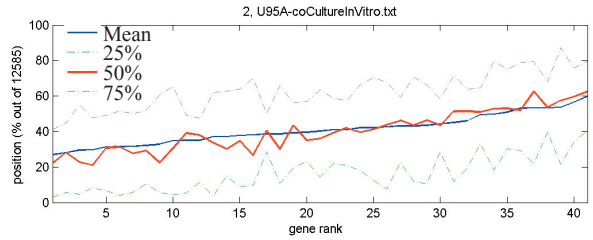
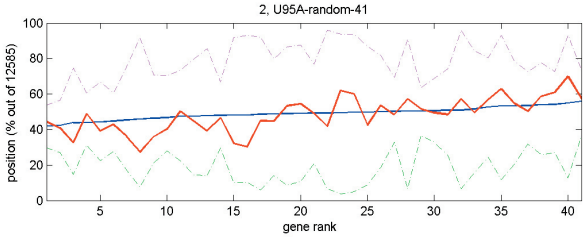

A
B
fold expression change
1000
100
10
1
0.1
0.01
4
8
24
Hours
48
Duration in co-culture
TNFα expression response in the Epithelial cell
Fold Response
1000
100
10
1
0.1
1 CC2008-8Hr
2 Ep with Fb CM
3 EGF+IL-8
4 EGF+LIF
5 TNFα
6 IL-8
7 LIF
8 EGF
9 ACTA
10 IL11
11 IL-6
12 TGFβ3
13 uPA
14 IFNγ
15 siR:IL6ST
16 siR:uPAR
17 siR:SMAD4
18 siR:STAT3
19 siR:SIFNGR1
20 siR:IL8RA
Treatments

Figure 5

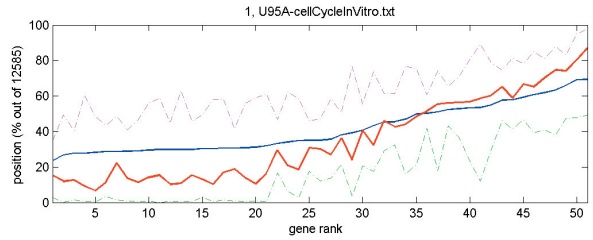
A 54 co-culture genes



B 54 random genes



C 56 cell-cycle genes



D 43 Premalignant Stomach and
64 Gastric Cancer expression profiles

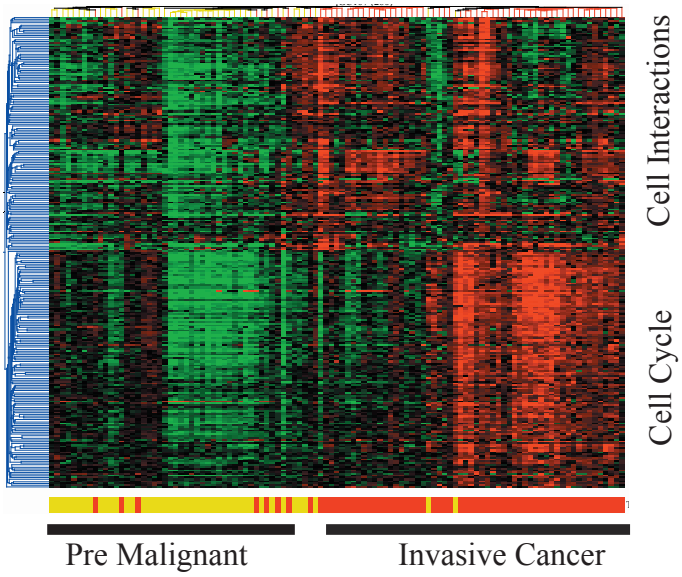
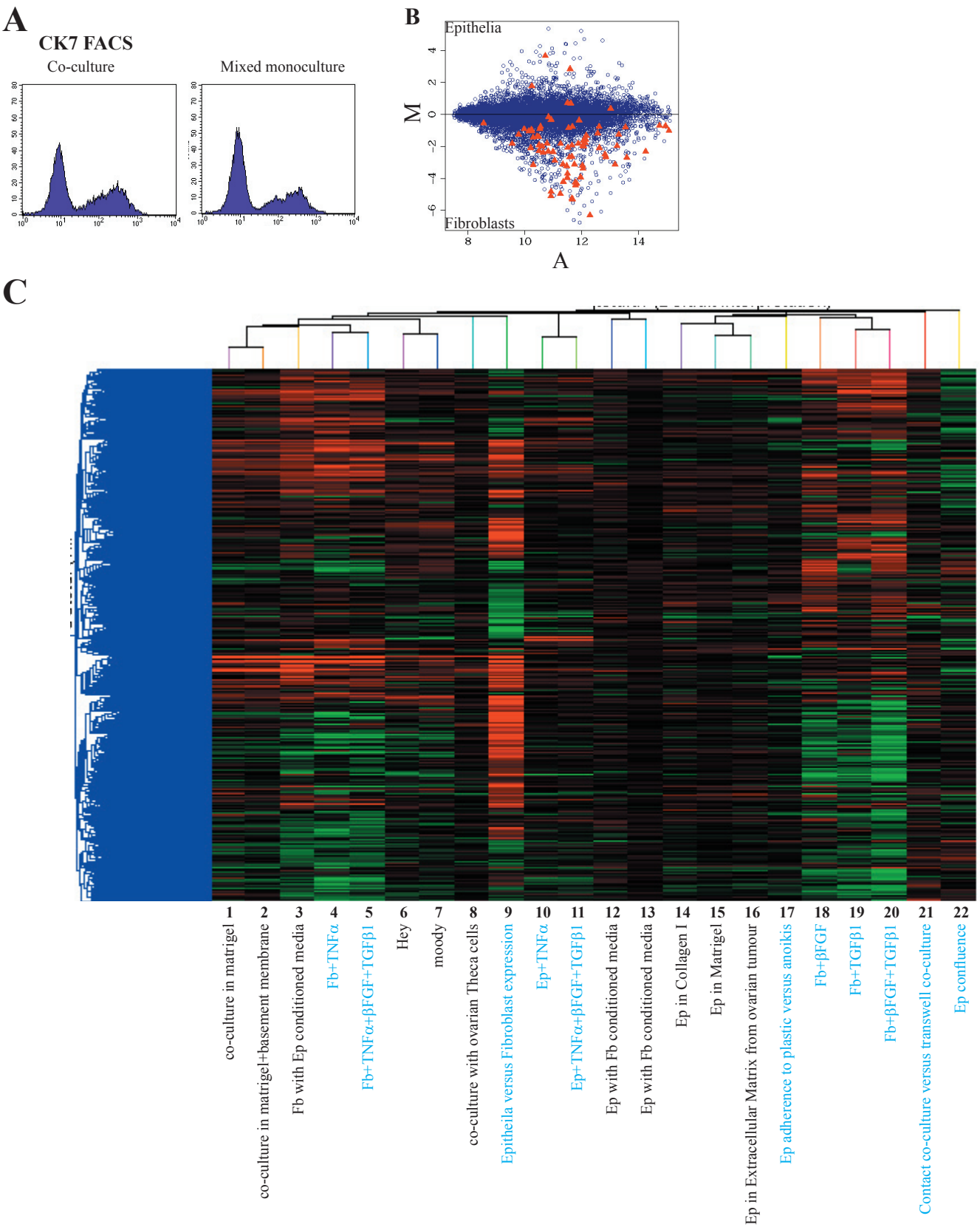
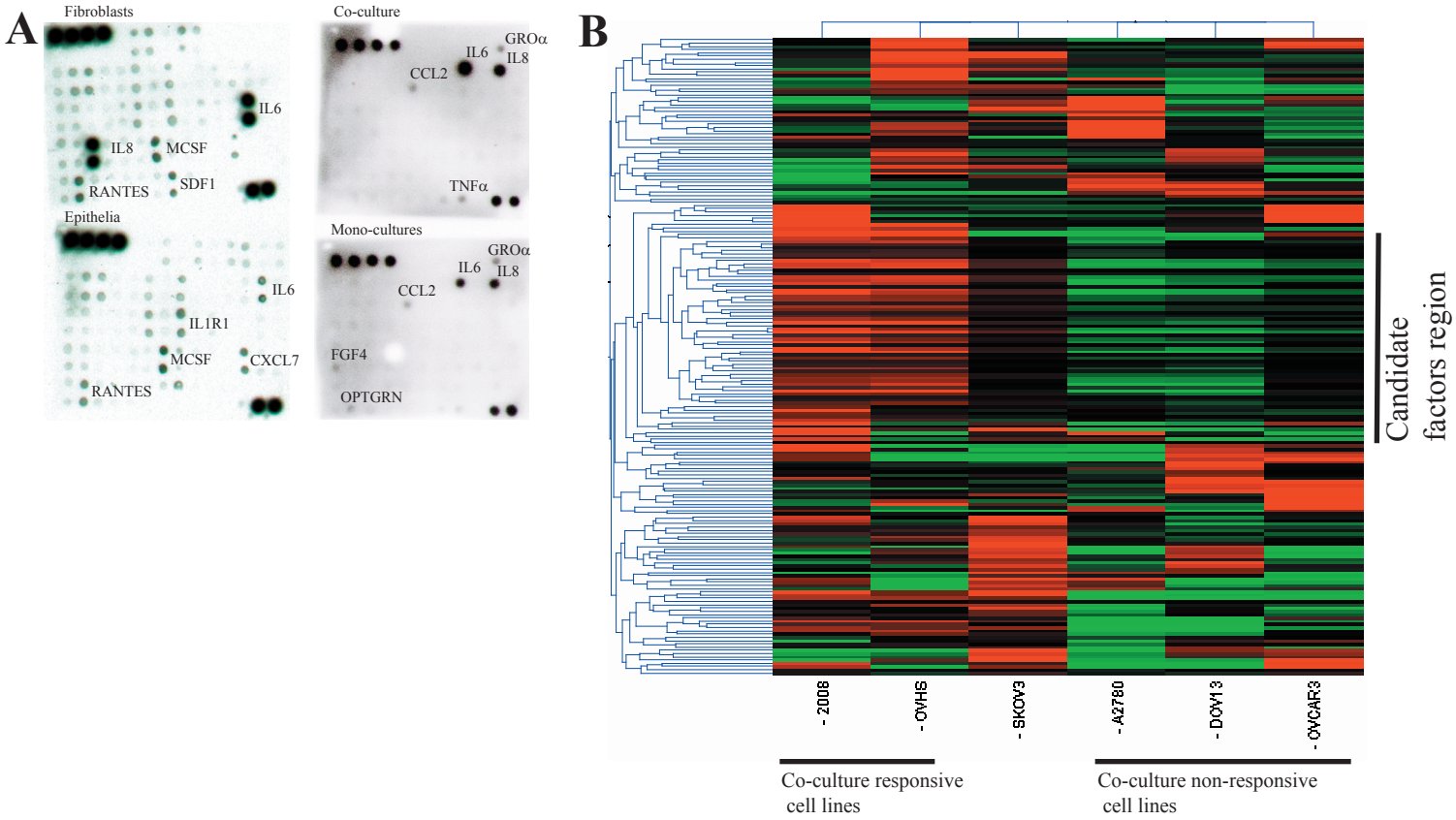


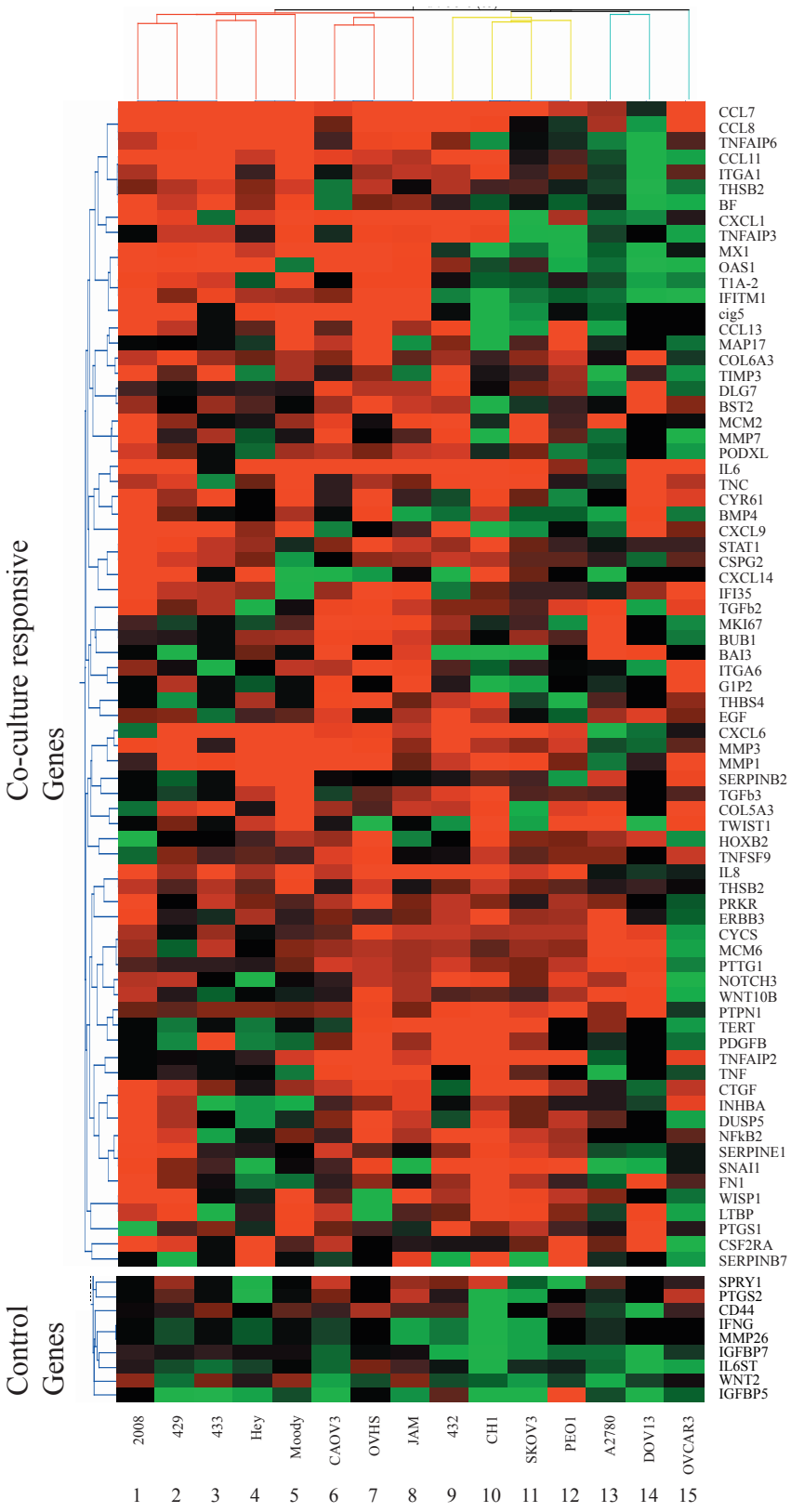
Figure 6



Supplemental Figure 1



Supplementary Figure 2



Supplementary Figure 3