Investigating the Role of Smad4 In TGF-ß Signaling using High Density Microarrays

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Synopsis: Agilent's high-density DNA microarrays are powerful tools in providing answers to complex biological questions. This poster brief presents data from both Human 1 cDNA microarrays and 60-mer oligo microarrays, which illustrate the complexity of the Transforming Growth Factor-ß (TGF-ß) induced transcriptional response in the absence and presence of Smad4. Smad4, a component of the pathway involved in TGF-ß signal transduction, has been identified as a putative tumor suppressor. Comparison of expression profiles for four tumor cell lines lacking endogenous Smad4 demonstrated that, although considerable heterogeneity exists in their TGF-ß induced transcriptional responses, different cells share a common set of Smad4-independent TGF-ß inducible genes. The effect of Smad4 on TGF-ß signaling was determined by profiling Smad4-transfected colon cancer cells, and both microarray types showed highly correlated data. Northern blotting was used to confirm a number of microarray measurements.

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ABSTRACT
Transforming growth factor-β is a multifunctional growth factor whose best-known function is to inhibit cell growth and suppress tumor formation. Loss of TGF-β growth inhibition is one of the most common cellular events in the pathogenesis of human breast, pancreatic and colon cancers. TGF-β signals through a heteromeric signaling complex consisting of Smad2, 3 and 4. Disruption of the Smad signaling complex often leads to tumor formation. We have used both 60-mer oligonucleotide and cDNA microarrays to investigate the role of Smad4 in the TGF-β controlled transcription program in tumor cells. These high density DNA microarrays, generated using Agilent's SurePrint inkjet technology, were used to profile global transcriptional regulation in breast, colon and pancreatic Smad4-null tumor cell lines in response to TGF-β . Data from both microarray types showed a high degree of correlation in demonstrating that TGF-β induces transcriptional activation and repression of genes involved in signal transduction, cell adhesion and transcriptional regulation across the range of cell lines tested. Data from a number of studies are presented comparing expression profiles from Smad4-null tumor cell lines to those from either Smad4-transfected cell lines or normal cell lines. These data indicate that the composition of the Smad signaling complex controls the specificity of TGF-β signaling.

Figure1: Smad4 in TGF-ß signal transduction. The TGF-β superfamily of secreted growth factors exerts extensive control over all aspects of development and homeostasis, and components of this pathway are often mutated in cancers and in several hereditary disorders. Smad4 is a common-mediator pathway component that forms a hetero-oligomeric complex with other Smads, which then translocates into the nucleus to function as a transcriptional co-modulator. Smad4 has been identified as a putative tumor suppressor with its role being the suppression of tumor angiogenesis. It is inactivated in more than 50% of pancreatic carcinomas and has been found to play a significant role in the malignant progression of colorectal and other tumors. Inherited inactivating mutations of Smad4 are responsible for predisposition to Juvenile Polyposis Syndrome.

Questions:

- Is Smad4 absolutely required for transmission of TGF-β signals?
- Does loss of Smad4 cause similar alterations in TGF-β signaling in different tumor cells?
- Is the specificity of TGF-β signaling altered in the presence or absence of Smad4?

Experimental Procedures

The Smad4-null tumor cell lines used in this study are listed in **Table 1**. All cell lines were obtained from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen). Smad4 activity was returned to SW480 cells using retroviral mediated gene transfer. Transfected cells, expressing recombinant Smad4, were selected using FACS sorting as illustrated in Figure **2A**. A western blot was performed using Smad4 antibody after the sorting as shown in Figure **2B**. The response to TGF-β stimulation was investigated by treating 5 x 10^7 cells with 100 pM TGF-β for 4 hours. After harvesting, total RNA was isolated using RNeasy columns (Qiagen) from untreated and treated cells and labeled using direct incorporation with either cyanine-3 or cyanine-5. Labeled target material representing untreated cells was then mixed with target representing treated cells and the mixture was hybridized to microarrays. Agilent's Human 1 cDNA microarrays (G4100A) were hybridized with cDNA targets which were generated from 10 µg of total RNA using Agilent's Fluorescent Direct Label Kit (G2557A). Agilent Human 1A Oligo microarrays (G4110A) were hybridized with cRNA targets which were genereated from 50 ng of total RNA using Agilent's Low Input Fluorescent Linear Amplification kit (5184-3523). Microarrays were manufactured using Agilent's SurePrint inkjet technology. Human 1 cDNA microarrays contain more than 12,000 sequence-verified cDNAs from the Incyte Genomics Human UniGene 1 and Human Drug Target clone sets. Human 1A Oligo microarrays consist of experimentally optimized and validated 60-mer oligonucleotide probes, representing more than 17,000 human genes. All experiments were done as dye-swaps with four replicate microarrays being processed for each dye-swap half. Microarrays were scanned using the Agilent dual laser DNA microarray scanner and data extracted using Agilent's Feature Extraction software.

Table 1. Tumor Cell Lines with Homozygous Smad4 Deletions Used in This Study

Cell line	Origin
MDA-MB468	Breast cancer cells
SW480	Colon cancer cells
CFPAC-1	Pancreatic cancer cells
Hs 766 T	Pancreatic cancer cells

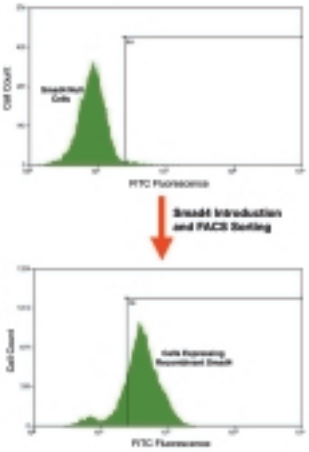


Figure 2A. Selection of cells expressing recombinant Smad4

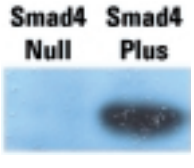
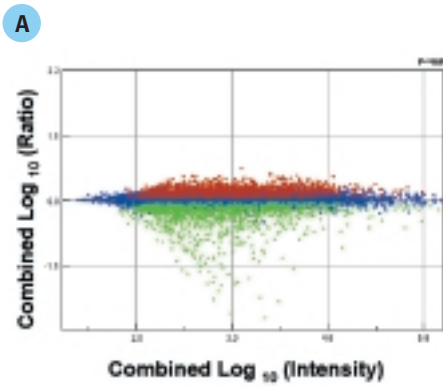


Figure 2B. Western Blot image using Smad4 antibody



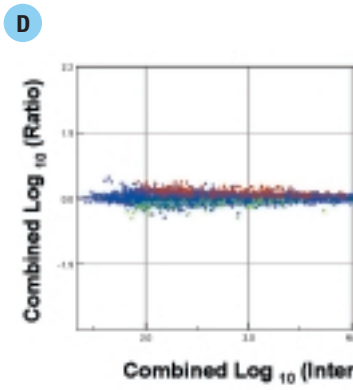


Figure 3: Expression profiles of Smad4-null tu
of SW480 **(A)**, Hs 766 T **(B)**, MDA-MB468 **(C)**
polarity). Data points represent Resolver-comb
against signal intensities. The Agilent Feature
each experiment. Log ratios colored blue are u
(significantly greater than 0) and those in gree

Figure **3E** presents a comparison of the numbe
different from 0 for SW480 and CFPAC-1 than f
genes were identified for all cell lines which ha

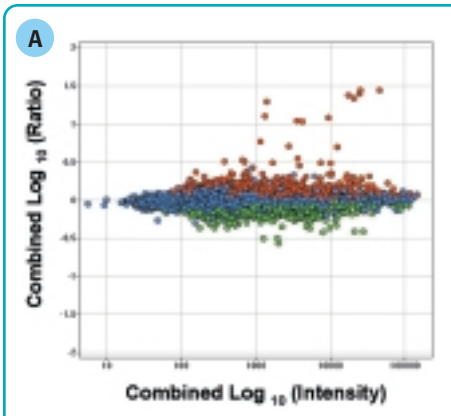
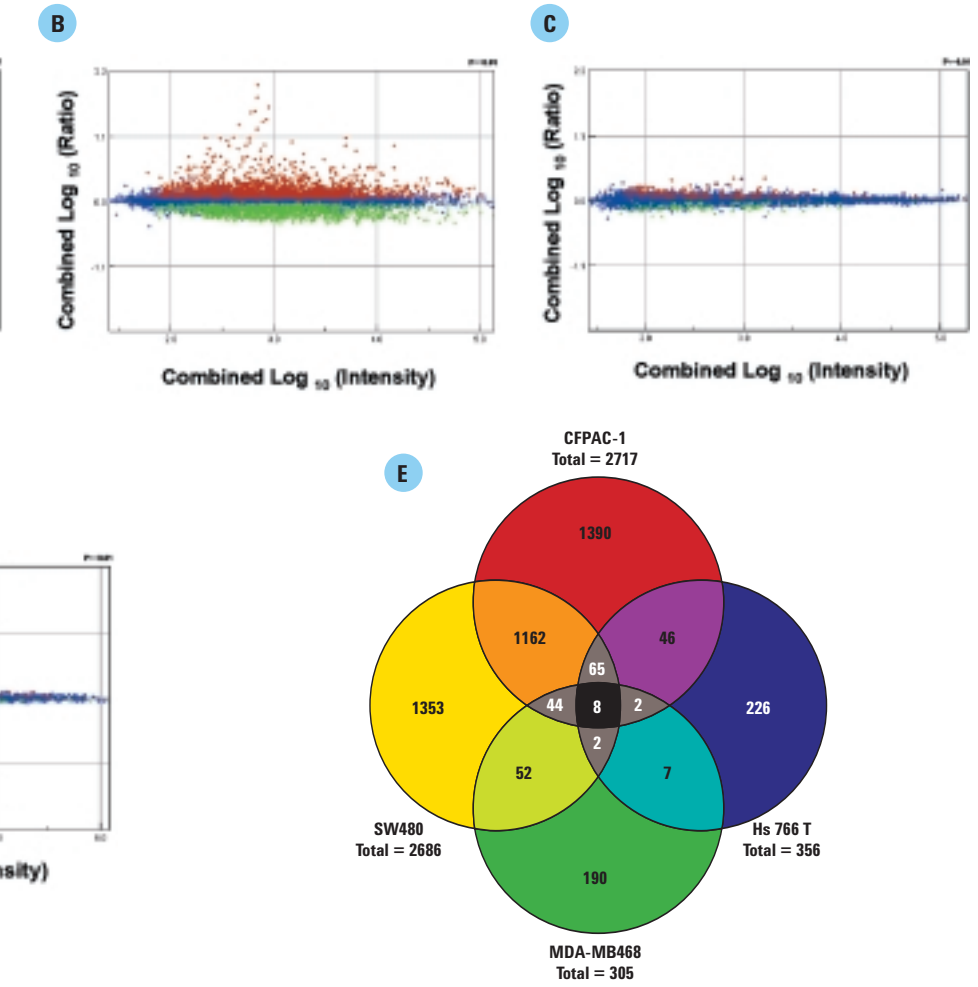
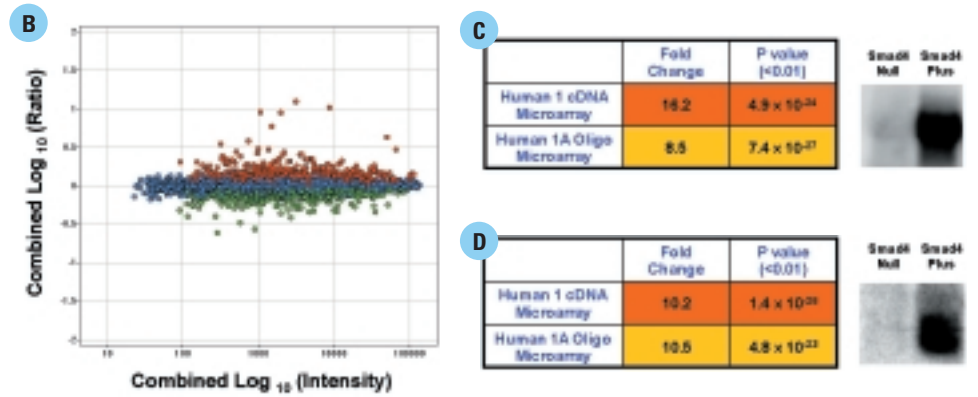


Figure 4: Measuring the effect of Smad4 on e
The effect of Smad4 on global gene expressior
both Human 1 cDNA microarrays **(A)** and Hum
significantly up-regulated in Smad4-positive ce
Although the resulting profiles show a low leve
by both microarray platforms. The differential e
regulated in the Smad4 expressing cells relativ
gene presented, Inhibitor of DNA binding 1 **(D)**



umor cell lines treated with TGF-β. The effect on gene expression of 4 h treatment with TGF-β in each and CFPAC-1 **(D)** was measured using 8 replicate Agilent Human 1 cDNA microarrays (4 in each ined log ratios for untreated cells labeled in one channel, and treated cells labeled in the other, plotted Extraction error model was utilized by Resolver to determine log ratio value significances ($p < 0.001$) for nchanged gene elements (not significantly different than 0), those shown in red are up-regulated n are down-regulated (significantly less than 0).

r of TGF-β responsive genes in the four cell lines. More log ratios were determined to be significantly for Hs7667 T and MDA-MB468, indicating overall differences in responsiveness to TGF-β . However ave previously been reported to be regulated by TGF-β.



xpression profiles in the absence of TGF-β using Human 1 cDNA and Human 1A Oligo microarrays. n in SW480 was investigated by hybridizing transfected Smad4-plus cells against Smad4-null cells using an 1A Oligo microarrays **(B)** using the scheme outlined in Figure 3. Data points colored red are lls relative to Smad4-null cells and data points shown in green represent down-regulated genes. el of differential expression, 209 genes which are regulated by the expression of Smad4 were identified expression data derived for Smad 4 itself are presented in figure **4C**. As expected, it is significantly up- e to the Smad4 null cells, and this is confirmed by the accompanying Northern blot. Data for the second), is even better correlated between the two platforms .

Figure 5: Identification of Smad4- dependent genes in colon cancer cells using both Human 1 cDNA microarrays and Human 1A Oligo microarrays. The response of Smad4-transfected cells to TGF-β stimulation was determined by hybridizing TGF-β treated cells against untreated cells to both Human 1 cDNA microarrays **(A)** and to Human 1A microarrays **(B)**. Expression data from both microarray platforms correlate very highly, with less than 0.4% of genes being anticorrelated. Differential expression measurements from both microarray types corresponding to a number of previously well-characterized TGF-β responsive genes are listed in **Table 2**. Data from this study indicate that, as well as being TGF-β independent, expression of these genes is Smad4- dependent.

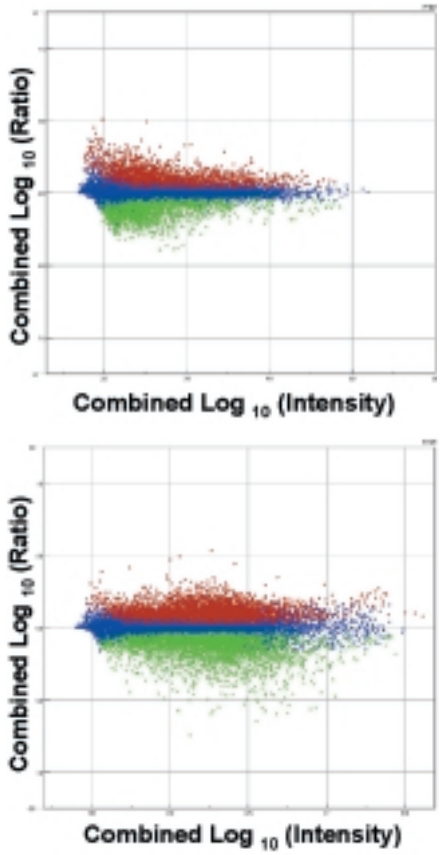


Table 2. Examples of TGF-β -Dependent Smad4-Dependent Genes

Gene	Response: Human 1 cDNA	Response: 60-mer Oligonucleotide
junB proto-oncogene	2.3-fold Up	2.7-fold Up
Smad7	2.5-fold Up	1.8-fold Up
Lamin alpha 3	3.2-fold Up	4.9-fold Up
Disintegrin/Zinc Metalloprotease	2.0-fold Down	1.6-fold Down

Conclusions

- Smad4 is not absolutely required for TGF-β induced signaling but does alter the specificity of the response.
- Different tumor cells exhibit considerable heterogeneity in their TGF-β induced transcriptional response but share a common set of Smad4-independent TGF-β inducible genes.
- The precision of Agilent's ink-jet microarrays enables accurate characterization of the TGF-β induced transcription response.

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