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Transformation by Oncogenic Ras Expands the Early Genomic Response to Transforming Growth Factor β in Intestinal Epithelial Cells[1]

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Abstract

A substantial body of evidence implicates TGFβ as a tumor promoter in epithelial cells that have become resistant to its tumor suppressor activity. To better understand early, genome-wide TGFβ responses in cells resistant to growth inhibition by TGFβ, we used microarray analysis in a well-defined cell culture system of sensitive and resistant intestinal epithelial cells. TGFβ-regulated gene expression in TGFβ-growth–sensitive, nontransformed rat intestinal epithelial cells (RIE-1) was compared to expression in TGFβ-growth–resistant RIE cells stably transformed by oncogenic Ras(12V). Treatment of RIE-1 cells with 2 ng/ml TGFβ1 for 1 hour increased the expression of eight gene sequences by 2.6-fold or more, whereas eight were down regulated 2.6-fold. In RIE-Ras(12V) cells, 42 gene sequences were up-regulated and only 3 were down-regulated. Comparison of RIE and RIE-Ras(12V) identified 37 gene sequences as unique, Ras-dependent genomic targets of TGFβ1. TGFβ-regulation of connective tissue growth factor and vascular endothelial growth factor, two genes up-regulated in RIE-Ras cells and previously implicated in tumor promotion, was independently confirmed and further characterized by Northern analysis. Our data indicate that overexpression of oncogenic Ras in intestinal epithelial cells confers a significantly expanded repertoire of robust, early transcriptional responses to TGFβ via signaling pathways yet to be fully elucidated but including the canonical Raf-1/MAPK/Erk pathway. Loss of sensitivity to growth inhibition by TGFβ does not abrogate TGFβ signaling and actually expands the early transcriptional response to TGFβ1. Expression of some of these genes may confer to Ras-transformed cells characteristics favorable for tumor promotion.

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Introduction

A large body of work during the past two decades has established transforming growth factor beta (TGFβ) as a potent suppressor of cellular proliferation in most experimental settings. Indeed, TGFβ is now distinguished as one of the most important tumor suppressors in human cancer biology [1–5]. Notwithstanding, for many years, it has been clear from *in vitro* studies that the contribution of TGFβ signaling to cell behavior is far more complex than negative regulation of cellular proliferation. In some contexts, TGFβ may contribute to the transformed phenotype. Several recent *in vivo* studies convincingly implicate TGFβ as a tumor promoter in transformed cells [6,7], leading to the hypothesis that the tumor-promoting effects of TGFβ increasingly predominate over growth-inhibitory effects during tumor progression. It seems that overexpression of genes associated with cellular proliferation cooperate with TGFβ to accelerate tumor formation and metastasis, presumably after cells have become resistant to TGFβ-mediated growth inhibition.

The most completely understood signal transduction pathway used by TGFβ is the canonical Smad pathway [8–10]. Transforming growth factor β receptor (TGFβR) activation results in serine phosphorylation of Smad2 and Smad3 and formation of heteromeric complexes with Smad4. The Smad complex accumulates in the nucleus

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by regulated nuclear import and binds to DNA in a sequence-specific manner in association with a large number of potential transcriptional coactivators and corepressors [11,12]. The specificity and complexity of the genomic response to TGFβ is attributable in part to the complex portfolio of coactivators and corepressors available under the specific conditions at the time of TGFβ exposure [3,10]. A large number of other signal transduction pathways, including Erk kinases, protein kinase C, Rho-like GTPases, p38, and c-Jun N-terminal kinases, have been identified as apparent Smad-independent signaling mechanisms, but activation of these depends to a significant extent on the cell system under study [13]. At present, it is not clear if tumor promotion by TGFβ occurs by these Smad-independent pathways or by a modification or attenuation of Smad signaling.

In intestinal neoplasia, loss of TGFβ tumor-suppressor activity occurs by a variety of well-described genetic and epigenetic defects. The small GTPase Ras is activated by mutation in many human cancers [14,15], including more than 50% of colorectal cancers [16]. Multiple cell culture studies have identified Ras activation as an epigenetic factor conferring resistance to growth inhibition by TGFβ [17]. We previously found that stable transformation of rat intestinal epithelial cells with oncogenic Ras(12V) did not attenuate Smad localization to the nucleus in response to TGFβ treatment but caused resistance to growth inhibition [18]. Furthermore, TGFβ activation of Smad binding element–driven reporter gene transcription in RIE-Ras(12V) cells was markedly reduced [19], indicating interference with Smad-dependent transcription. Notwithstanding this loss, or at least significant attenuation of Smad-mediated growth inhibitory signaling in the context of oncogenic Ras, it is clear that residual, albeit modified, TGFβ signaling continues to occur.

To examine more broadly the effects of Ras transformation on the early genomic response to TGFβ, we used DNA microarrays to compare induction of target gene transcription in nontransformed rat intestinal epithelial cells (RIE-1) and in cells stably transformed by Ras (12V) [*RIE-Ras(12V)*] 1 hour after treatment with TGFβ1. Cellular and specific Smad-responsive transcriptional responses to TGFβ have been extensively characterized in these cell lines, particularly in the context of Ras transformation [18–20]. Because growth inhibitory Smad signaling is markedly attenuated in RIE-Ras(12V) cells, we hypothesized that this apparent "resistance" to TGFβ would result in induction of fewer early transcriptional targets in Ras-transformed cells. However, to the contrary, we found that mutant oncogenic Ras expands the early genomic response to TGFβ, such that more than five times as many genes are activated in RIE-Ras(12V) cells than parental RIE cells at a cutoff threshold ≥2.6-fold induction. Microarray analysis of TGFβ-mediated gene transcription in RIE and RIE-Ras identifies a number of candidate genes, which may contribute to TGFβ actions as a tumor promoter, the setting of oncogenic Ras activation. Among these candidate genes, we examined in more detail the kinetics and signaling pathways involved in the induction of two differentially induced genes, vascular endothelial growth factor (*VEGF*) and connective tissue growth factor (*CTGF*).

Materials and Methods

Cell Lines and Reagents

RIE-1 rat intestinal epithelial cells (RIE) were obtained from Ken Brown (Cambridge, United Kingdom) and maintained in DMEM supplemented with 10% FCS. RIE cells are a diploid, nontransformed, TGFβ-growth–sensitive cell line derived from rat jejunum [18,20,21]. RIE-Ras cells were established by stable transfection of the parental cells with *pSV2-H-Ras(12V)* that contain human sequences encoding the constitutively active H-Ras(12V) protein [22]. The RIE-Ras transfectants were selected in G418 (Calbiochem, San Diego, CA). The cell line designated RIE-Ras(12V) in this study was established as a pooled population of stable clones and grown at all times in the presence of 50 μg/ml G418. For each experiment, RIE or RIE-Ras cells were plated in duplicate, grown to log phase, and treated with 2 ng/ml of TGFβ1 (BD Biosciences, Bedford, MA) or diluent for 1 hour, after which RNA was immediately collected.

RNA Isolation

For each array experiment, RNA was isolated from ~10^7 cells growing in log phase on four separate 10-cm^2 tissue culture dishes. RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), followed by purification with the RNEasy system (Qiagen, Valencia, CA). Each collection yielded approximately 100 μg of RNA. RNA integrity was verified using the Agilent Bioanalyzer in the Microarray Core Facility at the Columbus Children's Research Institute.

Probe Preparation and Array Hybridization

Complementary DNA (cDNA) probe was prepared by the Microarray Core Facility (Columbus Children's Research Institute) with Superscript III using oligo-dT primers and dNTPs supplemented with amino-allyl-UTP to improve hybridization characteristics and stability. The DNA samples were labeled with Cy3 or Cy5 then were purified. Purified probe was hybridized to the Agilent Rat Olio Microarray (Agilent, Cincinnati, OH) for 14 hours at 48°C according to the manufacturer's protocol. This array (Agilent G4130A) is composed of 20,500 rat genes, ESTs, and EST clusters printed on a glass slide as 60-mers. The gene list can be downloaded from www.agilent.com. Slide images were acquired using an Affymetrix 428 scanner with gain settings set so that 95% of spots were below saturation to yield the maximum dynamic range within an experiment. Images were converted into ".gpr" files using GenePix software (Axon, Union City, CA). Image files (.tif) were deposited onto the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo) with the Series Accession Number GSE2015.

Data Analysis

Four independent experiments from cell culture to chip hybridization were performed for both RIE and RIE-Ras. Data were analyzed using GeneTraffic 2.6 software (Iobion, La Jolla, CA). Lowess-global normalization was applied to all experiments. Flagging parameters were set as spot intensity lower than the intensity of local spot background, spot intensity lower than average background, and raw spot intensity less than 100. Flagged spots were not included in normalization or aggregate calculations. Only genes that were induced or repressed at a level of $\log_2 N > 1.4$ (2.6-fold) in at least three of the four independent analyses and hybridizations were identified as TGFβ-regulated genes. The NIA Array Analysis Tool (http://lgsun.grc.nia.nih.gov) was used to determine the false discovery rate (FDR), which is equivalent to a *P* value in experiments with multiple hypothesis testing. The maximum FDR was set at .05. The NIA Array Analysis Tool was also used to determine the correlation coefficient matrix for each of the RIE and RIE-Ras experiment sets.

Real-time Polymerase Chain Reaction and Northern Blot Analysis

Real-time polymerase chain reaction (PCR) and Northern blot analysis was used with a selected set of genes to verify results of the array experiments. RNA from two independent samples used to generate probes for the array experiments was used to generate cDNA for the real-time PCR reactions. First, 1 μg of total RNA was converted to cDNA using SuperScript II RT (Invitrogen). Serial 10-fold dilutions of the cDNA were used for each primer set in triplicate. Polymerase chain reaction reactions were carried out in 96-well plates with the with SybrGreen *Taq* polymerase (ABI, Foster City, CA) according to standard protocol with an ABI 7700 Sequence Detector machine. Primer sequences were determined using PrimerDesigner software (ABI) with published GenBank sequences. *Cyclophilin* and *GAPDH* were used as controls, and results are reported as $\Delta\Delta C_t$ relative to *GAPDH*. For each experiment, the ΔC_t was determined by the average of three identical samples. The values in Figure 1 represent the average $\Delta\Delta C_t$ from two independent experiments. Primer sequences are listed below:

- APC2-F: 5′-CGCTTCGGTACCTCAGACGA
- APC2-R: 5′-TGTCAATCTGCTCCAGGCG
- BHLHB2-F: 5′-CTGCCCAAAACGCCAGG
- BHLHB2-R: 5′-CACTTGGTACATGTGGGCAAA
- CYCLOPHILIN D-F: 5′-GGCCATGTATCCTTAGCAAG-TGTC
- CYCLOPHILIN D-R: 5′-GGTCAGCATTGCCGATGTC
- ERBIN-F: 5′-CACTCTGTGGCACCCTAAACAA
- ERBIN-R: 5′-CTGCACTCTCAGATCTTGGAGG
- FMR2-F: 5′-TGGTTTTTCCACAGTTATGGCA
- FMR2-R: 5′-CTGCAAAGACAGACCACCACAA
- GADD45B-F: 5′-ACTCCCCTCTCCTCGTCTCAG
- GADD45B-R: 5′-CTCAAAAGCTACCCTACCCGTG
- GADD45G-F: 5′-CCAGTCCAGGCGGCC
- GADD45G-R: 5′-GTGACTCAGCAAGCAGCCTTC
- GAPDH-F: 5′-ACAAGATGGTGAAGGTCGGTGT
- GAPDH-R: 5′-CAAGAGAAGGCAGCCCTGG
- JUNB-F: 5′-GAGGAGCAGGAGGGCTT
- JUNB-R: 5′-TCACGTGGTTCATCTTCTGCAG
- LRF-F: 5′-TGTGCCACAGTGCGGC
- LRF-R: 5′-GCACGGAAGTTCTTGCAGC
- LY94-F: 5′-GGGATCACACAGCCCAGAAT
- LY94-R: 5′-CAAAAGCCATACTAGAGCCATCAC
- SMAD7-F: 5′-GCGAAAGTGGGTACCACCTTC
- SMAD7-R: 5′-ATTCACGTACACCCCCCTCA
- SNON-F: 5′-GATCGTGAAGTCGCCCAAGA
- SNON-R: 5′-AGTCCTGCCAACCAAACACAG
- VEGF-F: 5′-GCCCTGGAGTGCGTGC
- VEGF-R: 5′-GTGAGGTTTGATCCGCATGA

Northern Blot Analysis

To generate nucleotide probe for *FMR2* sequence, cDNA was first generated from RIE mRNA according to a standard protocol using Superscript II Reverse Transcriptase (Invitrogen). An *FMR2* DNA probe was generated by amplification of *FMR2* sequence using probes (FMR2F and FMR2R) designed to amplify from nucleotide 1250 to 1531 of the sequence published with the accession number XM_219832 using an annealing temperature of 60°C. RIE and RIE-Ras cells were treated with 2 ng/ml TGFβ (BD Biosciences) from 0 to 24 hours, and RNA was isolated using Trizol (Invitrogen) with subsequent purification using the Qiagen RNEasy kit (Qiagen). Total RNA samples (1 mg per lane) were run on agarose gels, then blotted onto Hybond-XL (Amersham, Piscataway, NJ) membrane, which was hybridized with the 281-bp ^{32}P-labeled *FMR2* probe. The membrane was then washed and exposed to film, scanned, and

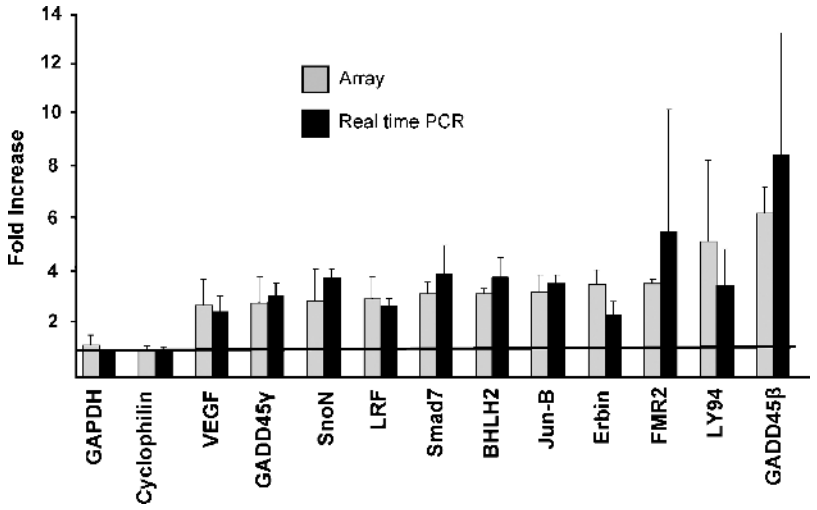

Figure 1. Real-time PCR of TGFβ target genes identified by DNA array. The change in gene expression of genes in RIE-Ras(12V) cells treated for 1 hour with 2 ng/ml TGFβ1 is shown by the results from the array experiments as well as by real-time PCR experiments. The average change in gene expression determined by the four independent array experiments is shown in light gray. Real-time PCR was used with a selected set of genes to verify the results of the array experiments. The $\Delta\Delta C_t$ for the genes relative to *GAPDH* represents fold-change after TGFβ treatment, shown in black. *GAPDH* and *cyclophilin* are housekeeping genes, the expression of which did not change significantly with TGFβ1 treatment. The genes identified by the array experiments as having increased transcription in RIE-Ras (12V) cells after TGFβ1 treatment were confirmed by real-time PCR experiments.

signal was quantitated with ImageQuant TL Image Analysis software (Amersham). The *FMR2* PCR probes were as follows: FMR2F: 5′-TGCAAAGACAGACCACCACAA-3′; FMR2R: 5′-AAAGTG-CGGGAGGGAACAG-3′.

For the expression of *VEGF* and *CTGF*, total cellular RNA was extracted using Trizol. RNA samples (20 μg per lane) were loaded into 1% agarose/formaldehyde gels, separated by electrophoresis, and blotted onto nitrocellose membranes. *VEGF* (a kind gift from Dr. Robert Coffey) and *CTGF* (a kind gift from Dr. David Brigstock) cDNA probes were labeled by random primer extension using Redivue with α-^{32} P-dCTP and the Rediprime DNA labeling system from Amersham Life Sciences (Arlington Heights, IL). After hybridization and washing, the membranes were subjected to autoradiography. Integrity and loading of the RNA samples were assessed by 18 rRNA signals or expression of cyclophilin, a constitutively expressed mRNA. For *VEGF*, mRNA results were confirmed by ELISA (VEGF Quantikine Kit; R&D Systems, Minneapolis, MN).

Results

Early Genomic Responses to Short-term TGFβ Exposure in RIE Cells

DNA microarray was used to quantitatively and qualitatively ascertain TGFβ1-regulated gene expression in RIE cells that are sensitive to growth inhibition by TGFβ and RIE cells stably transformed with oncogenic Ras(12V) that are resistant to growth inhibition by TGFβ [18]. We intentionally focused on early genomic responses because these most likely reflect the primary alterations in TGFβ signaling conferred by the expression of oncogenic Ras. Similarly, we imposed a stringent definition of a TGFβ1 target to identify as pure a profile of targets as possible.

The Agilent Rat Oligo microarray glass slide system was used to compare expression of 20,500 genes in each cell line at baseline and 1 hour after exposure to 2 ng/ml TGFβ1. Significantly regulated genes were defined by a minimum change in expression of $\log_2 N$ = 1.4 (2.6-fold) in at least three of the four array slides used for each cell line. Data tables for all real genes on the Agilent Rat Oligo chip for each of the four independent experiments from both the RIE and RIE-Ras cell lines are included in Supplemental Data. Data including fold change and log ratios are also available on the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo) with the Series Accession Number GSE2015. Reproducibility of the data from the individual array slides was verified using the NIA Array Analysis Tool, and correlation coefficients are shown in Table 1.

In RIE cells, eight genes were up-regulated by TGFβ, and eight genes were down-regulated. In RIE-Ras(12V) cells, 42 genes were up-regulated and 3 genes were down-regulated (Table 2, *A–D*). Of the 42 genes up-regulated by TGFβ in RIE-Ras(12V) cells, 5 were also up-regulated in RIE cells, all of which have previously been described as transcriptional targets of TGFβ: *GADD45β* [23], *VEGF* [24], *junB* [25], *BHLHB2* [26], and *Smad7* [27]. In our experiments, 37 transcripts were uniquely up-regulated in RIE-Ras cells. Of these, seven have unknown functions. The remainder fall into diverse functional clusters including genes whose products function as proteases (n = 3), transcription factors (n = 4), structural proteins (n = 5), cell cycle regulators (n = 1), cell signaling factors (n = 5), regulator of cell-mediated immunity (n = 1), and proteins with other cellular functions (n = 11). These Ras-specific genes are ideal candidate pro-oncogenic genes that contribute to TGFβ-mediated tumor progression and metastasis [1].

Table 1. Correlation Coefficient Matrices.

RIE: Experiment 1-4				
	1	2	3	4
1	1	.95	.94	.94
2	.95	1	.97	.94
3	.95	.97	1	.97
4	.91	.94	.97	1

RIE-RAS: Experiment 1-4				
	1	2	3	4
1	1	.95	.95	.91
2	.95	1	.96	.96
3	.93	.96	1	.95
4	.94	.96	.95	1

Confirmation of TGFβ-Regulated Gene Expression by mRNA Analysis

Eleven genes up-regulated by TGFβ1 treatment of Ras-transformed cells were selected for confirmation by real-time PCR because they were prominently regulated or were putative tumor promoters. In each instance, genes identified as transcriptional targets of TGFβ1 by microarray were similarly up-regulated when tested by real-time PCR (Figure 1). Several of the genes induced in both the RIE (5/8) and RIE-Ras(12V) (10/42) cells have been identified as TGFβ-induced genes in previous studies (Table 2), further supporting the validity of our results. The differential expression of *FMR2* was independently confirmed by a more extensive time course using Northern blot analysis. These results were also consistent with the results from the array experiments and were particularly interesting because expression was regulated in a binary fashion, being up-regulated in RIE-Ras cells treated with TGFβ1 and down-regulated in the parental cell line (Figure 2).

Regulation of VEGF *and* CTGF *Expression by TGFβ in RIE-Ras Cells*

Two genes, *VEGF* and *CTGF*, were selected for more intensive study because they were markedly induced in Ras-transformed RIE cells, they are abundantly expressed, and they are well known as TGFβ-induced genes. *VEGF* has been implicated as a potential contributor to the pro-oncogenic activities of TGFβ [5,28]. Northern blot analysis (Figure 3*A*) and signal quantification (Figure 3*B*, *right panel*) of an expanded time course of *VEGF* mRNA expression in intestinal epithelial cells lines show a fivefold induction in the TGFβ-growth–resistant, transformed RIE-Ras cell line as opposed to a twofold induction in the parental cell line. This more robust increase in Ras-transformed RIE-1 cells occurs despite a basal level of *VEGF* expression in RIE-Ras cells that significantly exceeds that in the RIE cells (Figure 3*B*, *right panel*). Similar observations were made for the VEGF protein measured by ELISA, again showing a more prominent induction of *VEGF* by TGFβ in the RIE-Ras cell line (Figure 3*C*). As prior work has found significantly reduced Smad-mediated transcription in Ras-transformed RIE cells [19,20], it is highly unlikely that TGFβ induction of *VEGF* in these cells occurs exclusively by Smad-dependent signaling. In support of this assertion, *VEGF* mRNA expression was also significantly induced in two human colon cancer cell lines, HT-29 and SW620, both of which have defective Smad-dependent signaling (not shown).

The potential contribution of alternate, Smad-independent signaling pathways in TGFβ-mediated *VEGF* induction was examined by

Table 2. Up-regulated and Down-regulated TGFβ-Responsive Genes in RIE and RIE-Ras Cells.

Name	Accession Number	$\log_2(N)$	FDR	Function
(A) RIE-Ras: up-regulated				
Unknown	AI599739	2.86	.000	Unknown
GADD45β*	CA509894	2.64	.000	Cdc2/cyclin B1 kinase inhibitor
Unknown	BE117708	2.48	.000	Unknown
Bile acid CoA ligase	NM_024143	2.39	.001	Conjugates bile acid with amino acid
Endothelial differentiation, sphingolipid G-protein–coupled receptor, 3	BF281109	2.26	.000	G-protein–coupled receptor with putative role in angiogenesis
Adenomatosis polyposis coli 2	CB547568	2.24	.000	Homolog of tumor-suppressor APC, with putative roles in transcription and signal transduction
C1q-related factor	AI716054	2.07	.000	Unknown
Sciellin	AI717110	2.05	.002	Structural protein
Lymphocyte antigen 94	NM_057199	2.03	.017	Lysis receptor of NK cells
Thyroglobulin*	NM_030988	1.95	.000	Catalyzes formation of thyroid hormone
Calcyon	NM_138915	1.9	.000	Membrane protein that interacts with dopamine receptor
Thrombospondin*	BE127095	1.87	.000	Extracellular matrix protein with putative role in angiogenesis
*Fragile X mental retardation 2**	CB546478	1.85	.000	Putative transcription factor
Erbb2 interacting protein	CB546222	1.83	.000	Inhibit EGF signaling by preventing the activation of the Raf-1 kinase by Ras
Down syndrome cell adhesion molecule	NM_133587	1.72	.000	Ig superfamily cell-adhesion molecule thought to function in neuronal development
Unknown (serine/threonine kinase domain)	AW527300	1.7	.000	Unknown
Isopeptidase T-3	AW521619	1.69	.000	Ubiquitin-specific protease
Jun B proto-oncogene*	NM_021836	1.69	.000	AP-1 family transcription factor
Basic helix-loop-helix domain containing, class B2*	NM_053328	1.68	.000	Transcription factor
MAD homolog 7*	NM_030858	1.68	.000	Inhibits Smad3/Smad4 signaling
FLJ00179 protein	BG673684	1.66	.000	Unknown
Myosin binding protein H	NM_031813	1.66	.014	Structural protein involved in myosin binding
Hypothetical C2H2 zinc finger protein	CB546651	1.65	.000	Putative transcription factor
Plasminogen activator inhibitor type 1[1]	NM_012620	1.62	.000	Major inhibitor of plasminogen activators
Gb3 synthase	NM_022240	1.62	.004	Catalyzes glycosphingolipid synthesis
Receptor type protein tyrosine phosphatase psi	U66566.1	1.6	.000	Transmembrane receptor–type protein tyrosine phosphatase with putative role in neuronal development
Neuraminidase 1	NM_031522	1.59	.000	Removes sialic acids from glycoproteins and gangliosides
Preproprolactin-releasing peptide	NM_022222	1.56	.000	Stimulates stress hormone secretion by either direct pituitary or indirect hypothalamic actions
Leukemia/lymphoma–related factor	CB546785	1.54	.000	Zing finger transcription factor
Tenascin XB	CB544736	1.54	.000	Extracellular matrix glycoprotein
Connective tissue growth factor*	NM_022266	1.53	.000	Matricellular protein that plays an essential role in the formation of blood vessels, bone, and connective tissue
Unknown function	CB544526	1.52	.000	Unknown
Unknown function	AI409493	1.47	.000	Unknown
Ski-related novel protein N*	CB548407	1.46	.000	Smad 2/Smad 3 repressor
Hypothetical intermediate filament proteins containing protein	BM389145	1.45	.002	Putative structural protein in cytoskeleton or nuclear envelope
GADD45γ*	BF419904	1.45	.000	Cdc2/cyclin B1 kinase inhibitor
Olfactory protein	M64376.1	1.45	.004	Seven transmembrane domain protease
Eferin	CB545653	1.45	.032	ADP-ribosylation factor binding protein of unknown function
45-kDa secretory protein	AJ132352.1	1.44	.000	Lipid binding protein of unknown function
Activator of G-protein signaling 3	NM_144745	1.43	.001	Guanine nucleotide dissociation inhibitor that contains G protein regulatory motifs in its C-terminal domain
Vascular endothelial growth factor*	NM_031836	1.4	.000	Vascular growth factor
(B) RIE-Ras: down-regulated				
S100A6	NM_053485	−2.38	.000	Calcium binding protein of unknown function
Cadherin 15	CB544279	−2.15	.020	Cell-cell adhesion molecule
Stomatin-like 1	CB544301	−1.39	.049	Putative sterol-binding protein
(C) RIE: up-regulated				
MAD homolog 7*	NM_030858	2.16	.000	Inhibits Smad3/Smad4 signaling
GADD45β*	CA509894	2.03	.000	Cdc2/cyclin B1 kinase inhibitor
Basic helix-loop-helix domain containing, class B2*	NM_053328	1.87	.000	Transcription factor
Unknown	CB545096	1.77	.002	Unknown
Vascular endothelial growth factor*	NM_031836	1.64	.000	Vascular growth factor
Jun B proto-oncogene*	NM_021836	1.55	.004	AP-1 family transcription factor
Hairy and enhancer of split 1	NM_024360	1.4	.000	Notch pathway homeobox transcription factor
Unknown	CB546780	1.4	.000	Zinc finger domain, unknown function
(D) RIE: Down-regulated				
Nuclear distribution gene C homolog	NM_017271	−2.04	.000	Regulates movement of nuclei after mitosis
Ribosomal protein L27	NM_022514	−1.9	.001	Ribosomal protein
Ribosomal protein S29	NM_012876	−1.83	.004	Ribosomal protein
Glutathione S-transferase P subunit	NM_138974	−1.7	.002	Component of glutathione *S*-transferase
Helicase DDX32	CB548436	−1.59	.006	DEAH family helicase
Hypothetical protein FLJ12800	BE106894	−1.56	.009	Unknown
*Fragile X mental retardation 2**	CB546478	−1.5	.017	Putative transcription factor
Unknown	BF390720	−1.45	.000	Putative component of Golgi complex

*Previously identified as TGFβ-regulated.

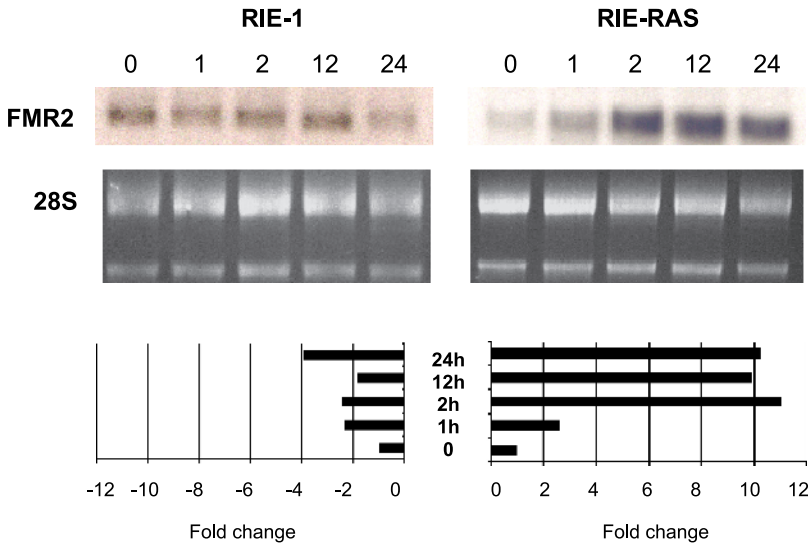

Figure 2. RIE and RIE-Ras Northern blots show differential expression of *FMR2* in response to TGFβ1. The RIE gene expression array showed inhibition of *FMR2* expression after 1 hour of incubation with 2 ng/ml TGFβ1, whereas the RIE-Ras(12V) array showed activation of *FMR2* gene expression. These findings were supported by Northern blot analysis of *FMR2* gene expression as shown in the top panel. In the middle panel, equivalent RNA loading was confirmed verified by ethidium bromide staining of 18 and 28S ribosomal RNA. RNA induction by TGFβ1 was quantified by scanning the autoradiographs and analyzing band intensity with densitometry (bottom panel). Results from these Northern blots are consistent with *FMR2* expression results obtained in the microarray experiments and show a marked differential effect on *FMR2* expression, with 11-fold maximal induction in Ras-transformed cells and a fourfold repression in the parental cell line. In these graphs, band intensity is reported as the signal at any given time point relative to the signal of the control RNA or "0 hour" sample.

use of specific small molecule inhibitors. Inhibition of the p38 MAPK pathway and the phosphoinositol 3-kinase pathway, two signaling cascades previously implicated in Smad-independent responses to TGFβ [13,29], did not attenuate induction of *VEGF* by TGFβ1 (Figure 3*D*). Inhibition of MAP kinase kinase (MEK) with U-0126 and Raf-1 kinase with BAY-439006 blocked induction of *VEGF* by TGFβ, implicating TGFβ activation of Raf-1/Erk signaling as a potential Smad-independent pathway for TGFβ signaling in Ras-transformed RIE-1 cells. Interestingly, in the setting of Raf-1 kinase inhibition, levels of VEGF were not further increased by TGFβ1, but basal levels of *VEGF* were elevated above control.

The role for *CTGF* in tumorigenesis is not clear, but in certain cellular contexts, *CTGF* is a pro-oncogenic, profibrogenic growth factor [30]. It is tumor-suppressive in other contexts [31]. *CTGF* is also frequently identified as a prominently regulated gene in genomic profiles of TGFβ action [32,33] a finding that we confirm herein. *CTGF* expression was also differentially and prominently up-regulated in RIE-Ras cells, with maximal expression 31-fold above basal levels occurring 2 hours after treatment with TGFβ in RIE-Ras cells compared with a 3.5-fold induction in the parental RIE-1 line (Figure 4*A*). *CTGF* expression was also robustly induced in HT-29 cells, a Smad4-deficient human colon carcinoma cell line that is resistant to growth inhibition by TGFβ (not shown), implying Smad independence. Figure 4*B* shows markedly reduced basal levels of *CTGF* in exponentially growing RIE-Ras(12V) cells compared with the parental line. The original of this differential expression is being explored separately. In contrast to the involvement of the canonical MAPK pathway in *VEGF* induction by TGFβ in Ras-transformed cells (Figure 3*A*), induction of *CTGF* was not blocked by inhibitors of the p38 MAPK, phosphoinositol 3-kinase, MEK, or Raf-1 kinase pathways (Figure 4*C*). Thus, the Smad4-independent signaling pathway responsible for TGFβ induction of *CTGF* in Ras-transformed intestinal remains unclear.

Discussion

Until recent years, the pro-oncogenic effects of TGFβ could only be indirectly inferred [34–36] from studies that describe increased TGFβ levels in metastatic tumors [37,38] or report increased expression of TGFβ as a risk factor for recurrence and reduced survival [39]. For instance, colorectal cancers with mutant, inactivated TGFβRII have presumably no potential for pro-oncogenic signaling and a prognosis that is better than cancers with retention of one allele [40]. Conversely, restoration of TGFβRII expression in cells that carry a mutation in this gene increases cell invasiveness *in vitro* [35]. Thus, once tumors escape growth regulation by TGFβ, if the potential remains for residual TGFβ signaling, the result is an enhanced potential for tumorigenesis. Furthermore, the apparent duality of TGFβ signaling has been observed *in vivo*; in bitransgenic mice, mutant activated TGFβ is associated with delayed tumor onset in early stages, whereas in later stages, it leads to an increase in metastatic foci [7,41].

It is clear that oncogenic events such as transformation by mutant activated Ras may "adversely" interact with growth inhibitory TGFβ signaling. In Ras-transformed cells, loss of an autocrine growth-inhibitory GFβ signaling occurs coincident with a synergistic stimulation of angiogenic molecules [42], matrix molecules [43], and epithelial to mesenchymal transition [35], each of which is believed to be important for tumor growth and metastasis *in vivo*. Inducible Ras in an *in vitro* model using the same rat intestinal epithelial cell line used in our current study conferred characteristics, such as invasiveness, consistent with tumor promotion and metastasis [44]. Ras

overexpression also activates transcription of the *TGFβ1* gene [45]. Increased levels of TGFβ in this context may further contribute to tumor-promoting signaling in growth-resistant cells.

In the present study, we found that oncogenic Ras confers to TGFβ a significant gain of robust early genomic responses in cultured rat intestinal epithelial cells that are resistant to growth inhibition. Although we found five immediate transcriptional targets of TGFβ activated in both RIE and RIE-Ras(12V) cells, a larger number of genes are up-regulated by TGFβ only in the context of Ras activation. These findings imply a model in which Ras-dependent TGFβ early genomic targets may be responsible for pro-oncogenic activities of TGFβ and potentially contribute to resistance to growth inhibition as well.

The well-recognized reduction of Smad-dependent signaling that is characteristic of Ras-transformed epithelial cells [19,20,46,47] indicates that the expanded genomic response to TGFβ occurs by an as yet unidentified Smad-independent pathway or by an unrecognized modification or interaction with the canonical Smad signaling pathway. Several published reports compare transcriptional responses in epithelial cell lines that are sensitive and resistant to growth inhibition by TGFβ [32,48]. The conflicting findings in the aforementioned reports, both of which examined the consequences of silenced Smad4 on TGFβ-mediated gene expression, serve to further underscore the complexity of TGFβ signaling and reinforce the necessity for further investigation.

Although our own experimental approach was not designed to specifically determine functional profiles of genes induced by TGFβ1, it is unexpected that the *GADD45β*, a gene product generally associated with apoptosis and cell cycle arrest, was prominently induced in both TGFβ-growth–sensitive parental cells and growth-resistant Ras-transformed cells (Table 2), again emphasizing the complexity and intricacy of TGFβ signaling. Transforming growth factor β also regulates distinct genomic profiles in HaCaT cells with Smad2 and Smad3 individually attenuated by specific antisense molecules [49].

In a similar study examining transcriptional responses in a control cell line *versus* a Ras-transformed line, Chen et al. [33] found that four of the five genes up-regulated in our studies of RIE and RIE-Ras(12V) cells were also up-regulated by TGFβ exposure for 2 to 4 hours in a nontumorigenic mammary cell line (MCF-10A) that is sensitive to growth inhibition by TGFβ. Those same four genes were also up-regulated by TGFβ in MCF-10A cells dually transformed with activated c-Ha-*ras* and c-*erbB2*, as well as in a human breast cancer cell line with hyperactive Ras (MDA-MG-231) both of

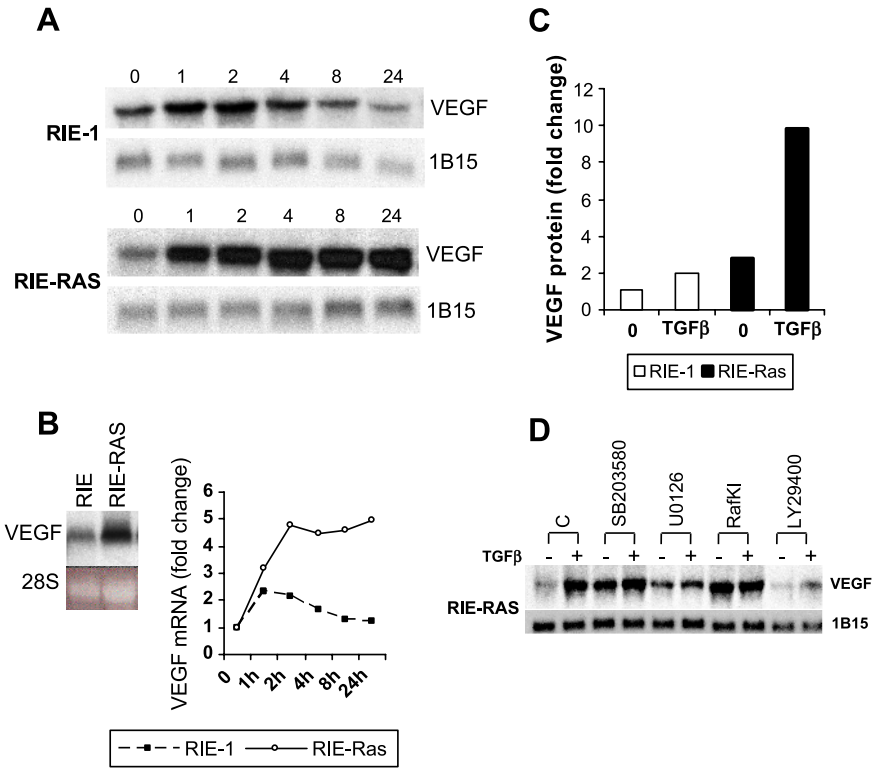

Figure 3. Induction of *VEGF* by TGFβ in RIE and RIE-Ras(12V) cells. (A) Northern blots were prepared from RNA isolated from RIE and RIE-Ras(12V) cells treated with 2 ng/ml of TGFβ1 for the time intervals shown and probed for *VEGF* expression. Equivalent loading was confirmed by the signal intensity of IB15 (*cyclophilin*) a constitutively expressed gene. In (B), basal expression of *VEGF* is shown in RIE and RIE-Ras(12V) cells, confirming markedly increased basal expression in the Ras-transformed line. The bottom right shows densitometric values obtained from the Northern shown in the top panel with expression shown as a fold change overexpression at time 0. As shown in (C) VEGF protein was also differentially induced in RIE-Ras(12V) cells. Whole-cell protein lysates were obtained from RIE-1 and RIE-Ras(12V) cells treated with TGFβ for 24 hours. VEGF protein was quantified by ELISA, as described in the Materials and Methods section. (D) Effect of signaling pathway inhibitors on induction of *VEGF* by 2 ng/ml TGFβ1. Cells were treated with 10 μM SB203580 (p38 kinase inhibitor), 10 μM U0126 (a MEK inhibitor), 1 μM RafKI (BAY-439006, a Raf-1 kinase inhibitor), and 20 μM LY294002 (phosphoinositol 3-kinase inhibitor) for 24 hours and then treated with 2 ng/ml TGFβ1 for 1 hour before isolation of RNA according to procedures described in the Materials and Methods section. Equivalent loading was confirmed by hybridization with a cDNA probe complementary to *cyclophilin*. The results shown are representative of three separate experiments.

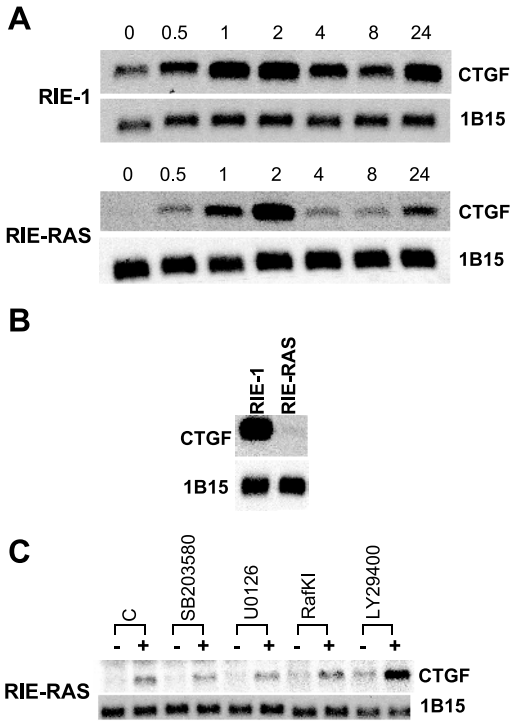

Figure 4. Induction of *CTGF* by TGFβ in RIE and RIE-Ras(12V) cells. (A) Northern blots were prepared from RNA isolated from RIE and RIE-Ras(12V) cells treated with 2 ng/ml of TGFβ1 for the time intervals shown and probed for *CTGF* expression. Equivalent loading was confirmed by the signal intensity of *1B15*, a constitutively expressed gene. (B) Relative expression of *CTGF* in RIE and RIE-Ras (12V) cells shows markedly reduced basal expression in the Ras-transformed line. Equivalent loading is confirmed by expression of *1B15*, a constitutively expressed RNA species. (C) Organized identically to Figure 3*D*, and the RNA samples were identical to those used in Figure 3*D*. Cells were treated with 10 μM SB203580 (p38 kinase inhibitor), 10 μM U0126 (a MEK inhibitor), 1 μM RafKI (BAY-439006, a Raf-1 kinase inhibitor), and 20 μM LY294002 (phosphoinositol 3-kinase inhibitor) for 24 hours and then treated with 2 ng/ml TGFβ1 for 1 hour before isolation of RNA according to procedures described in the Materials and Methods section. Equivalent loading was confirmed by hybridization with a cDNA probe complementary to *cyclophilin*. The result shown is representative of three separate experiments.

which are resistant to growth suppression by TGFβ [33]. The relative magnitude of the genomic response was not provided in the report by Chen et al, except to allude to the fact that many responses were generated in the Ras-transformed lines. These results, along with others [50,51], indicate that despite resistance to TGFβ growth inhibition, transformed epithelial cell lines from multiple epithelial lineages retain at least some common TGFβ transcriptional targets as well as an expanded repertoire of genomic responses. Thus, the results observed in the present study and others reported to date seem to be generalizable.

Individual Ras-specific, TGFβ up-regulated genes identified in our study have been implicated in tumor progression. For example, plasminogen activator inhibitor type 1 (PAI-1) is up-regulated in human cancer and correlates with metastatic behavior [52]. Thrombospondin and VEGF are associated with angiogenesis and metastasis (reviewed in Bergers and Benjamin [53]). The ErbB2-interacting protein (Erbin) is a PDZ domain-containing protein that binds the C-terminus of ErbB2 that is essential for epithelial integrity. Recent studies show that Erbin suppresses Ras activation of Erk signaling while preserving activation of other Ras effectors such as Akt. A recent expression profile of genes expressed in breast cancer includes Erbin as a "poor prognosis" marker [54].

A homolog of the tumor-suppressor gene adenomatosis polyposis coli (*APC*), *APC2*, was also specifically up-regulated in the RIE-Ras (12V) cell line. *APC1* and *APC2* have overlapping roles in Wingless pathway signaling in *Drosophila* and mice [55]. Loss of expression of *APC* has been associated with most sporadic colon adenocarcinomas leading to overactivation of the Wingless/Wnt signaling pathway (reviewed in Grady and Markowitz [56]). It is not clear how overexpression of *APC2* might contribute to tumorigenesis; however, its expression has recently been found to be up-regulated in lung adenomas and lung adenocarcinomas compared to normal lung [57]. Neither *APC* nor *APC2* has previously been identified as a transcriptional target of TGFβ.

An intriguing finding in our analysis is that fragile X mental retardation gene 2 (*FMR2*) was among the most repressed genes in RIE cells and among the most activated in RIE-Ras(12V) cells. Northern blot analysis of *FMR2* expression at multiple time points verified the finding of the array that *FMR2* is induced by TGFβ in RIE cells and is inhibited in RIE-Ras cells (Figure 2, *A* and *B*). *FMR2* was originally cloned in an attempt to identify the basis for FRAXE-linked mental retardation. Subsequent analysis localized *FMR2* to the nucleus and identified it as a transcriptional activator (reviewed in Gu and Nelson [58]). *LAF4*, an *FMR2* family member, was identified through chromosomal translocations that resulted in Burkitt lymphoma [59]. Rearrangements of *LAF4*, as well as two other *FMR2* family members, *AF4* and *AF5q21*, with the mixed leukemia lineage (*MLL*) gene have been identified in infant acute lymphoblastic leukemia, indicating that this gene family may have a function in cell proliferation and oncogenesis [60–62]. *Lilliputian*, an FMR2 *Drosophila* homologue, is involved in both TGFβ [63] and Erk signaling [64], which is intriguing in light of our observation that *FMR2* expression was repressed by TGFβ in RIE cells and induced by TGFβ in the setting of oncogenic Ras. The role of this gene in the cellular response to TGFβ merits further study.

VEGF and *CTGF* expression in response to TGFβ exposure was examined in more detail in our study. These genes are more robustly regulated in RIE-Ras cells than the parental line, raising the important question of the signaling mechanics involved. Because Smad-dependent signaling is attenuated in Ras-transformed RIE cells [19], it is unlikely that this pathway accounts for the accentuated expression of these TGFβ-inducible genes. Our results indicate that activation of the canonical Raf-1/MAPK/Erk pathway may contribute to *VEGF* but not *CTGF* induction. Additional candidate pathways deserve further exploration.

In summary, ours is the first study designed to identify and characterize the TGFβ-induced transcriptome in intestinal epithelial cells transformed by oncogenic Ras. Despite the attenuation of Smad-dependent, growth-inhibitory signaling in intestinal epithelial cells that overexpress oncogenic Ras, TGFβ induces a significantly expanded repertoire of highly regulated (≥2.6-fold induction) genomic targets in these cells. This finding is analogous to other existing work that shows an expanded genomic profile when specific components of the Smad pathway are abrogated. The biologic properties of many of these TGFβ-induced genes are consistent with a tumor-promoting role for TGFβ and deserve further scrutiny as pro-oncogenic factors.

A critical consideration is the extent to which this unique transcriptome occurs because of Smad-independent signaling, Smad signaling that is modified in an unrecognized manner by hyperactive Ras activity, or both.

Supplemental Data

Raw Data

Raw image files (.tif) for each experiment are available at the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo) with the Series Accession Number GSE2015. These images are the original chip scans used to generate the data in this study.

Complete Data Tables

Data tables are listed as RIE *versus* RIE-TGFβ × 1 hour and RAS *versus* RAS-TGFβ (for the RIE-Ras experiments), each with worksheets #1 to #4 corresponding to results from experiments #1 to 4. The tables summarize results including raw signal intensity from each channel (Lex.R corresponds to control, Lex.E corresponds to experimental), raw signal intensity with background subtracted, normalized signal intensity, fold change (experimental/control), flag status, Agilent gene description, Agilent probe identification, and GenBank Accession Number. Results are listed in ascending order of Agilent probe identification. Control spots on the Agilent Rat Oligo chip (Agilent G4130A) not corresponding to sequence from real genes were excluded from the table. Data including fold change and log ratios are also available at the NCBI GEO Web site (http://www.ncbi.nlm.nih.gov/geo) with the Series Accession Number GSE2015.

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