A FoxO–Smad synexpression group in human keratinocytes

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Transforming growth factor β (TGF-β) signals through activation of Smad transcription factors. Activated Smad proteins associate with different DNA-binding cofactors for the recognition and regulation of specific target genes. Members of the forkhead box O family (FoxO1, FoxO3, and FoxO4) play such a role in the induction of the cyclin-dependent kinase inhibitors p15Ink4b and p21Cip1. To delineate the organization of the TGF-β response in human keratinocytes, we defined the set of genes whose activation by TGF-β requires both FoxO and Smad functions. FoxO factors are shown to be essential for 11 of the 115 immediate gene activation responses to TGF-β in these cells. FoxO1, FoxO3, and FoxO4 act redundantly as mediators of these effects. Smad4, which functions as a partner of receptor-phosphorylated Smad2/3, is required for all of these responses. These results define a FoxO–Smad synexpression group or group of genes that are jointly induced by a common mechanism in response to TGF-β. In addition to *p15INK4b* and *p21CIP1*, these genes include mediators of stress responses (*GADD45A*, *GADD45B*, and *IER1*) and adaptive cell signaling responses (*CTGF*, *JAG1*, *LEMD3*, *SGK*, *CDC42EP3*, and *OVOL1*). Bioinformatic analysis of the promoter region of these genes reveals diverse configurations of Smad and FoxO binding elements, implying differences in the regulatory properties of this group of genes. Indeed, a subset of FoxO/Smad-dependent TGF-β gene responses additionally require the transcription factor CCAAT/enhancer-binding protein β. The composition of the FoxO–Smad synexpression group suggests that stress reactions and adaptive functions accompany the cytostatic response of keratinocytes to TGF-β.

Forkhead | TGF-β | transcription

Transforming growth factor β (TGF-β) is a member of a large family of secreted growth factors of central importance in metazoan development and homeostasis (1–5). TGF-β signaling induces a large set of gene responses that control cell behavior and fate. These responses are susceptible to disruption in inherited and somatic disorders including cancer (1, 3, 4). Delineating the organization of TGF-β transcriptional programs is therefore important for understanding the basis for the multifunctional action of TGF-β.

TGF-β activates a membrane receptor serine/threonine kinase complex that phosphorylates the transcription factors Smad2 and Smad3 (6). Thus activated, Smad2/3 accumulate in the nucleus and bind Smad4, which is essential for many, but not all, Smad-dependent responses (7–9). Smad proteins bind DNA, preferentially at the sequence AGAC, denoted the Smad-binding element (SBE) (6, 10). Alone, the affinity of Smad proteins for the SBE is insufficient to support binding to endogenous promoters *in vivo* except in genes with multiple SBE clusters. In most cases, activated Smad complexes must associate with other DNA-binding proteins and cooperatively bind compound elements in gene regulatory regions (11). Members of diverse families of DNA-binding proteins fulfill this role as Smad partners. Based on this model, one can envision repertoires of Smad transcriptional complexes regulating distinct subset of genes in a cell type-specific manner. The specificity of TGF-β action would thus depend on the Smad cofactors and chromatin status provided by the developmental state and environmental context of the target cell.

This model predicts that the transcriptional response to TGF-β in a given cell type could be parsed into groups of genes that are controlled by specific Smad–cofactor combinations. Each group of genes would be regulated in a unified manner. Groups of genes that are synchronously regulated by a common signal, referred to as "synexpression groups," have been described during embryo development (12). Synexpression groups may support coordinated events for the completion of a developmental step. By their nature, Smad–cofactor combinations could provide a mechanistic basis for the coordinated regulation of selected gene sets and, therefore, a mechanism-based definition of synexpression groups. Evidence for this role has been provided in the action of the TGF-β family member BMP4 (bone morphogenetic protein 4) in *Xenopus* development (13).

In the present studies we tested these predictions by focusing on FoxO factors (FoxO1, FoxO3, and FoxO4) as Smad partners. FoxO factors are critical players in growth inhibitory responses to stress in human cells and the control of starvation responses and longevity in lower organisms (14, 15). We recently found that FoxO factors act as Smad partners in the induction of *p21CIP1* and *p15INK4b* as part of the cytostatic response of epithelial cells (16, 17). By means of FoxO and Smad4 genetic depletion and gene expression profiling in human keratinocytes, we have now defined a set of genes that are transcriptionally induced by TGF-β through the FoxO–Smad combination. This FoxO–Smad synexpression group includes cytostatic, stress, and adaptive activities, providing insights into the organization of the overall TGF-β response in this cell type.

Results

Identification of FoxO-Dependent TGF-β Gene Responses. *p21CIP1* and *p15INK4b* are activated by TGF-β in a Smad- and FoxO-dependent manner (16, 17). To identify other genes in this class, we subjected the human keratinocyte cell line HaCaT to RNAi-mediated depletion of FoxO factors. As FoxO1, FoxO3, and FoxO4 can act redundantly as Smad cofactors (16), we transfected cells with a mixture of siRNA oligonucleotides targeting these three transcripts (Fig. 1*B*). The FoxO3 RNAi also targets FoxO6. Cells were incubated with or without TGF-β1 for 3 h in serum-free media and then subjected to transcriptomic analysis using Affymetrix (Santa Clara, CA) HG-U133A microarrays (21,608 probe sets). Analysis of the data from cells transfected with a control siRNA (targeting GFP) revealed 115 genes whose signal was increased

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Abbreviations: shRNA, short hairpin RNA; FHBE, forkhead-binding element; SBE, Smad-binding element; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative real-time PCR; CDK, cyclin-dependent kinase.

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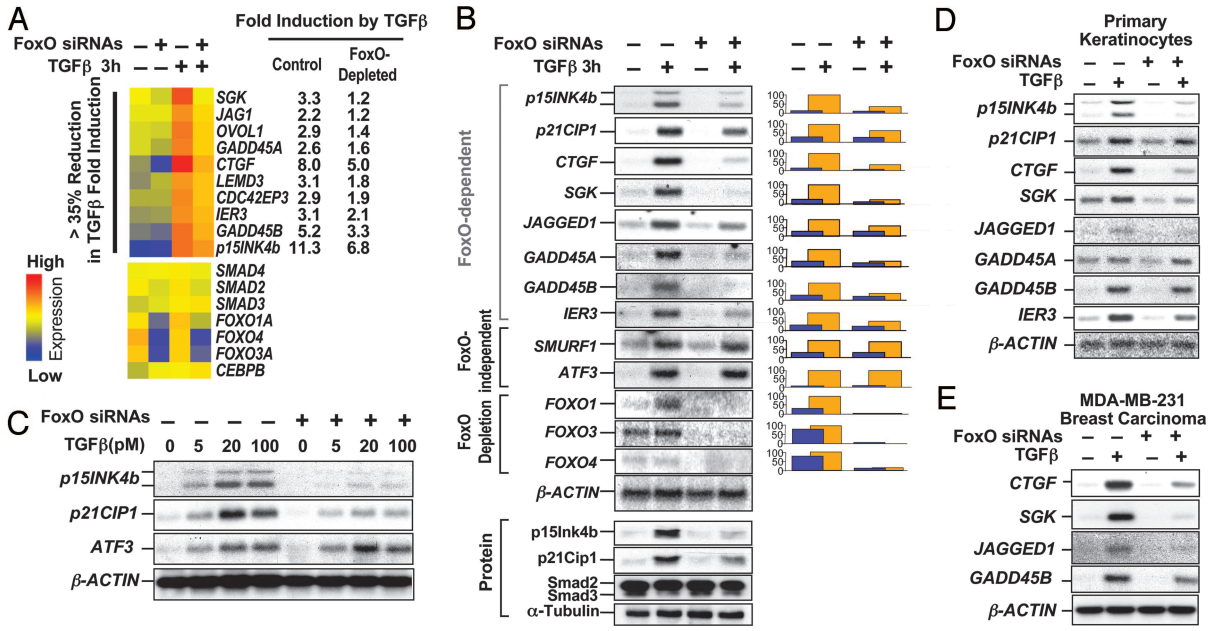

Fig. 1. FoxO-dependent TGF-β gene responses. (*A*) Control and FoxO-depleted HaCaT cells were incubated with TGF-β (100 pM) for 3 h and then total RNA was subjected to Affymetrix analysis with the U133-A microarray (21,608 probe sets). (*Left*) The heat map plot represents the expression level of genes identified as members of the FoxO–Smad synexpression group in response to TGF-β and the levels of Smads, FoxOs, and C/EBPβ. (*Right*) The fold induction for each gene by TGF-β is shown. (*B*) Control and FoxO-depleted HaCaT cells were incubated with TGF-β for 3 h. Total RNA was subjected to Northern blot analysis with the indicated probes. RNA quantitation by phosphor screen (Molecular Dynamics, Portland, OR) was plotted. Whole-cell extracts were probed with the antibodies against the indicated proteins. (*C*) Control and FoxO-depleted HaCaT cells were incubated with the indicated concentrations of TGF-β for 3 h. Total RNA was subjected to Northern blot analysis with the indicated probes. (*D* and *E*) Control and FoxO-depleted primary keratinocytes (*D*) and MDA-MB-231 mammary epithelial cells (*E*) were incubated with TGF-β for 3 h. Total RNA was subjected to Northern blot analysis with the indicated probes.

>2-fold by TGF-β, as reported with WT HaCaT cells (18). We then filtered the data for responses that were decreased by >35% in FoxO-depleted cells compared with control cells. Ten of the 115 TGF-β activated genes met these criteria (Fig. 1*A*). The FoxO dependence of these gene responses was confirmed by Northern analysis (Fig. 1*B* and data not shown). The extent of activation after 3 h of treatment with TGF-β ranged from 2-fold (e.g., in *JAG1*) to >10-fold (*p15INK4b*) over basal levels. As FoxO depletion was not complete under our conditions, our transcriptomic data analysis likely missed some FoxO-dependent responses. Indeed, *p21CIP1* activation did not score in this analysis even though it was attenuated in FoxO-depleted cells at the mRNA and protein levels (Fig. 1*B*) and at different TGF-β concentrations (Fig. 1*C*). Among these TGF-β gene responses, six (*p15INK4B*, *p21CIP1*, *SGK*, *GADD45B*, *CTGF*, and *JAG1*) were conserved when the experiments were performed in the presence of serum (data not shown). siRNA-mediated knockdown of individual FoxO factors had little or no effect on the expression of these genes (Fig. 5, which is published as supporting information on the PNAS web site), suggesting functional redundancy and providing further verification that the effect of the triple knockdown was specific. Many of these TGF-β gene responses also showed FoxO dependence in primary human keratinocytes (Fig. 1*D*) and MDA-MB-231 breast carcinoma cells (Fig. 1*E*). Exceptions included the *GADD45A* and *GADD45B* responses, which showed little or no attenuation in FoxO-depleted primary keratinocytes.

These FoxO-dependent TGF-β responsive genes comprise a functionally diverse group that includes the cyclin-dependent kinase (CDK) inhibitor *p15INK4b* (also known as *CDKN2B*), which is a key participant in the TGF-β cytostatic response along with *p21CIP1* (*CDKN1A*) (19); the paracrine signaling factors, connective tissue growth factor (*CTGF*), which is a mediator of fibrogenesis and angiogenesis (20), and *JAG1*, which is a ligand for Notch receptors controlling cell fate (21); *GADD45A* and *GADD45B*, which are transcriptional mediators of stress responses to DNA damage (15) and TGF-β (22, 23); *IER1*, which is an early responder to inflammatory and stress signals (24); *LEMD3*, which encodes the negative regulator of Smad signaling MAN1 (25, 26); *SGK* (serum and glucocorticoid-activated protein kinase-1), which mediates FoxO phosphorylation (27, 28); *CDC42EP3*, which is an effector of Cdc42 in actin cytoskeleton control (29); and *OVOL1*, which is of unknown function in this context (30). Thus, this group includes mediators of cytostatic and stress responses, paracrine factors, and potential mediators of feedback control.

A FoxO/Smad4 Synexpression Group. Smad4 is a central partner of receptor-phosphorylated Smads 2 and 3 in the induction of TGF-β responses. To test the necessity of Smad4 in these FoxO-dependent gene responses, we used a series of HaCaT derivatives with different knockdown levels of Smad4 by stable expression of anti-*SMAD4* short hairpin RNA (shRNA) vectors (Fig. 2*A*). Cells with lower levels of Smad4 had a decreased ability to induce the expression of *p15INK4b* and *p21CIP1* and another TGF-β target gene, plasminogen activator inhibitor 1 (*PAI-1*) (Fig. 2*A*). The *p15INK4b* and *p21CIP1* responses were more resistant to Smad4 depletion than was the *PAI-1* response (Fig. 2 *A* and *B*). Overexpression of a kinase-inactive mutant form of the TGF-β type I receptor [TβR-I(K232R)] (31) also caused a more profound inhibition of the *PAI-1* response than of the *p15INK4b* and *p21CIP1* responses (Fig. 2*A*). The high sensitivity of *p15INK4b* and *p21CIP1* to residual TGF-β and Smad4 signaling activity likely explains the apparent absence of Smad4 necessity reported by others (32).

Further evidence of Smad4 dependence was provided by the ability to rescue the *p15INK4b* and *p21CIP1* responses by ectopic expression of WT Smad4 in the *Smad4*-defective cancer cell line MDA-MB-468 (Fig. 2*C*). Upon TGF-β treatment of MDA-MB-468 cells, neither *p15Ink4b* nor *p21Cip1* was induced (Fig. 2*C*). To firmly establish the requirement for induction of these two gene

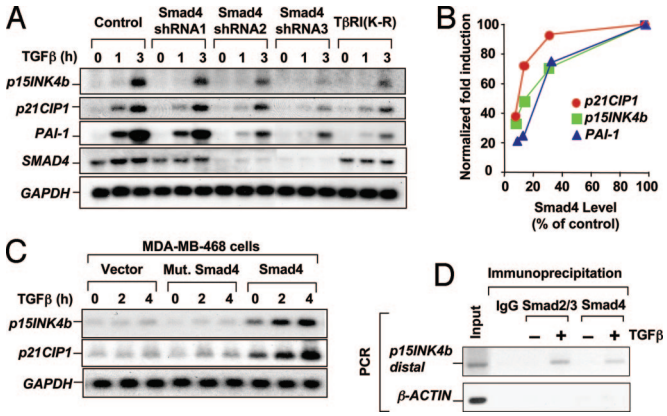

Fig. 2. Induction of CDK inhibitors is Smad4-dependent. (*A* and *B*) Control and Smad4-depleted HaCaT cells were incubated with TGF-β for the indicated times. (*A*) Total RNA was subjected to Northern blot analysis with the indicated probes. (*B*) The normalized RNA values were plotted versus the relative Smad4 protein level. (*C*) MDA-MB-468 cells were stably transfected with an empty vector, a vector expressing Smad4–MH1 domain (1–363Aa), or the full-length SMAD4. Cells were incubated with TGF-β (100 pM) for the indicated times. Total RNA was subjected to Northern blot analysis with the indicated probes. Note that only the lower band for *p15INK4b* is shown. (*D*) HaCaT cells were incubated with or without TGF-β for 90 min and subjected to ChIP assays with the indicated antibodies and PCR primers. The β-actin promoter was used as a negative control.

responses in MDA-MB-468, these cells were stably transfected with either a construct encoding a Smad4 fragment of 1–363 aa, including the MH1 domain and the linker region, or a full-length cDNA. Only when Smad4 full-length cDNA was reintroduced, both gene responses to TGF-β were restored, demonstrating that the lack of Smad4 leads to the loss of TGF-β–mediated induction of *p15Ink4b* and *p21Cip1* in this particular cell line (Fig. 2*C*).

Evidence of a TGF-β-directed interaction of the Smad endogenous proteins with the *p15INK4b* promoter was obtained by chromatin immunoprecipitation (ChIP) analysis (Fig. 2*D*). TGF-β induced the rapid binding of endogenous Smad2/3 and Smad4 (Fig. 2*D*) to the distal promoter region of *p15INK4b* containing the Smad-binding region. Note that the antibodies do not distinguish between Smads 2 and 3. No binding was observed with the promoter region of β-actin, which served as an internal control (Fig. 2*D*). The response of the other genes in the FoxO–Smad synexpression group was also attenuated by Smad4 knockdown HaCaT cells, as determined by microarray analysis (Fig. 3*A*) and quantitative real-time PCR (qRT-PCR) (Fig. 3*B*). Collectively, these results define a FoxO–Smad synexpression group that includes two CDK inhibitors and a functionally diverse set of other genes.

Heterogeneous Configuration of FoxO and Smad Binding Sites. The TGF-β-responsive region of the *p21CIP1* promoter comprises one forkhead-binding element (FBHE) followed by three closely spaced SBEs (16). In contrast, the corresponding region in the *p15INK4b* promoter in human and mouse includes two separate SBEs, one flanked by a FBHE and the other flanked by a binding site for the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) (17) (Fig. 4*A*). C/EBPβ is a member of the basic leucine zipper family and plays important roles in cell proliferation, differentiation, and senescence (33). To determine whether other members of FoxO–Smad synexpression group shared either of these configurations, we searched the potential regulator regions of these genes. Noncoding regions conserved among human, mouse, and rat were extracted in the vicinity of the genes of interest by using the University of California, Santa Cruz gene browser database (from 4 kb upstream of the transcription start site until the stop codon, including introns). These sequences were then examined for regions that contain FHBE sites [(G/A)(T/C)AAA(T/C)A] and SBE sites (AGAC) within 100 nt upstream and downstream of the FHBE by using a dual site matching program (http://cbio.mskcc.org/cgi-bin/lash/dualsite). Once identified the human Smad/Forkhead site-derived sequences were BLAST-compared (34) against the 4-kb upstream regions of mouse genes homologous to the synexpression group to further confirm conservation (BLAST outputs for significant matches are available on request).

By applying this analysis to the region from 4 kb upstream of the transcriptional start site through the start of the 3′ UTR of the FoxO/Smad target genes, we identified segments with >75% homology in human and mouse (Fig. 4*A*). Assessment of these computationally predicted, putative binding sites in light of current genome builds as well as comparative, phylogenetic footprinting data (e.g., conservation track available on the University of California, Santa Cruz genome browser) may provide more accurate predictions of actual functional binding to enhancer regions. In seven of the genes, these conserved regions contained clusters of FHBEs with neighboring SBEs. None of these clusters shared the same configuration. Some of the genes contained multiple con-

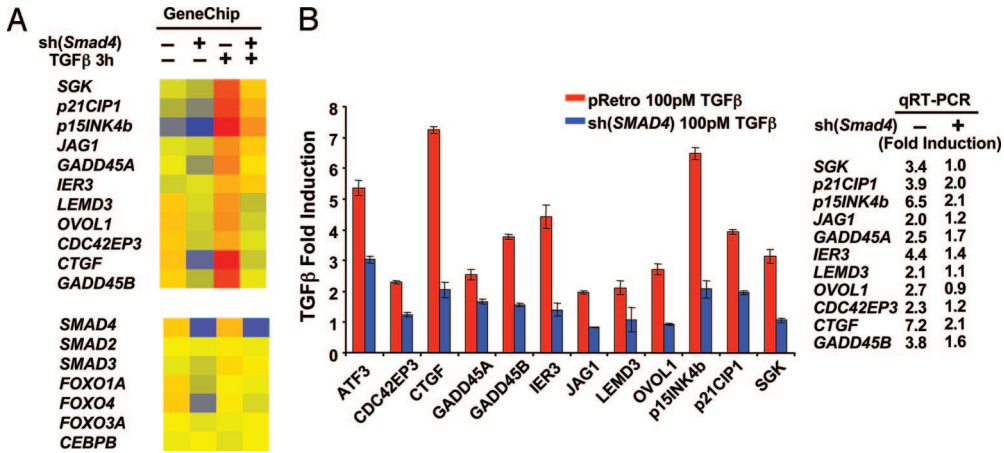

Fig. 3. FoxO–Smad-dependent TGF-β gene responses. (*A*) Control and Smad4-depleted HaCaT cells were incubated with TGF-β for 3 h, and then total RNA was subjected to Affymetrix analysis with the U133-A microarray. The heat map plot represents the expression level of genes identified as members of the FoxO–Smad synexpression group in response to TGF-β and the levels of Smads, FoxOs, and C/EBPβ. (*B*) Control and Smad4-depleted HaCaT cells were incubated with or without TGF-β for 3 h. qRT-PCR was used to measure RNA levels of the indicated genes. The fold increase in mRNA level is indicated. Data are mean ± SD of three independent experiments.

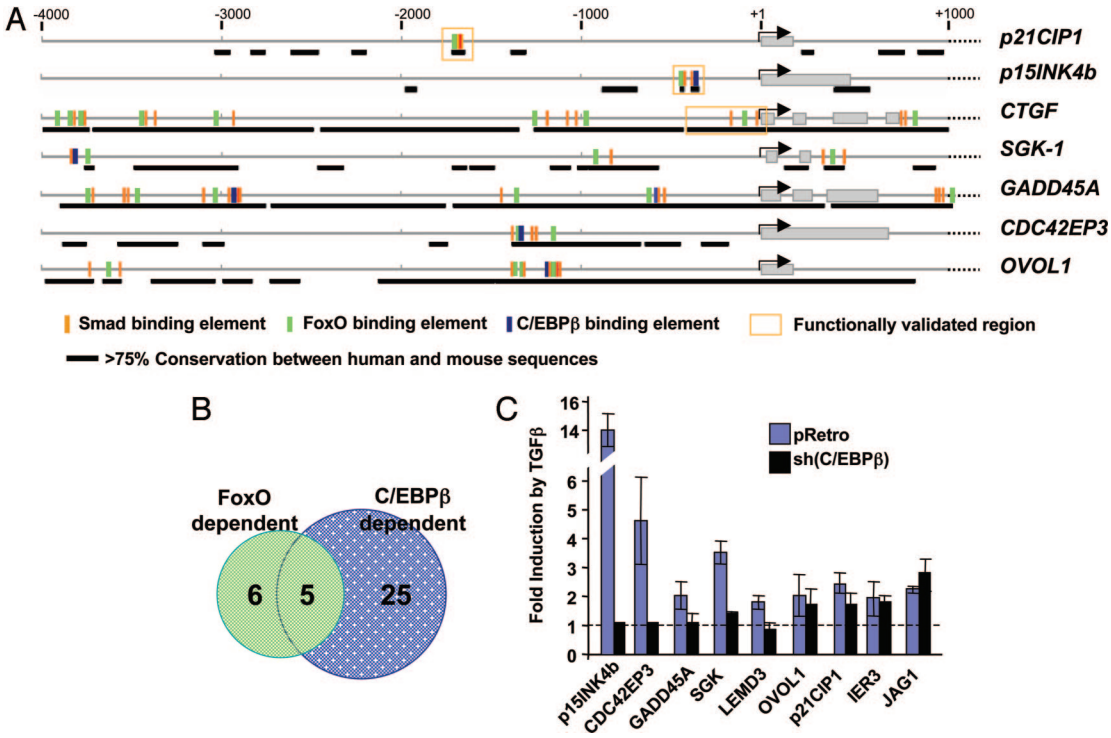

Fig. 4. Variability in promoter configuration and regulation in the FoxO–Smad synexpression group. (*A*) A graphic comparison of the promoter regions of the FoxO–Smad synexpression genes is shown. The conserved FHBEs (green) surrounded by SBEs (orange) within 100 nt and the C/EBPβ binding elements (C/EBPβBE, blue) identified by computational sequence analysis are indicated. The track (black) indicates mouse sequence segments that are similar to its human ortholog. Percent identities of the segments ranged from 75% to 100%. Shaded boxes (orange) identify the functionally validated TGF-β responsive promoter regions. (*B*) Venn diagram depicting the overlap between FoxO-dependent and C/EBPβ-dependent TGF-β gene responses in HaCaT keratinocytes. (*C*) Effect of TGF-β on FoxO–Smad responsive genes in control and C/EBPβ-depleted HaCaT cells. Cells were incubated with or without TGF-β for 3 h. qRT-PCR was used to measure mRNA levels of the indicated genes. The fold increase of these levels by TGF-β is indicated. Data are mean $\pm$ SD ($n = 3$) of three independent experiments.

served FBHE/SBE clusters (Fig. 4*A*). Transcriptional assays of luciferase reporter constructs driven by different FBHE/SBE-rich regions of the human *CTGF* promoter showed TGF-β response only in the proximal FBHE/SBE-rich region ($-400/+73$ region, Fig. 4*A*) (data not shown).

Variant C/EBPβ-Dependent Regulation in the FoxO–Smad Synexpression Group. Five genes (*p15INK4b*, *SGK*, *GADD45A*, *JAG1*, *OVOL1*, and *CDC42EP3*) contain conserved C/EBPβ sites within FHBE/SBE clusters in the promoter region (Fig. 4). C/EBPβ plays a critical role in the *p15INK4b* response to TGF-β but not in the *p21CIP1* response (17). C/EBPβ is required for *p15INK4b* induction by TGF-β, as determined by RNAi-mediated knockdown of C/EBPβ. TGF-β stimulation induces the binding of C/EBPβ along with FoxO and Smad proteins to the *p15INK4b* promoter in ChIP assays. TGF-β also induces the formation of a C/EBPβ–Smad2/3 complex. The ectopic overexpression of C/EBPβ increases the transcriptional activity of the *p15INK4b* promoter, and the C/EBPβ binding element is required for this response. Other family members, C/EBPα and C/EBPγ, do not mimic these effects of C/EBPβ (17).

To test the relevance of C/EBPβ in other TGF-β responses, we used HaCat cells stably harboring a shRNA vector that knocks down *C/EBPβ* expression by >85% (17). We determined the transcriptional response to TGF-β (3-h stimulation) by microarray profiling and compared it with the response in control knockdown HaCaT cells. Thirty of the genes whose signal was increased by TGF-β in control cells showed a blunting of this response by >50% in C/EBPβ-depleted cells (Fig. 4*B*). Five of these genes (*p15INK4b*, *SGK*, *GADD45A*, *CDC42EP3*, and *LEMD3*) overlap the FoxO–Smad synexpression group, and four (*p15INK4b*, *SGK*, *GADD45A*, and *CDC42EP3*) contain conserved C/EBPβ sites within FHBE/SBE clusters (see Fig. 4*A*). Using qRT-PCR, we confirmed that these five gene responses were absent in C/EBPβ knockdown cells, whereas other FoxO-dependent TGF-β responses showed little or no dependence on C/EBPβ (Fig. 4*C*). These results suggest the presence of regulatory variability within the FoxO–Smad synexpression group, with a subset of these genes additionally requiring C/EBPβ for their response to TGF-β.

Discussion

The present results collectively describe the transcriptional response to TGF-β in human epithelial cells and provide insights on the organization of this response. By means of RNAi-mediated depletion of FoxO1, FoxO2, and FoxO3, coupled with transcriptomic profiling and subsequent validation, we provide evidence that FoxO factors mediate at least 11 of the 115 gene-activation responses induced by TGF-β in human keratinocytes. In addition to the previously known FoxO–Smad target, *p21CIP1* (16), these genes include *p15INK4b*, several mediators of stress responses (*GADD45A*, *GADD45B*, and *IER1*) and mediators of adaptive responses (*CTGF*, *JAG1*, *LEMD3*, *SGK*, *CDC42EP3*, and *OVOL1*). All of these genes are activated in a FoxO-dependent and Smad4-dependent manner. It has been previously proposed that the induction of *p21CIP1* and *p15INK4b* by TGF-β does not require Smad4, because these responses persisted after a RNAi-mediated knockdown of *Smad4* (32). However, this negative result was likely caused by a higher sensitivity of these two genes to limiting amounts of Smad4. Using a series of knockdown cell lines, we show that a more complete knockdown of Smad4 levels strongly inhibits induction of these two gene responses.

The functional diversity of the group of genes that are jointly controlled by FoxO and Smad points at diverse cellular activities that are programmed to be enacted as cells process a TGF-β growth inhibitory input. Groups of genes that are coordinately regulated by a common signal, or synexpression groups (12), are thought to provide a balanced regulation of diverse cellular functions for the successful completion of a developmental step or stress response. Originally, synexpression groups were defined as genes with shared, complex expression pattern during embryogenesis (13, 35). In the present study the term is used in a broader sense to describe clusters of functionally diverse genes that are coregulated by a common transcription factor complex. That is, we identify as a synexpression group a set of genes that are expressed at the same time, in response to a common signal, and through a common set of transcriptional factors. Despite the commonalities, a synexpression group may be specific to a given cell type owing to differences in other enhancer elements in the gene promoters and cell-specific differences in available transcription factors. Indeed, the FoxO–Smad synexpression group defined in human keratinocytes includes different FoxO and Smad site configurations, different requirements for C/EBPβ, and distinct gene composition in different epithelial cell types.

The identification of groups of genes that share regulatory properties is of general interest, and it may be achieved by searching genomes for shared regulatory sequence motifs (36, 37). However, our results establish the feasibility of using RNAi-mediated depletion of Smad cofactors to functionally identify groups of genes that are jointly controlled by a given Smad–cofactor combination in a given cell type. Using a functional approach, we have identified a FoxO–Smad synexpression group whose members contain FBHE sites near clusters of SBE sites in most cases. The number and relative position of individual FoxO and Smad binding elements in these clusters differ substantially from gene to gene. For example, the configuration consisting of one FHBE followed by three SBEs that is present in *p21CIP1* (16) is not found in any other member of this synexpression group. Moreover, several genes in this group contain multiple such clusters. Systematic analysis of these regions may provide in the future a better definition of the rules governing the TGF-β responsiveness of these genes.

The variability in FoxO–Smad binding site configurations identified here implies differences in the regulation of these genes. A detailed analysis of the two CDK inhibitory genes in this group, *p21CIP1* and *p15INK4b*, has recently revealed the existence of two subsets of FoxO–Smad target genes that differ in their requirement of C/EBPβ (17). The distal region of the *p15INK4b* promoter contains a C/EBPβ binding site that is required, together with the Smad and FoxO sites, for the TGF-β response of this promoter. This requirement of C/EBPβ is shared by one subset of FoxO–Smad responsive genes represented by *p15INK4b* and also including *SGK*, *GADD45A*, *LEMD3*, and *CDC42EP3*. Another subset of FoxO–Smad responsive genes represented by *p21CIP1* does not have this requirement. Thus, C/EBPβ-dependent regulation provides variability within a FoxO–Smad synexpression group.

The Smad signaling pathway integrates cellular signals at multiple levels, allowing cells to properly read these signals. As Smad partners in target gene selection, the FoxO proteins provide a platform for coordination of different gene response to TGF-β. FoxO factors are under negative regulation by the phosphatidylinositol 3-kinase/AKT pathway (15, 28, 38), the telencephalic factor FoxG1 (16), and other pathways. In revealing the complex regulatory properties of these FoxO-and Smad-target genes, the present results may additionally pave the way for the identification of components of this pathway that are vulnerable to disruption in cancer and other disorders.

Experimental Procedures

Cell Lines, Transfection, and Proliferation Assays. HaCaT keratinocytes and MDA-MB-468 cells were maintained in DMEM supplemented with 10% FBS. Cell culture media also contained 100 units/ml penicillin/streptomycin, 2 mM L-glutamine, and 1 μg/ml fungizone. Primary keratinocytes were purchased from Cambrex (East Rutherford, NJ) and maintained in the manufacturer's medium. Cell lines were transfected with DNA by using Lipofectamine following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Luciferase reporter assays were performed as described (39). A CMV-Renilla luciferase plasmid (Promega, Madison, WI) was included to control for transfection efficiency. For TGF-β treatment we used 100 pM TGF-β1 (R & D Systems, Minneapolis, MN) unless indicated. ^{125}I-deoxyuridine incorporation assays were performed as described (40).

siRNA and shRNA. siRNA duplexes targeting FoxO1, FoxO3, and FoxO4 were obtained from the Memorial Sloan–Kettering Cancer Center High Throughput Screening Core Facility. The sense strands of the siRNA were: FoxO1, CCGCGCAAGAGCAGCTCGT, TGTGCGCCTGGACTCTTGA; FoxO3, GGGCGACAGCAACAGCTCT; and FoxO4, CCCGACCAGAGATCGCTAATT. Cell lines and primary keratinocytes were transfected with siRNA by using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). The siRNA-expressing H1 retroviral system was generously provided by R. Bernards (Netherlands Cancer Institute, Amsterdam). To generate the pRetrosuper sh(*Smad4*) and sh(*C/EBPβ*) vectors, the pRetrosuper vector was digested with BglII and HindIII and ligated with the annealed oligos. The Smad4 and C/EBPβ siRNA oligo pairs have been described (17, 41).

Oligonucleotide Array Assays. RNA sample collection and generation of biotinylated cRNA probe were carried out essentially as described in the standard Affymetrix (Santa Clara, CA) GeneChip protocol. Ten micrograms of total RNA was used to prepare cRNA probe with a Custom Superscript Kit (Invitrogen) and the BioArray High Yield TNA transcript labeling Kit (Enzo, New York, NY). Each sample was hybridized with an Affymetrix Human Genome U133A microarray at the Memorial Sloan–Kettering Cancer Center Genomics Core Laboratory. Genes whose expression level was marked as absent in the control plus TGF-β were discarded. Only those genes whose expression level was changed by >2-fold were scored as TGF-β-regulated genes. Venn diagram and heat maps analysis were carried out by using GeneSpring (Silicon Genetics, Mountain View, CA) software to identify genes loss in the FoxO-depleted and Smad4-depleted HaCaT cells.

Retroviral Infection. H29 packaging cell lines were transfected with the pRetroSuper or pBabe-Puro retroviral constructs by using Lipofectamine following the manufacturer's instructions (Invitrogen). Viral supernatants were harvested 48 h posttransfection, filtered (0.45 μM), then concentrated at 40,000 $\times$ *g* for 2 h and used for overnight infections of HaCaT cells or MDA-MB-468 cells in the presence of 8 μg/ml of polybrene. Cells were then recovered 24 h with fresh media and then selected by 5 μg/ml puromycin for 48 h.

Northern Blot. Cells were incubated with TGF-β as indicated, and total RNA was extracted by using the Qiagen (Valencia, CA) RNAeasy kit. Five micrograms of total RNA was used for Northern blot analysis. RNA was fractionated through a 1% agarose gel and transferred to Hybond N$^+$ nylon membranes (Amersham Pharmacia, Piscataway, NJ). Blots were probed with probes corresponding to the indicated genes. Data were quantified with a Phosphor screen in a STORM 840 scanner and Scanner Control version 4.1 software (Amersham Pharmacia)

ChIP. HaCaT cells were grown to 70% confluence, incubated in the presence or absence of TGF-β for 90 min, and subsequently cross-linked with 1% formaldehyde at room temperature for 15 min. ChIP was performed as described (42). The antibodies used

were anti-Smad2/3 (43) and anti-Smad4. A 308-bp segment of the distal region of the *p15INK4b* promoter (nucleotides −547 to −239) was amplified with the following primers: 5′-TATGGTTGACTAATTCAAACA-3′ (sense) and 5′-AATATTTTGGGAATGTTCACCA-3′ (antisense). As a negative control, a 166-bp region of the β-actin promoter (nucleotides 29–195) was amplified with the following primers: 5′-TCGAGCCATAAAAGGCAACTT-3′ (sense) and 5′-AAACTCTCCCTCCTCCTCTTCC-3′ (antisense).

qRT-PCR Assays. For qRT-PCR assays, cDNA was synthesized from 1 μg of purified RNA by using the SuperScript III First-Strand Synthesis System for RT-PCR from Invitrogen following the manufacturer's protocol. qRT-PCR was performed with a 7900HT instrument (Applied Biosystems, Foster City, CA). All reactions were performed in a volume of 10 μl containing 1 μl of cDNA template (20 ng), 0.1 μM primers, and 5 μl of the SYBR Green I Master Mix (Applied Biosystems). Each sample was analyzed in quadruplicate. A no-template control was included for each primer set used (Table 1, which is published as supporting information on the PNAS web site). PCR cycling parameters were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15 s, 60°C for 1 min. Data analysis was done by using the comparative C_T method in software SDS2.2.2 (Applied Biosystems).

Genomic Sequence Analysis. Human and mouse genomic sequences were extracted from 4 kb upstream of the transcription start site until the stop codon, including introns, for the genes of interest by using the University of California Santa Cruz genome browser from human genome release hg17 [National Center for Biotechnology Information (NCBI) Build 35, July 23, 2004] and mouse genome release mm6 (NCBI Build 34, May 4, 2005), except for mouse gene Cdkn2b (*p15INK4b*), which we extracted from release mm8 (NCBI Build 36, May 10, 2006). The human sequences were then examined for regions that contain FHBEs [(G/A)(T/C)AAA(T/C)A] and SBEs (AGAC) within 100 nt upstream and downstream of the FHBE by using a dual site matching program (http://cbio.mskcc.org/cgi-bin/lash/dualsite). Once identified the human Smad/Forkhead site-derived sequences were BLAST-compared (34) against the 4-kb upstream regions of mouse genes homologous to the synexpression group to further confirm conservation.

To further identify conserved C/EBPβ binding sites we followed a similar method. C/EBPβ and Smad binding sites [C/EBPβBE: T(G/T)NNG(A/C)AA(G/T) and SBE: AGAC] were identified by using the dual site matching program on the FoxO–Smad synexpression genes. Co-occurrence of the sites within 100 nt was identified. Then the previously generated FHBE–SBE sequences were BLAST-compared to a BLAST database created from the C/EBPβBE-SBE sequences, and a subset of regions containing one FHBE, one C/EBPβ, and one or more SBE binding sites in the human FoxO–Smad synexpression group were identified. The 10 significant matches of these two sequence sets are available on request. The 4 kb upstream of the transcription start site until the stop codon sequences for each human and mouse gene pair was BLAST-compared by using the bl2seq program on the National Center for Biotechnology Information web site or using word size 7 and default settings otherwise. The pairwise alignment file was then parsed by using a custom program to generate a track indicating mouse sequence segments that were similar to its human ortholog. Percent identities of the segments ranged from 75% for one segment in SGK to 100% for several segments on GADD45A.

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