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Tumor suppressor and oncogene actions of TGFβ1 occur early in skin carcinogenesis and are mediated by Smad3

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Abstract

Interactions between TGFβ1 and *ras* signaling pathways play an important role in cancer development. Here we show that in primary mouse keratinocytes, *v-ras*^{Ha} does not block the early biochemical events of TGFβ1 signal transduction but does alter global TGFβ1 mediated gene expression in a gene specific manner. Expression of Smad3 dependent TGFβ1 early response genes and the TGFβ1 cytostatic gene expression response were not altered by *v-ras*^{Ha} consistent with an intact TGFβ1 growth arrest. However, TGFβ1 and *v-ras*^{Ha} cause significant alteration in genes regulating matrix remodeling as the TGFβ1 induction of extracellular matrix genes was blocked by *v-ras*^{Ha} but specific matrix proteases associated with cancer progression were elevated. Smad3 deletion in keratinocytes repressed normal differentiation marker expression and caused expression of Keratin 8 a simple epithelial keratin and marker of malignant conversion. Smad3 was required for the TGFβ1 cytostatic response in *v-ras*^{Ha} keratinocytes, but also for protease induction, keratinocyte attachment and migration. These results show that pro-oncogenic activities of TGFβ1 can occur early in carcinogenesis before loss of its tumor suppressive function and that selective regulation rather than complete inactivation of Smad3 function may be crucial for tumor progression.

Keywords

TGFβ1; *ras* oncogene; skin carcinogenesis; Smad3; keratinocytes; transcriptional profiling

Introduction

Transforming growth factor β1 (TGFβ1) is a cytokine with context dependent tumor suppressor and tumor promoting activity in cancer development. For most normal epithelial cells, and at early stages of tumor development TGFβ1 is believed to regulate gene expression in ways that are consistent with its function as a tumor suppressor [1]. This largely occurs through activation of the surface serine-threonine receptor complex and phosphorylation intracellular Smads, although other signaling pathways are also engaged [2]. In contrast elevated expression of TGFβ1 may enhance malignant properties of tumor cells through effects on invasion, metastasis, epithelial to mesenchymal transition or anti-tumor immunity [3]. This alteration in function of TGFβ1 during tumor progression may in part depend on the interaction with other signaling pathways such as *ras* that become

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activated during tumor progression [3]. Thus overexpression of an oncogenic *ras* or activation of the ERK pathway has been reported to block TGF β 1 or TGF β superfamily signaling through several distinct mechanisms [4–9]. However, oncogenic *ras* and TGF β 1 can cooperate to enhance epithelial to mesenchymal transition and invasive behavior of neoplastic breast cancer cells [10,11]. However it is not clear if these studies done in immortal and cancer cell lines are applicable to normal epithelial cells.

Two-stage chemical carcinogenesis of the mouse epidermis is a widely used model to study the interaction of TGF β 1 and oncogenic *ras*. Mutations in the c-Ha-*ras* gene occur in most benign and malignant squamous tumors derived from 7,12-di methylbenz[*a*]anthracene (DMBA) initiation and 12-O-tetradecanoylphorbol-13-acetate (TPA) promotion [12], and this can be modeled *in vitro* using a retrovirus expressing v-*ras*^{Ha} to infect primary mouse keratinocytes (MEK). v-*ras*^{Ha} causes MEK hyperproliferation, altered expression of differentiation-related genes [13], and formation of papillomas in a skin graft [14]. TGF β signaling can suppress squamous tumor formation and progression initiated by an activated cellular or viral *ras* oncogene [15–18] but overexpression of TGF β 1 in normal skin causes formation of highly malignant spindle cell carcinomas [19] and enhances a metastatic phenotype of benign squamous tumor cells [20]. The different roles of TGF β 1 in skin carcinogenesis may depend on the interaction of Smad2 and Smad3 with *ras* signaling. Both Smad2 and Smad3 expression are lost during progression of chemically induced tumors [21], and expression of v-*ras*^{Ha} in Smad3 null MEK causes rapid progression to squamous cell carcinoma in a skin graft system [22]. However other studies have shown that Smad3 is required for tumor formation in the 2-stage carcinogenesis model [23], suggesting context dependent interactions between *ras* and Smads that are critical for tumor suppression or progression in this model.

In this report we have used normal and Smad3 null MEK to examine the effect of oncogenic *ras* on TGF β 1 signaling in a primary epithelial cell and identify how Smad3 impacts cancer progression. Here we show that oncogenic *ras* alters TGF β 1 regulated gene expression in a gene specific manner producing a phenotype with characteristics of tumor suppression and tumor progression. Loss of Smad3 both enhances and inhibits TGF β 1 responses associated with progression, suggesting that selective rather than global inactivation of key TGF β 1 responses is an essential component of tumor progression.

Materials and Methods

Cell Culture

Primary keratinocytes were isolated from newborn BALB/c mice and cultured as described previously [24]. On day 3 of culture, the keratinocytes were transduced with a replication-defective retrovirus containing the v-*ras*^{Ha} gene [14] at a moi of 1–2 and are referred to as v-*ras*^{Ha} keratinocytes. The high titer of this retrovirus ensures that all keratinocytes in the culture are infected without selection. Smad3 wildtype and null keratinocytes (C57Bl/6 $\times$ SVJ) were isolated and cultured as described [22]. For induction of differentiation, confluent cultures were switched to medium containing 0.12 mM CaCl₂ for 24 hours which induces a normal pattern of squamous differentiation *in vitro* [25]. In all experiments keratinocytes were treated with 1 ng/ml TGF β 1 (R&D) after 4 days of v-*ras*^{Ha} infection and RNA or protein was harvested at the indicated times. Luciferase assays to measure TGF β 1 induced gene expression were done by transfecting MEK in quadruplicate with the luciferase reporter pGL-SBE4-luc containing a TGF β 1 responsive Smad binding element [26] and a renilla luciferase internal control. Cells were treated with or without 1 ng/ml TGF β 1 for 24 hrs before analysis of luciferase activity using a dual luciferase system (Promega).

Gelatin Zymography

Conditioned media was made by incubating keratinocytes in 0.2% chelex-treated serum and 0.05mM Ca^{2+} with or without TGF β 1 (1ng/ml) for 24 hours. Equal volumes of supernatant was added to non-reducing sample buffer (2% SDS, 50mM Tris-HCl, 20% glycerol, and 0.01% bromophenol blue) and protease activity was detected using 10% SDS-PAGE gels copolymerized with gelatin (1mg/ml) as described [27].

^3H -Thymidine Incorporation

DNA synthesis in MEK or v-*ras*^{Ha} MEK of indicated genotypes treated with 1 ng/ml TGF β 1 for 24 hrs was measured in quadruplicate as previously described [22].

Migration Assay

To measure keratinocyte migration, confluent monolayers of either normal or v-*ras*^{Ha} transduced MEK were treated for 3 hrs with 10ug/ml mitomycin C (Sigma) in PBS to prevent cell proliferation and then washed and allowed to recover for 1 hr in media. A scratch was made in the monolayer using a plastic pipette tip, and a photographic record made of the scratch distance using a 10X objective and a Spot digital camera (Nikon). Cells were treated with 1 ng/ml TGF β 1 and photographs were taken at the same location as the zero time measurement to record distance migrated into the scratch. Gap closure was measured in photographs using Spot Advanced Software, and the distance migrated was determined by subtracting the gap distance measured at 10 or 24 hrs from the initial measurement divided by 2. Replicates were measured at two different regions along the gap with at least 8 measurements made at each region. Each treatment group was done in triplicate and the experiment was repeated twice.

Attachment Assay

4 days after virus infection keratinocytes and controls were treated with 1 ng/ml TGF β 1 for 24 hrs, and then trypsinized, and plated at 5×10^4 cells/well into 96 well trays that had been precoated overnight with purified type I laminin, type IV collagen or fibronectin (BD Biosciences) at 10 ug/well and blocked with BSA. After incubation at 37 degrees for 3 hrs in a CO₂ incubator, the wells were washed and attached cells were measured using an MTT assay (Promega). Each treatment group was done in quadruplicate.

Immunostaining

Cells were fixed with ice-cold methanol for 10 min, blocked with 5% donkey serum in 1% BSA-TBS, and stained with Smad2/3 (N-19, Santa Cruz) or H-*Ras* (C-20, Santa Cruz) antibodies overnight at 4 degrees. After incubation with labeled secondary antibodies cells were mounted with VECTASHIELD with DAPI (Vector Labs.) and analyzed by Zeiss LSM 510 microscope (Confocal Core Facility, NCI).

Immunoblotting

Nuclear and cytoplasmic extracts were isolated and immunoblotting was carried out as described [22]. Total cell extracts to measure keratins were isolated as described [25]. The primary antibodies used were as follows: Smad2, Smad3, and Smad4 (Zymed); H-*Ras* (C-20), Lamin A/C (Santa Cruz); pERK1/2, ERK1/2, pSmad2 (Cell Signaling Technology); β -actin (Chemicon); Keratin 8 (University of Iowa Hybridoma bank); and Keratin 1, 10, Loricrin (Covance).

Microarray Analysis

Total RNA was isolated from 5 independent cultures using TRIzol (Gibco BRL) and purified using the QIAGEN RNeasy Midi Kit (Qiagen), with on column DNase digestion. RNA from primary and *ras* MEK treated for 1, 6, 24, and 48 hrs with TGF β 1 at 1ng/ml was reverse transcribed and compared in dual fluor hybridizations to RNA from the corresponding untreated control using NCI 9000 feature cDNA microarrays. For each time point sample from primary and *ras* cells, 5–8 arrays including dye swaps were analyzed. To examine the role of Smad3 in the effects of oncogenic *ras* on TGF β 1 mediated transcriptional response, RNA was isolated from primary keratinocytes derived from Smad3 wildtype and null mice and mock infected or infected with the *v-ras*^{Ha} retrovirus. Microarray analyses were performed according to a published protocol [28]. Signal intensity was detected using the Axon GenePix 4000 scanner and analyzed with GenePix Pro 5.0 software (Axon). The image and data were uploaded into the NCI microarray database system supported by the Center for Information Technology of NIH for data interpretation and visualization. Gene expression ratios for each time point for a given gene were averaged after preliminary filtering, and then TGF β 1 regulated genes were identified as those with ratios greater than 1.8 or less than 0.56-fold in at least 1/8 averaged arrays across the time course. For Smad3 wild type and null data set, genes with expression ratio greater than 1.8 or less than 0.56 in at least 1/4 averaged arrays were identified as the Smad3 data set. Two independent RNA samples were analyzed for this data set. The data were further analyzed using K-means clustering to identify similar and/or distinct expression patterns in primary and *ras* keratinocytes following TGF β 1 treatment. All array data are deposited for public viewing in the Gene Expression Omnibus (GEO) at www.ncbi.nlm.nih.gov/geo under GEO accession GSE 3228. Expression of selected genes was measured using the LightCycler (Roche) or MyIQ system (BioRad) to validate the results of microarray data as described previously [28]. The primers for quantitative PCR were designed by PrimerSelect (DNASTAR Inc.) and sequences used are available in Supplemental Table I. Expression of *Gapdh* or β -actin was used as a normalization control for quantitative PCR.

Results

Oncogenic *ras* does not block initial TGF β 1 induced biochemical responses in primary mouse keratinocytes

To examine the effects of oncogenic *ras* on the TGF β 1 signaling pathway in a normal epithelial cell, we infected primary mouse epidermal keratinocytes (MEK) with a replication defective retrovirus expressing *v-ras*^{Ha} [14] and analyzed the initial Smad dependent steps in the TGF β 1 signal transduction pathway. High levels of Ras protein and hyperactivation of ERK1/2 were confirmed by immunoblotting (Fig.1A) and elevated Ras protein expression in all cells was demonstrated by indirect immunofluorescence (Fig.1D). Figure 1B shows that as expected *v-ras*^{Ha} expression elevated mRNA levels of several cell growth associated genes such as proliferin (4-fold), TGF α (17-fold), and cyclin D1 (6-fold). When control and *v-ras*^{Ha} transduced MEK were treated with TGF β 1 there was an increase Smad2, 3 and 4, increased phosphorylation of p-Smad2 that followed similar patterns of induction and decay in both cell types (Fig.1C), and similar nuclear translocation of Smad2/3 in both normal and *v-ras*^{Ha} transformed keratinocytes (Fig.1D). Western analysis (Fig.1C) showed that the total levels of Smad3 and 4 were slightly elevated in the *v-ras*^{Ha} MEK, but no change in levels of p-ERK1/2 were observed after treatment with TGF β 1. Thus at this level of analysis oncogenic *ras* does not alter TGF β 1 induced Smad phosphorylation or translocation, and TGF β 1 does not alter *v-ras*^{Ha} induced hyperactivation of ERK1/2.

Gene specific effects of oncogenic ras on TGF β 1 signaling

To assess the effects of *v-ras*^{Ha} on generic TGF β 1-induced gene expression we transfected MEK and *v-ras*^{Ha} transduced MEK with a SBE (Smad binding element)-luciferase reporter that is dependent on TGF β 1 and Smad signaling for activation. TGF β 1 stimulated SBE driven luciferase activity 6 fold in the MEK but there was only a 2 fold increase in the *v-ras*^{Ha} transduced MEK (Fig. 2), suggesting that despite similar immediate biochemical responses as observed in Figure 1, *v-ras*^{Ha} was able to inhibit TGF β 1 mediated transcriptional activation. To determine if oncogenic ras blocked all or a subset of TGF β 1 transcriptional responses we analyzed global patterns of TGF β 1 mediated gene expression in the presence or absence of *v-ras*^{Ha}. Gene expression patterns were compared in control and *v-ras*^{Ha} expressing keratinocytes after 1, 6, 24 and 48 hrs of treatment with TGF β 1. 274 TGF β 1-responsive genes were identified (Supplemental Table IA) using the average expression ratio from at least 5 arrays for each treatment and filtering for genes whose expression ratio was greater than 1.8 or less than 0.56 for at least 1 time point. A hierarchical clustering method utilizing the K-means algorithm was used to group genes based on similar expression patterns throughout the time course (Fig. 3A). For each cluster the average expression ratio of all the genes was calculated and plotted against time, as shown in Figure 3B. Two major clusters were observed: the first cluster containing 94 genes whose induction (cluster 1, n=42 genes) or repression (cluster 3, n=52 genes) by TGF β 1 was not altered by *v-ras*^{Ha}, and there was no statistically significant difference in the average expression ratio of genes in these clusters associated with the presence or absence of *v-ras*^{Ha} and a second cluster of 140 genes whose induction (cluster 2, n=66 genes) or suppression (cluster 4, n=74 genes) by TGF β 1 was reduced or blocked by *v-ras*^{Ha}. For these clusters by 48 hrs post TGF β 1 treatment where maximal induction or repression of gene expression by TGF β 1 occurred, the presence of *v-ras*^{Ha} significantly altered the expression ratio ($p < .001$). Thus these results show that rather than simply suppressing all TGF β 1 signaling, ras oncogene signaling in keratinocytes alters TGF β 1 mediated gene expression in a gene specific manner.

Early transcriptional responses to TGF β 1 are not blocked by oncogenic Ras

Several studies have identified a set of genes that are rapidly activated or repressed following activation of TGF β 1 signaling [29]. Figure 4 shows results from the microarray analysis (A) and quantitative PCR (B) expressed as log₂ ratios for each treated time point relative to untreated control for several TGF β early response genes. The initial pattern of TGF β 1 induction or repression of *Skil*, *Smad7*, *Tiegl*, *Irb1*, *Irb2*, *Ier3* and *c-Myc* was not significantly altered by *v-ras*^{Ha}, although *Smad7* expression was sustained through 6 hrs and there was a more rapid return of *c-Myc* levels to baseline in the *v-ras*^{Ha} keratinocytes. Since previous studies have shown that these TGF β 1 primary response genes are largely dependent on Smad3 we tested how loss of Smad3 altered gene expression of *Smad7* in the *v-ras*^{Ha} keratinocytes. TGF β 1 induced a 4 fold increase in *smad7* expression within 1 hr in the Smad3 wildtype *v-ras*^{Ha} MEK that was significantly reduced in the Smad3 null *v-ras*^{Ha} MEK as well as control MEK (Fig. 4C). Although non-Smad3 pathways may contribute to the slight induction of *Smad7* by TGF β 1 in the Smad3 null keratinocytes, these results show that the TGF β 1 induction of *Smad7* is not affected by overexpression of oncogenic ras.

Expression of matrix remodeling but not cell cycle genes are blocked by ras

TGF β 1 is a potent inhibitor of normal epithelial cell proliferation and this is mediated in part through downregulation of c-myc and induction of several cyclin dependent kinase inhibitors [30]. Cell cycle associated genes were highly represented in the cluster of genes downregulated in both primary MEK and *v-ras*^{Ha} MEK, including *Ccnb1*, *Ccnd2*, *Cdc2a*, *Cenpa*, *Gmnn*, *Mad2l1*, *Mcm5*, *Mki67*, *Odc*, *Plk1*, *Tk1*, *Top2a*, *Ung*, and *Tpx2* (Fig. 5A,B), and suggest that *v-ras*^{Ha} cannot abrogate the TGF β 1 cell cycle arrest program. To test this

we treated keratinocytes with different doses of TGF β 1 for 24 hrs and measured DNA synthesis using ^3H -thymidine incorporation. Despite somewhat altered kinetics of *Smad7* and *c-Myc* response to TGF β 1 in the *v-ras*^{Ha} MEK (Fig. 4A,B), Figure 5C shows that TGF β 1 inhibited DNA synthesis in the primary keratinocytes and *v-ras*^{Ha} MEK with the same dose response, indicating that the cytostatic response program was largely intact despite high levels of *v-ras*^{Ha} expression.

While cell cycle genes were unaffected, *v-ras*^{Ha} blocked the TGF β 1-mediated induction of most genes encoding extracellular matrix proteins including *Bgn*, *Col1A1*, *Col5A1*, *Pcdh7*, *Itbp1* and *Sparc*. Figure 6A shows the averaged log₂ expression ratio of multiple ECM genes relative to untreated cells, at 1, 6 24 and 48 hrs after treatment with TGF β 1 from microarray analysis. Figure 6B shows validation for expression of 7 individual genes at 24 hrs post TGF β 1 treatment by quantitative PCR. TGF β 1 induced an approximately 3 fold increase in expression for these genes by 48 hrs and this induction was blocked by *v-ras*^{Ha} (Fig. 6A). In contrast, gene expression of two proteases critical in matrix remodeling and tumor cell invasion, *Urokinase* (uPA) and *Mmp9* were significantly upregulated in the *v-ras*^{Ha} keratinocytes following treatment with TGF β 1, while induction of tissue inhibitor of matrix metalloproteinases (*Timp1*) was blocked by *v-ras*^{Ha} (Fig. 6C). We collected conditioned media from MEK and *v-ras*^{Ha} MEK treated with TGF β 1 for 24 hrs and analyzed protease activity using gelatin zymography. Figure 6D shows that TGF β 1 treated *v-ras*^{Ha} MEK expressed much higher levels compared to *v-ras*^{Ha} MEK and control or TGF β 1 treated MEK of a gelatinase that is likely to be pro-MMP9 based on co-migration with a recombinant standard. Higher molecular weight bands detected on the zymogram likely represent aggregates of MMP9. In addition we detected a band of gelatinase activity at 35Kd specifically in the TGF β 1 treated *v-ras*^{Ha} MEK that could represent a distinct MMP, although the exact identity remains to be determined. Since changes in matrix and protease expression are often linked to cell migration, we used a monolayer scratch assay to measure effects of TGF β 1 on MEK migration. *v-ras*^{Ha} transduced MEK migrated faster into the scratch compared to normal MEK (Fig. 6E). TGF β 1 treatment inhibited migration of MEK ($p=.001$) but the migration of *v-ras*^{Ha} MEK was reproducibly stimulated 1.5 fold by TGF β 1. Despite this we did not observe any TGF β 1 enhancement of keratinocyte invasion through a matrigel coated membrane irrespective of the presence or absence of *v-ras*^{Ha} (data not shown).

Tumor suppressive and prooncogenic functions of Smad3

We previously showed that Smad3 null keratinocytes transduced with the *v-ras*^{Ha} retrovirus undergo rapid malignant conversion in a skin grafting bioassay [22], but others have demonstrated that Smad3 null mice are resistant to 2-stage chemical carcinogenesis [23]. To test how Smad3 functions to promote or suppress oncogenic functions of ras we examined responses to TGF β 1 in Smad3 wildtype and null keratinocytes expressing *v-ras*^{Ha}. Microarray analysis of Smad3 wildtype and null keratinocytes treated with TGF β 1 for 48 hours showed that most cell cycle associated genes analyzed in the earlier data set (Fig. 5) were downregulated by TGF β 1 in both Smad3 wildtype, Smad3 null and Smad3 wildtype/*v-ras*^{Ha} keratinocytes but this downregulation was abolished in Smad3 null/*v-ras*^{Ha} keratinocytes (Fig. 7A), and Supplemental Table II. In agreement with these gene expression data, TGF β 1 treatment caused 95% inhibition of DNA synthesis in Smad3 wildtype and wildtype/*v-ras*^{Ha} MEK and 80% inhibition of DNA synthesis in Smad3 null MEK. In contrast, while the Smad3 null/*v-ras*^{Ha} had only a slight increase in baseline levels of DNA synthesis compared to the Smad3 wildtype/*v-ras*^{Ha} cells, growth inhibition by TGF β 1 was only 40% (Fig. 7B). The microarray studies also suggested that keratinocyte differentiation was altered in the absence of Smad3 (Supplemental Table II). Expression of normal differentiation markers such as Keratins 1 and 10 are lost during progression of mouse

squamous tumors and Keratin 8, a marker for simple epithelia that is reexpressed upon malignant conversion of keratinocytes [31,32]. To test if Smad3 regulates keratinocytes differentiation *in vitro*, we increased media calcium from 0.05mM to 0.12mM and measured expression of differentiation markers by immunoblotting. As expected Smad3 wildtype MEK switched to 0.12 mM calcium expressed elevated levels of the normal differentiation markers Keratins 1, 10 and Loricrin, but not Keratin 8. In contrast, Smad3 null MEK expressed detectable levels of Keratin 8 under basal conditions and this was induced following a calcium switch, while the induction of Keratins 1 and 10 and Loricrin was blocked (Fig. 7C). *v-ras*^{Ha} MEK expressed high levels of Keratin 8 under all conditions and did not show induction of these differentiation markers (data not shown).

Unlike the cell cycle response, the loss of Smad3 did not significantly alter the reduction in ECM gene expression levels caused by expression of *v-ras*^{Ha} (Supplemental Table II). Thus for both biglycan (*Bgn*) and *Ltbp1*, the levels of expression in both Smad3 wildtype/*v-ras*^{Ha} and Smad3null/*v-ras*^{Ha} keratinocytes were 5 fold lower than corresponding control cells and there was no induction by TGFβ1 (data not shown). In contrast, Figure 8A shows that the ability of TGFβ1 to induce either *Mmp9* or *uPA* gene expression in *v-ras*^{Ha} MEK was blocked by the loss of Smad3. It is likely that slight differences between kinetics and level of induction of these genes compared to Figure 6C reflects strain specific differences between Balb/c and Smad3 wildtype keratinocytes. In addition, TGFβ1 stimulated attachment of *v-ras*^{Ha} MEK was dependent on Smad3. Figure 8B shows that TGFβ1 caused a 1.5 to 2-fold increase in attachment of Smad3 wildtype/*v-ras*^{Ha} keratinocytes to untreated, laminin I, type IV collagen, and fibronectin coated tissue culture plastic. For all substrates attachment of TGFβ1 treated *v-ras*^{Ha} MEK ranged from 1.4 to 1.8 fold higher than TGFβ1 treated MEK (data not shown). Thus *v-ras*^{Ha} by itself enhances TGFβ1 induced attachment. In the absence of Smad3, attachment to fibronectin increased slightly, nevertheless the TGFβ1 stimulated increase was abolished or significantly reduced for each matrix substrate tested (Fig. 8B). Paralleling the accelerated wound healing in the Smad3 null mouse [33] Smad3 null MEK migrated faster than the wildtype cells in the scratch assay (data not shown) but TGFβ1 suppressed the migration of the Smad3 null/*v-ras*^{Ha} MEK but not Smad3 wildtype *v-ras*^{Ha} MEK (Fig. 8C). Taken together, these data show that Smad3 functions to both suppress and promote an oncogenic ras driven cancer phenotype.

Discussion

Activation of ras in normal and neoplastic epithelial cell lines can antagonize and synergize with TGFβ1 signaling [4,6,7,10,34] providing a mechanism for subversion of TGFβ1 tumor suppressor function and pro-oncogenic switching of TGFβ1. Despite these studies, TGFβ1 clearly suppresses tumor development *in vivo* where *ras* is the activating oncogene, suggesting that immortalized cell lines may not illuminate the complex interactions between these two pathways. In this report we have utilized a primary culture model in which mouse epidermal keratinocytes are transduced with a high titer retrovirus expressing *v-ras*^{Ha}, to more clearly define interactions between ras and TGFβ1 signaling at the earliest stages of cancer development. Our data show that oncogenic ras and hyperactivation of the ERK1/2 pathway did not block Smad2 phosphorylation, nuclear translocation or regulation of Smad3 dependent early response genes. However, over a 48 hr time course *ras* significantly altered the pattern of TGFβ1 mediated gene expression, with 50% of TGFβ1 regulated genes (both induced and suppressed) blocked while 34% were unaffected by oncogenic *ras*. This is similar to repression of 32% of TGFβ1 induced genes in 3T3 fibroblasts by activated ras oncogene [35]. In agreement with previous studies [36,29], our data show that in primary keratinocytes the majority of TGFβ1 regulated gene expression is Smad3 dependent, since TGFβ1 induction or repression of these genes is blocked, or significantly reduced in Smad3 null keratinocytes. Surprisingly, Smad3/4 dependent early response genes [29] are

unaffected by oncogenic *ras*, while the induction of many extracellular matrix genes, that are well defined primary targets of TGF β 1 and Smad3 is blocked by *v-ras*^{Ha}. These data suggest that additional factors may be important in targeting specific Smad3 dependent genes for suppression, or that secondary events occur in a TGF β 1 gene expression cascade that contribute to blocked expression of specific Smad3 dependent genes. In contrast to the blocked ECM gene expression observed in this study under conditions of hyperactivated *ras* and ERK1/2, TGF β 1 treatment of *ras* transformed 3T3 fibroblasts reversed the inhibition of matrix gene expression and the transformed phenotype caused by *ras* [35]. Furthermore, an array study of the HaCaT human keratinocyte cell line showed that many of these same matrix genes were dependent on ERK1/2 for responsiveness to TGF β 1 [37]. Taken together, these studies suggest that the context of ERK1/2 activation distinctly alters the readout of the TGF β 1 transcriptome.

Within the complex changes in the TGF β 1 transcriptome caused by *ras* we find compelling evidence that components of both the tumor suppressor and pro-oncogenic functions of TGF β 1 occur at the same time in this early stage cancer model. Thus while *ras* does alter the kinetics of *c-myc* downregulation and *smad7* induction by TGF β 1, suggesting a degree of alteration in the growth arrest program, nearly all identifiable cell cycle associated genes were similarly downregulated by TGF β 1 in the presence or absence of *v-ras*^{Ha}, and TGF β 1 caused a nearly identical dose dependent growth arrest in both cell types. This is consistent with reduced tumor formation in mouse models of TGF β 1 overexpression [17,38], and suggests that at the earliest stage of cancer development modeled by expression of *v-ras*^{Ha} in primary epidermal keratinocytes, the central cytostatic effect of TGF β 1 that correlates with homeostatic growth control and tumor suppression is intact.

Regulation of extracellular matrix remodeling is one of the most significant functions of TGF β 1 that has important functions in wound healing, inflammation, fibrosis and cancer development [39]. Our data show that *v-ras*^{Ha} caused significant suppression of both basal and TGF β 1 induced extracellular matrix gene expression in primary keratinocytes. This decrease in matrix gene expression was accompanied by induction of at least two protease genes urokinase and *Mmp9*, increased gelatinolytic activity in the conditioned media of TGF β 1 treated *v-ras*^{Ha} MEK, and inhibition of TGF β 1 induced TIMP1. Furthermore, we found that in an *in vitro* monolayer migration assay, TGF β 1 stimulated *v-ras*^{Ha} MEK migration but blocked migration of normal MEK, the latter consistent with effects of TGF β 1 signaling in studies of keratinocyte migration and *in vivo* wound healing [40–42]. Similar induction of urokinase and *Mmp9* by TGF β 1 has been linked to enhanced migration in transformed keratinocytes cell lines [43,44] but this is the first report to link this induction to the earliest stage of cancer development in keratinocytes. How *ras* signaling switches the migration response to TGF β 1 from inhibition to stimulation is not yet clear but is likely related both to extracellular matrix remodeling and to changes in expression of integrins such as $\alpha 6 \beta 4$ which are crucial for enhanced migration and invasion of skin cancer cells [45]. Nevertheless, these results show that in the context of a *ras* oncogene, TGF β 1 causes significant changes expression of genes involved in matrix protein expression and matrix remodeling enzymes that are associated with the phenotype of many neoplastic cells *in vivo* [46]. Thus at the earliest stage of cancer development components of TGF β 1 tumor suppressive and oncogenic activities are present.

While Smad3 is a critical mediator of TGF β 1 transcriptional responses [29], it is not frequently mutated in human cancers [1]. Here we find that deletion of Smad3 both enhances and inhibits expression of a tumor progression phenotype. This is similar to xenograft and transgenic models of breast cancer, where inhibition of Smad2/3 or inactivation of the TGF β receptor enhanced tumorigenesis of premalignant cells but blocked metastasis of malignant cell lines [50,51]. Thus while loss of Smad3 by itself modestly

reduces responsiveness of keratinocytes to TGFβ1 mediated growth inhibition it significantly alters keratinocyte differentiation, blocking calcium mediated induction of suprabasal markers Keratin 1 and 10 and the granular layer marker Loricrin. Most importantly, loss of Smad3 causes induction of Keratin 8 a marker of simple epithelia and malignant conversion of squamous tumors [48]. Similar effects of Smad7, cripto-1 and the Alk5 inhibitor SB431542 on keratinocyte differentiation have been reported by us [49], and suggest that TGFβ1 through Smad3 is important for maintaining squamous differentiation and suppressing simple epithelial differentiation. Our results also show that in *ras* expressing keratinocytes Smad3 is essential for the TGFβ1 cytostatic response, but is also essential for the induction of protease genes, cell attachment and migration by TGFβ1. The observation that deletion of Smad3 does not completely block TGFβ1 mediated growth inhibition suggests that Smad2 or other non-Smad signaling pathways are also essential for the TGFβ1 cytostatic response and that Smad3 may be primarily responsible for regulating squamous differentiation. Indeed it is possible that *ras* activation acts on these other pathways but this is only evident in the absence of Smad3. In a previous study we showed that *v-ras*^{Ha} transduced Smad3 null keratinocytes rapidly progress to squamous cell carcinoma in an *in vivo* graft model due in part to abrogation of a TGFβ1 induced senescence response [22]. In contrast, Smad3 null mice are resistant to tumor promotion and skin tumor induction [47], suggesting that the effects of Smad3 on cancer progression in the skin model are complex. It is possible that loss of TGFβ1 responsiveness, altered oncogene induced senescence and suppressed squamous differentiation in grafted Smad3 null/*v-ras*^{Ha} keratinocytes may be sufficient to accelerate malignant conversion this may not be sufficient within the context of an intact epidermis. Taken together these data suggest that squamous tumor progression requires selective rather than complete inactivation of Smad3 function such that the cytostatic and differentiation arms of TGFβ1 signaling are lost but matrix remodeling and migratory phenotype are retained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Oncogenic *v-ras*^{Ha} does not block Smad phosphorylation or translocation
 Balb/c primary keratinocytes were transduced with the *v-ras*^{Ha} retrovirus and then control and *v-ras*^{Ha} keratinocytes treated with TGFβ1 at 1ng/ml and samples taken for immunoblotting, quantitative PCR or immunofluorescence. **A.** Immunoblotting of cellular extracts showing high levels of ras protein expression in transduced keratinocytes and elevated phosphorylation of ERK1/2 that is not altered by TGFβ1 treatment. **B.** Increased expression of proliferation associated genes in MEK expressing *v-ras*^{Ha}. RNA was isolated from control and *v-ras*^{Ha} transduced cells and expression of the indicated genes determined by quantitative PCR as described in Materials and Methods. The level of each transcript was normalized to that of *Gapdh*, and expressed as relative to the expression level in control keratinocytes. **C.** TGFβ1 induces similar alterations in Smad levels and phosphorylation in control and *v-ras*^{Ha} transduced keratinocytes. Nuclear extracts were isolated from keratinocytes at the indicated times after treatment with TGFβ1 and immunoblotted for the indicated Smads. **D.** *v-ras*^{Ha} does not prevent nuclear translocation of Smad2/3. Indirect immunofluorescence for Smad2/3 (FITC) or ras (Texas Red) in MEK or *v-ras*^{Ha} transduced MEK treated with TGFβ1 (1 ng/ml) for 1 hour. Nuclei were detected using DAPI staining.

Figure 2. Oncogenic *ras* suppresses SBE-luciferase reporter transactivation by TGFβ1
 MEK and v-*ras*^{Ha} transduced MEK were transfected with pGL-SBE4-luc and pRLTK and luciferase activity measured in a dual luciferase assay after 24 hrs in the presence or absence of 1ng/ml TGFβ1. RLU represent SBE-luciferase values normalized to renilla luciferase for each well.

Figure 3. Gene specific alteration of TGFβ1 transcriptome by v-ras^{Ha}

Primary and v-ras^{Ha} transduced keratinocytes were treated with TGFβ1 and microarray analyses were performed on RNA isolated at the indicated times as described in Materials and Methods. **A** Representative portions of major clusters of expression patterns obtained using the K-means cluster program from the NCI mAdbweb site. Cluster1: Similar induction by TGFβ1 in control and v-ras^{Ha} expressing MEK; Cluster 2: Induction by TGFβ1 suppressed in v-ras^{Ha} expressing MEK; Cluster 3: Similar repression by TGFβ1 in control and v-ras^{Ha} expressing MEK; Cluster 4: Suppression by TGFβ1 blocked in v-ras^{Ha} expressing MEK. Each time point in the heat map represents the averaged (5–8 arrays) log₂ transformed expression ratio calculated from TGFβ1 treated cells over untreated control cells. Red indicates overexpression in TGFβ1 treated cells while green indicates underexpression in TGFβ1 treated cells as compared to controls. **B**. Plots of the average log₂ expression ratio at each time point for all genes within the corresponding cluster in A. N is the number of genes in each cluster used for the analysis, and p values were computed using a nonparametric T test from the values of the 48 hr time points.

Figure 4. Oncogenic ras does not block TGFβ1 early gene response

A Log₂ expression ratio (TGFβ1 treated/control) from microarrays of specific early response genes at 1, 6, 24 and 48 hrs (left to right) after TGFβ1 treatment. Each histogram represents the average expression ratio expressed as treated over control from at least 5 arrays per time point analyzed. **B.** Quantitative PCR validation of microarray response at 1 and 6 hrs after TGFβ1 treatment for specific early response genes. Normalized quantitative PCR data is expressed as a ratio of TGFβ1 treated/control for comparison to microarray. ■ MEK, ▨ *v-ras*^{Ha} transduced MEK. **C.** Smad7 induction by TGFβ1 is Smad3 dependent in keratinocytes. Quantitative PCR analysis of Smad7 transcript levels in Smad3 wt and Smad3 null control and *v-ras*^{Ha} transduced MEK treated with TGFβ1 (1 ng/ml) for 1 hour.

Figure 5. Ras does not block TGFβ1 cytostatic response

A. Histogram representing the average log₂ microarray expression ratio (TGFβ1 treated/untreated) of cell cycle genes in MEK and v-ras^{Ha} transduced MEK at the indicated times after treatment. Genes in this set include: *Ccnb1*, *Ccnb2*, *Cdc2a*, *Cenpa*, *Gmnn*, *Mad2l1*, *Mcm5*, *Mki67*, *Odc*, *Plk1*, *Tk1*, *Top2a*, *Ung*. **B.** Quantitative PCR validation of *Ccnb1*, *Top2a* and *Tpx2* downregulation by TGFβ1 in MEK and v-ras^{Ha} transduced MEK at 24 hrs. Histograms represent normalized expression levels expressed relative to untreated cells. **C.** Oncogenic ras does not block TGFβ1 cytostatic response. Normal and v-ras^{Ha} transduced MEK were treated in triplicate with the indicated concentrations of TGFβ1 and

incorporation of ^3H -thymidine measured after 24 hrs. Percent incorporation was measured relative to untreated control for each cell type.

Figure 6. Ras alters TGFβ1 regulation of extracellular matrix and keratinocyte migration

A. TGFβ1 mediated induction of extracellular matrix genes is blocked by oncogenic *ras*. Each histogram represents the average log₂ microarray expression ratio (TGFβ1 treated/untreated) for a set of ECM genes induced by TGFβ1 at the indicated times after treatment. Genes in this set include: *Bgn*, *Col1a1*, *Col1a2*, *Col4a1*, *Col4a2*, *Col5a1*, *Col6a2*, *Efemp2*, *Fbln5*, *Itga5*, *Ltp1*, *Nid1*, *Sparc*, *Tnn*. **B.** Quantitative PCR validation of the inhibition of ECM gene induction by *v-ras*^{Ha} for indicated genes at 24 hrs after TGFβ1 treatment. Histograms represent normalized expression levels expressed relative to untreated cells, ■ - *v-ras*^{Ha}, ▨ + *v-ras*^{Ha}. **C.** Quantitative PCR analysis of MMP9, urokinase (uPA) and TIMP1 transcript levels in MEK and *v-ras*^{Ha} MEK treated with TGFβ1. Cells were treated with TGFβ1 (1ng/ml) and RNAs were isolated at the indicated times. Expression levels for each gene were normalized to β-actin levels. **D.** Gelatin zymography of conditioned media from MEK and *v-ras*^{Ha} MEK treated with TGFβ1 (1 ng/ml) for 24 hrs. Equal volumes of conditioned media were loaded on a gelatin zymogram and position of proteases determined as described in Materials and Methods. Identity of MMP9 was based on co-migration with a recombinant MMP9, and relative size based on molecular wt standards. Other arrows indicate additional bands of gelatinase activity. **E.** TGFβ1 inhibits migration of MEK but stimulates migration of *v-ras*^{Ha} MEK. Data show migration of the indicated cell type cultured in the presence or absence of TGFβ1 for 18 hrs. Confluent monolayers were treated with mitomycin C, scratched and treated with TGFβ1 for 18 hrs, and distance migrated calculated as described in Materials and Methods. Each histogram represents the average distance migrated in two defined fields for each of 4 wells per treatment group. Data are representative of three independent experiments. * significantly different from untreated MEK at p=.001, ** significantly different from untreated *v-ras*^{Ha} MEK, as determined by unpaired Students T Test.

Figure 7. Loss of Smad3 suppresses TGFβ1 cytostatic response and promotes aberrant differentiation

A. Downregulation of cell cycle genes by TGFβ1 is lost only in Smad3 null/v-ras keratinocytes. Averaged log₂ expression ratio (TGFβ1 treated/control) of cell cycle genes identified on microarrays of the indicated genotypes. Genes in this set include: *Mad2l*, *Pmscl1*, *Birc5*, *Tk1*, *Mcm5*, *Ung*, *Top2a*, *Cdc2a*, *Plk*, *Nusap1*, *Ccnb1*, *Stk6*, *Cenpa*. **B.** Cytostatic response to TGFβ1 in Smad3 wildtype and null keratinocytes. DNA synthesis was measured using a ³H-thymidine incorporation assay in control or TGFβ1 treated cells of the indicated genotypes as described in Materials and Methods. **C.** Loss of Smad3 blocks normal differentiation and causes abnormal induction of Keratin 8. Smad3 wildtype or null

MEK were cultured in 0.05 mM calcium media until confluent then switched to 0.12 mM calcium medium to induce differentiation. Total cellular lysates were harvested after 24 hrs and keratin expression determined by immunoblotting with antibodies specific for mouse Loricrin or Keratin 1, 10 or 8.

Figure 8. Smad3 loss blocks TGFβ1 induction of proteases and migration in *v-ras*^{Ha} transduced keratinocytes

A. Measurement of the relative transcript levels of urokinase (uPA) and MMP9 in Smad3 wt and Smad3 null MEK expressing *v-ras*^{Ha} treated with TGFβ1 (1 ng/ml) for the indicated times by Quantitative PCR. Histograms show the relative transcript level normalized to β-actin. **B.** Attachment of Smad3 wildtype and null *v-ras*^{Ha} MEK to the indicated substrates in the presence or absence of TGFβ1 (1 ng/ml). Histograms represent the average attached cells in quadruplicate wells measured as indicated in Materials and Methods, and normalized to the attachment of uninfected wildtype keratinocytes. Experiment was repeated twice with similar results. * : significantly different from untreated on same matrix using a two-tailed T-test at $p < .001$, ** significantly different from untreated on same matrix at $p = .006$. **C.** Distance migrated into a denuded monolayer of Smad3 wildtype and null *v-ras*^{Ha} MEK in the presence and absence of TGFβ1 (1 ng/ml). Migration was measured in triplicate wells as indicated in Materials and Methods 10 hours after scratching the cell monolayer and treating with TGFβ1. * : significantly different from Smad3 wildtype/*v-ras*^{Ha} using a two-tailed T-test at $P < .05$.