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TITLE: The Effects of IGFBP3 Induction by TFG-B in Breast Tumorigenesis

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The goal of this research is to study the role of the antagonistic relationship between					
a hypothesis that the	induction of IGFBP-3 b	n mammary curmor. v TGF-R in stroma	igenesis. al fibrobli	asts is a mechanism	
by which TGF-ß regulat	tes the growth of breas	t epithelial cell	ls. To do	this, a model cell	
culture system was est	ablished in which the	effects of TGF-ß	through I	GFBP-3 could be	
studied. Our results	show that recombinant	IGFBP-3 is able t	to block I	GF-induced growth of	
breast cancer cells, r	nowever the effects of	secreted IGFBP-3	trom fibre	oblast media are	
cloud IGFBP-3's contri	bution. Additionally.	we set out to de	fine the i	mechanisms by which	
TGF-ß induces IGFBP-3	in MRC-9 cells. We de	termined that the	e gene is :	regulated by TGF-ß	
at the level of transc	cription, and not throu	gh mRNA stability	7. Analys	is of the promoter	
(-1800 bp) for TGF-ß regulatory elements, however, showed that TGF-b did not significantly					
induce IGFBP-3 promoter activity in MRC-9 fibroblasts. Therefore, the location of the TGF-					
14 SUBJECT TERMS					
Breast Cancer, TGF-ß, IGF, IGFBP-3, transcription, promoter, tumorigenesis, stromal/epith			nelial cells	84	
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# **ACCOMPANYING LETTER**

Ms. Anita J. Borton Box 3813 Department of Pharmacology Duke University Medical Center Durham, NC 27705 October 20, 2000

To whom it may concern:

The enclosed documents reflect work that Ms. Ellisa M. Rougier-Chapman and I have done as graduate students in the laboratory of Dr. Xiao Fan Wang in the Department of Pharmacology at Duke University. Ms. Rougier-Chapman was primarily concerned with the role of TGF- $\beta$  in countering the growth stimulatory effects of IGF in breast epithelial cells through inducing the transcription of IGFBP3. Upon completion of her degree, the grant was transferred to me for the final year of funding. In that year, we have addressed the mechanism of IGFBP3 regulation by TGF- $\beta$  through the over-expression of Smad proteins. In addition, we have begun further avenues of research involving the potential involvement of Smads in the adhesion of breast cancer cells to bone. We believe that studying this process could lead to novel treatments for breast cancer bone metastasis.

Although TGF-ß has been previously implicated as a major growth factor in bone, our research defines Smad3 as a major transducer of these effects. In addition our data suggests an important role for TGF-ß, and potentially Smads, in breast cancer metastasis to bone. Since this data is unpublished, we would appreciate your cooperation in protecting this information. All pages containing unpublished data which should be protected are marked by circling the page number in red. These pages include, but are not limited to, all of the data in the manuscript entitled "An Essential Role for Smad3 in Bone Formation Through Regulation of Osteoblast Differentiation."

As a graduate student, I have appreciated the support of the Department of Defense Breast Cancer Research Program and the interaction with breast cancer survivors provided by the Era of Hope Meeting. As a woman, I appreciate the federal government's dedication to advancing research that could lead to better treatments or a potential cure for this terrible disease. Thank you for your time and consideration.

Sincerely

Ms. Anita J. Borton

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### INTRODUCTION

The overall goal of the proposed project was to examine the roles and interactions of the TGF-B and IGF growth signals in breast tumorigenesis. TGF-B is a multifunctional peptide that plays a role in a wide variety of normal cellular functions including the regulation of proliferation, differentiation, extracellular matrix deposition, cell adhesion and migration (1). However, the effects of TGF-B can be highly cell-type and even cell-state specific. In normal mammary epithelial cells, TGF-B has been shown to be a potent inhibitor of growth through paracrine and autocrine pathways. TGF-ß acts through a direct mechanism as an inhibitor of proliferation by inducing the expression of the cyclin/cdk inhibitors such as p15, p21, and p27 in many cell types. Therefore, lesions to TGF-ß signaling pathways that disrupt the negative growth regulation of breast epithelia may contribute to mammary carcinogenesis and represent an obligatory step in neoplastic progression of breast epithelia. Indeed, loss of TGF-B responsiveness of MCF7 breast cancer cells has been shown to correlate with a loss of expression in these cells of the TGF-ß signaling receptors (2). Loss of such autocrine control by TGF-ß represents an opportunity for malignant epithelia to increase proliferation in response to other positive growth factors, like IGF, and hormones, like estrogen.

Available evidence strongly suggests that an another aspect of TGF- $\beta$ -induced growth inhibition of breast cancer epithelia may involve countering the growth stimulatory effects of IGF by inducing the secretion of IGFBP-3. IGFBP's are a family of molecules which sequester and prevent IGF's from binding and transducing mitogenic signals through the IGF receptors. As interactions between breast epithelial and stromal cells have been implicated as being important in the genesis and proliferation of breast cancer, we wanted to test whether fibroblasts affect the growth of breast epithelia by secreting IGFBP-3 in response to TGF- $\beta$ . Such a phenomena, if perturbed, could contribute to mammary carcinogenesis.

Therefore, we initiated a study aimed at determining the roles IGF, IGFBP-3, and TGF- $\beta$  have in breast tumorigenesis. Over the past several years, our work has been focused on two main areas: analysis of the interactions between breast epithelial and fibroblast cells mediated by IGF, IGFBP-3 and TGF- $\beta$  in cell culture and characterization of the molecular mechanism by which the expression of IGFBP-3 is regulated by TGF- $\beta$ . These studies were proposed as Technical Objectives 1 & 2 in the original proposal. Based upon the results of these experiments, we decided that the best method to further advances in the area of breast cancer research was to pursue alternate lines of investigation. We have begun to investigate the role of Smads in TGF- $\beta$  signaling and to determine the role of Smads in breast cancer metastasis to the bone.

#### BODY

### A. Optimization of Model Cell Culture System

As discussed above, TGF-ß may repress the growth of mammary epithelial cells through its induction of IGFBP-3 from stromal fibroblasts under normal and malignant conditions (3). This activity of TGF-ß may represent an indispensable mechanism by which the actions of growth stimulatory factors such as IGF are inhibited at the time when the cell cycle progression of epithelial cells is blocked by the TGF-ß signal. Furthermore, the effects of TGF-ß on the activity of IGF through its function of IGFBP-3 may represent an important aspect of interactions between mammary epithelial cells and stromal fibroblasts during normal mammary development as well as carcinogenesis. While several lines of evidence supported this notion in the literature (4,5,6), during the first year of this research we tested this hypothesis in a more direct fashion by establishing a model cell culture system using two MCF7 lines and MRC-9 fibroblasts as model epithelial and stromal cells. In this system, TGF-ß induces IGFBP3 in the stromal fibroblasts (MRC-9) but not in epithelial cells (MCF7) (Figure 1A).

Further research time was dedicated to optimizing the established cell culture system in an effort to conclude whether the secreted IGFBP-3 from the fibroblasts is adversely affecting the growth of breast cancer cells. Indeed, the results of initial studies showed that conditioned media from MRC-9 cells containing IGFBP-3 induced by TGF- ß could function to block IGF mediated proliferation in MCF7 cells (Figure 1B and 1C). Thus, it appeared that the conditioned media from the TGF-B did have an anti-proliferative effect, which may be mediated by the secreted IGFBP-3. However, TGF- ß was still present in the conditioned media from MRC-9 cells and could be responsible for the observed growth inhibitory effect on the MCF7 cells. Although the basal proliferation of MCF7 cells increased when the conditioned media was added, the relative growth inhibition between the control -/+ TGF- ß and conditioned media -/+ TGF-ß treated MCF7 cells were not significantly different at 64% and 50%, respectively. Therefore, we tried to precipitate out the residual TGF-ß with specific antibodies, in an effort to show that part if not all of the observed growth inhibition was due to IGFBP-3 in the conditioned media experiments. Interestingly, anti-TGF-B antibodies had a growth stimulatory effect when added to the conditioned media, an unexpected and confusing result. Overall, it appears that there are many growth factors present in the conditioned media besides TGF-B, which makes interpretation of this data difficult. To determine if the growth inhibitory response is even partially due to IGFBP-3, we tried to use a neutralizing antibody against IGFBP-3 to block the observed growth inhibitory response. We hypothesized that it was possible that the proliferation would increase significantly with the addition of such a neutralizing antibody. Unfortunately, anti-IGFBP-3 antibodies failed to demonstrate this effect in our cell culture system.

One explanation of the data is that, in contrast to the recombinant IGFBP-3, the secreted IGFBP-3 from MRC-9 cells in inactive. It is possible that MCR-9 cells not only secrete IGFBP-3 but also the protease that inactivates it. Since the initiation of this study, there have been reports in the literature identifying specific IGFBP proteases being present in the media of some cell

types. These proteases inactivate IGFBPs, preventing them from binding and sequestering IGFs. Hence, such proteases represent another level of regulation of IGFs and IGFBPs that had not been considered until recently. In fact, there is evidence to suggest that such proteases are upregulated by certain cancers to overcome the anti-proliferative effects of IGFBPs.

#### Recommendations:

The above mentioned experimental challenges and limitations have led us to abandon further study of the cell culture system to analyze the role of secreted IGFBP-3. We feel the only way to truly elucidate the role of IGFBP-3 in TGF-8 mediated negative growth regulation of breast epithelial cells

would be to engineer a transgenic mouse that either overexpressed or deleted IGFBP-3 in a breast tissue specific manner. Such an endeavor, however, is beyond the scope of this study and will not be attempted.

# **B.** Promoter Analysis of IGFBP-3

In addition to the finding that TGF-ß potently induces IGFBP3 protein in MRC-9 cells (Figure 1A), we also determined that TGF-ß increases IGFPB3 transcript (Figures 2 and 3). RNAse protection analysis reveals IGFBP3 mRNA is induced in MRC-9 cells after 26 hrs of TGF-ß treatment (Figure 2A). This induction of mRNA can occur within six hours (Figure 2B) and is not dependent on new protein synthesis which is blocked by cyclohexamide (Figure 2B). Therefore, the data suggests TGF-B's effect on the IGFBP-3 gene is direct. As the IGFBP-3 gene has been shown to be transcriptionally up-regulated by the tumor suppressor gene p53, regulation of IGFBP-3 expression at the transcriptional level may be a general mechanism through which the expression of IGFBP-3 can be induced by anti-proliferative signals. To determine whether the increase in IGFBP-3 mRNA is due to transcriptional activation by TGF-B, nuclei from MRC-9 cells treated with or without TGF-ß were harvested and a nuclear transcription run-off assay was performed (Figure 3A). As a control, total RNA from those treated cells was also saved and an RNAse protection assay was performed (Figure 3B). This assay allows for the direct measurement of RNA transcripts that have already been initiated at the time of cell harvesting. The result of the run-off assay showed that TGF-B did alter the rate of IGFBP-3 gene transcription after 6 and 24 hours of treatment by approximately 1.8-3 fold. Thus, the increase in IGFBP-3 mRNA after 6 and 24 hours of TGF-B treatment is due to an increase in transcription. Consistent with this conclusion, mRNA stability does not appear to be affected by TGF-B treatment. Following TGF-ß treatment of MRC-9 cells for 8 hours, actinomycin D treatment had little to no effect on the half-life of the IGFBP-3 message compared to control (data not shown).

The first step in defining the mechanism by which TGF-ß transcriptionally regulates IGFBP-3 gene expression is to determine the regions of the IGFBP-3 promoter responsible for transcriptional activation. To this end, we obtained approximately 1.8kb of the IGFBP-3 promoter upstream of the transcriptional start site, and cloned it into a reporter vector which drives the expression of a luciferase reporter gene (pGL2-Basic). Figure 4A diagrams the 5' deletions which were made of this promoter, in an effort to determine the TGF-ß responsive region. These constructs were transiently transfected into MRC-9 cells and TGF-ß induced

luciferase activity was assayed. Unfortunately, after many attempts and conditions, no significant induction by TGF- $\beta$  was observed for any of the IGFBP-3 promoter constructs (Figure 4B). In contrast, as a positive control, the common TGF- $\beta$  responsive reporter, p3TP-lux, gave a 5 fold induction of luciferase activity. From this data, it appears that the TGF- $\beta$  responsive region for MRC-9 cells is not located within –1.8 kb of the promoter. Furthermore, sequences we obtained from another lab that corresponded to regions in introns 1 and 2 were not responsive (data not shown). To test whether this promoter region would be active in another cell type, these promoter regions were transfected into primary human aortic smooth muscle cells. These cells were also shown to respond to TGF- $\beta$  by inducing IGFBP-3 protein and mRNA. The result of these experiments yielded similar results as the MRC-9 cells, showing insignificant induction of the promoter by TGF- $\beta$  (data not shown).

To further pursue the region of the IGFBP3 promoter which is TGF-ß responsive in MRC-9 cells, we performed a genomic library screen on a human placental library in the effort to obtain more sequence from introns 1-4, as many genes are known to contain enhancer

elements in intronic sequences, as well as in sequences many kb away from their promoters. We screened the library using 800bp of IGFBP-3 coding sequence, and obtained genomic sequence that appeared to correlate with introns 1-3. These sequences have proven too difficult to isolate and amplify by PCR as they are high in G-C content, and have thus not been analyzed for TGF-ß response

in MRC-9 cells. Using promoter sequence as a probe, we were also not able to isolate sequences of the IGFBP-3 gene beyond 1.8kb to analyze. This screen yielded only sequences we already had. Potentially, our library has been amplified too many times and has lost representation of all sequences as a result.

In other experiments with available promoter fragments, we did observe significant induction of IGFBP3 promoter activity by TGF- $\beta$  in HaCat cells (Figure 5A). Using this human keratinocyte cell line, we attempted to address the mechanism for IGFPB3 induction by TGF- $\beta$ . Major signal transducers for TGF- $\beta$  are the members of the Smad family of proteins Smad2, Smad3, and Smad4. Therefore, we over-expressed combinations of Smad2, Smad3, or Smad4 using adenoviral constructs obtained from collaboration with Glaxo-Welcome. However, we saw no elevation of basal promoter activity in the presence of exogenous Smads, nor was TGF- $\beta$  induced promoter activity enhanced (Figure 5A). In addition over-expression of adenoviral Smads in the presence or absence of TGF- $\beta$  in MRC-9 cells does not enhance basal or TGF- $\beta$  induced expression of IGBP-3 mRNA as analyzed by RNAse protection (Figure 5B). As a control, cell lysates were analyzed for expression of various Smad proteins by Western blot analysis (Figure 5C), revealing successful infections. In combination, the data suggests that TGF- $\beta$  effects on the IGFBP-3 promoter are not Smad mediated.

#### Recommendations:

Even though IGFBP-3 transcript and protein are strongly induced by TGF- $\beta$  in MRC-9 cells, the promoter region is not. One explanation is that the TGF-b regulatory element used in MRC-9 cells is not present within –1.8kb of the promoter and is located elsewhere, possibly in distant sequences outside of the IGFBP-3 gene. If this is the case, much effort and time would be

required to make another genomic library, which is not our expertise, and undertake more genomic library screens and cloning experiments to locate this elusive regulatory region. Therefore, we felt we could gain a better understanding of TGF-ß's complex role in breast cancer progression by refocusing the statement of work and specific aims of this study. Specifically, we have begun to investigate the role of the recently identified Smad molecules in mediating TGF-ß signaling.

## C. The Role of Smads in Breast Cancer Bone Metastasis

The Smads are a family of proteins consisting of at least eight members. Smad2 and Smad3 are downstream effectors of TGF- $\beta$ . In response to TGF- $\beta$ , Smad2 and Smad3 are phosphorylated by the type I receptor kinase and translocate to the nucleus in a complex with Smad4. While in the nucleus they act as sequence specific transcription factors at the consensus site: GTCTAGAC. Several genes to date are known to be regulated by Smads at similar sequences including PAI-1, p3TP-Lux, COL7A1, JunB, and c-Jun. Smads are also thought to synergize or act in combination with other transcription factors such as the Vitamin D receptor, AP-1, Sp-1, p300/CBP, and MuE3 (TFE3) to activate transcription.

A role for Smads as tumor suppressors has been suggested by the observation that Smad2 and Smad4 are commonly deleted in a variety of cancers including the majority of human pancreatic, certain colon cancers, and some breast cancers. Thus, in light of their role as transcription factors, Smads may regulate genes which are critical for TGF- $\beta$  mediated growth inhibition. Consistent with this hypothesis, mouse embryo fibroblasts and lymphoid cells derived from Smad3 deficient mice have been found to be impaired in TGF- $\beta$  mediated growth inhibition. Although there is only one report implicating Smads in regulating the known TGF- $\beta$ growth inhibition pathway through p21 promoter induction, it is likely that other yet identified genes important for cell growth will be found to be linked to transcriptional regulation by Smads.

While TGF $\beta$  induces cell cycle arrest and Smads are known tumor suppressors, late stage breast cancers produce large amounts of TGF $\beta$  and are refractory to its growth inhibitory effects. Since TGF $\beta$  can also stimulate cell invasion of breast cancer cells, TGF $\beta$  may initially act to suppress tumor formation but promote metastasis in later stages of disease. Indeed, recent evidence suggests that the signal transduction cascade may be required for breast cancer metastasis to the bone. A reduction in osteolytic metastasis is observed when MDA-MB-231 cells over-expressing dominant negative type II receptor are injected into nude mice (7). The loss of TGF $\beta$  signal transduction results in fewer tumors

with reduced numbers of osteoclasts and increased animal lifespan. Additionally, overexpression of constitutively active TGF $\beta$  type I receptor to potentiate the signal cascade results in increased metastasis with a corresponding decrease in survival (7).

Two genes potentially involved in the ability of TGF $\beta$  to promote bone metastasis are parathyroid hormone related protein (PTHrP) and interleukin 11 (IL-II), which are both expressed by metastatic MDA-MB-231 cells (8,9). PTHrP increases bone resorption (10) and increases osteolytic metastasis when over-expressed in breast cancer cells (11). TGF $\beta$  treatment increases PTHrP expression *in vitro* (12), and over-expression of TGF $\beta$  type I receptor *in vivo* results in increased PTHrP (7). IL-11 promotes bone resorption *in vitro* by increasing osteoclast formation (13), and secretion of the cytokine is increased in osteolytic bone metastasis induced in nude mice (14). TGF $\beta$  can activate the IL-II promoter through a mechanism involving AP1 family members (15) and Smad3 (data not shown). Recombinant human interleukin-II is being used in clinical trials in breast cancer patients to prevent thrombocytopenia induced by chemotherapy (16-17), so elucidation of the potential role of interleukin-11 in osteolytic metastasis is of immediate clinical importance.

We are testing the hypothesis that Smad molecules are required for the ability of breast cancer cells to metastasize to bone. Initial experiments in this area were supported by the final year of funding on this grant and were used to determine a method for evaluating the loss of Smad3 signaling on the ability of breast cancer cells to adhere to bone in vitro. We have established a model system in which adherent osteoblasts can be easily distinguished from adherent breast cancer cells and the effects of TGF $\beta$  are easily observed. To establish this model, osteoblast-like cells derived from osteosarcomas were plated at similar densities and cultured in the presence of differentiation inducing media. This media contains  $\beta$ -glycerolphosphate to facilitate mineral deposistion and ascorbic acid to facilitate collagen production for matrix deposition. After several days, the cells were fixed with 1% paraformaldehyde and washed multiple times with phosphate buffered saline solution. MDA-MB-231 breast cancer cells suspended in serum free media were then allowed to attached to the osteoblast matrix for three hours. The wells were then washed again with PBS and stained for 10 min with trypan blue. Since the trypan blue stain will only enter dead cells, the fixed osteoblasts are stained whereas the breast cancer cells remain clear. The number of adherent cells can then be easily visually quantitated. Using this system we have been able to show TGFB dependent effects on the number of breast cancer cells adhering to osteoblasts (Figure 6).

The creation of Smad3 null mice by our laboratory provides a valuable tool to determine if TGFB mediated signal transduction in the bone is required for breast cancer metastasis. We would like to use isolated primary osteoblasts which lack Smad3 in the model discussed above. In order to achieve that goal, we have established methods for culturing primary osteoblasts isolated from the calvaria of Smad3 null mice. In this procedure, the skulls of sacrificed new born pups are dissected, washed in phosphate buffered saline, and incubated overnight in media. Animal tails are digested with proteinase K and the remaining DNA is used for PCR to determine the genotype of the animals. The calvaria of wild-type and Smad3 null littermates are then digested with collagenase to isolate osteoblasts. These isolated primary osteoblasts express differentiation markers such as osteocalcin and will form calcium nodules in vitro (see attached manuscript). Studies with these osteoblasts and further in vivo experiments to define the role of Smad3 in breast cancer bone metastasis are beyond the scope of this grant, but will continue through alternate funding sources. Based on the comments of previous reviewers, we have characterized the bone phenotype of Smad3 null mice (see attached manuscript) to demonstrate a requirement for Smad3 in bone. A revised statement of work for the continuation of this project is as follows:

# STATEMENT OF WORK

**Title**: The Role of Transforming Growth Factor Beta and its Downstream Signaling Effector, Smad 3, in Breast Cancer Bone Metastasis

Task 1: Evaluate the effects of the loss of TGF $\beta$  signal transducer Smad3 in bone and in breast on the ability of breast cancer cells to metastasize *in vitro* (months 1-18).

- a. Establish an *in vitro* model for breast cancer adhesion to bone.
- b. Assess ability of MDA-MB-231 cells to adhere to wild type and Smad3 null bone.
- c. Assess ability of wild type and Smad3 null breast cancer cells to adhere to bone.

Task 2: Establish the role of TGF $\beta$  signaling through Smad3 in the ability of breast cancer cells to metastasize *in vivo* (months 10-30).

- a. Cardiac inject irradiated wild type and Smad3 null mice with breast cancer cells.
- b. Evaluate osteolytic lesions in the bones of injected nude mice through X-ray and histomorphometric analysis.
- c. Assess plasma levels of PTHrP and IL-ll.

Task 3: Evaluate the role of Smad3 in the regulation of PTHrP and IL-ll expression (months 26-36).

- a. Compare levels of PTHrP and IL-11 in wild type and Smad3 null breast cancer cells.
- b. Evaluate the ability of TGF $\beta$  to activate PTHrP and IL-II promoters in the absence of Smad3 through the use of primary wild type and Smad3 null cells.
- c. Determine if Smad3 can directly bind the DNA of PTHrP and IL-ll promoters.

# **KEY RESEARCH ACCOMPLISHMENTS**

- Recombinant IGFBP-3 effectively blocks IGF-1 stimulated proliferation of MCF7 cells in culture, and represents a potential key mediator of mediated growth inhibition of breast epithelial cells.
- TGF- $\beta$  strongly induces IGFBP-3 protein and mRNA in MRC9 fibroblasts
- The rapid induction of IGFBP-3 mRNA by TGF $\beta$  does not require new protein synthesis, and is a result of transcription and not mRNA stability
- The TGF $\beta$  cis-regulatory elements do not appear to reside within -1.8kb of the IGFBP-3 proximal promoter
- Smads are not involved in IGFBP3 induction by TGFβ

• • •

• A model for breast cancer adhesion to bone was established in vitro, and TGF $\beta$  was determined to alter MDA-MB-231 adhesion to osteosarcoma cells in this system

# **REPORTABLE OUTCOMES**

- Elissa Rougier-Chapman (Principal Investigator) obtained Master of Science degree in Molecular Cancer Biology from Duke University, September, 1999.
- Anita J. Borton (Principal Investigator for final year of funding) has completed and passed all preliminary examinations for a degree in Pharmacology at Duke University
- Publications:
- Wong C, Rougier-Chapman EM, Frederick JP, Datto MB, Liberati NT, Li Jian-Ming, Wang X-F. 1999. Smad3/Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by Transforming Growth Factor-ß. Mol. Cell. Bio. 19(3): 1821-1830.
- Liberati NT, Datto MB, Frederick JP, Shen X, Wong C, Rougier-Chapman EM, Wang X-F. 1999. Smads bind directly to the Jun family of AP-1 transcription factors. Proc. Natl. Acad. Sci. 96: 4844-4849.
- Datto M.B., Frederick JF, Borton AJ, Zhuang Y, and Wang X-F. 1999. Targeted disruption of Smad3 Reveals an Essential Role in Transforming Growth Factor β-mediated signal transduction. Mol Cell Bio. 19: 2495-2504.
- Meeting abstracts:
- The Effects of IGFBP-3 Induction by TGF-β in Breast Tumorigenesis E.M. Rougier-Chapman, H.S. Symonds, A.J. Borton and X.F. Wang Department of Defense Era of Hope Meeting 2000
- An Essetial Role for Smad3 in Bone Formation Through the Regulation of Osteoblast Differentiation
   A.J. Borton, J.P. Frederick, M.B. Datto, X.F. Wang, and R.S. Weinstein American Society for Bone and Mineral Research Meeting 2000

No Patents or Inventions to Report

# CONCLUSIONS

Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a potent growth inhibitor of normal breast epithelia. Evidence is accumulating that TGF-B activity is critical to maintaining the negative paracrine and autocrine regulation of breast epithelial growth. Therefore, defects in the TGF-B signaling pathways may result in uncontrolled growth of mammary epithelial cells and consequently contribute to carcinogenesis of the breast. Available evidence suggests that TGF-ß is able to indirectly mediate its growth inhibitory effects on breast epithelia by inducing the secretion of insulin-like growth factor binding protein 3 (IGFBP-3). Our goal was to test a hypothesis that the induction of IGFBP-3 by TGF-b in stromal fibroblasts is a mechanism by which TGF-b regulates the growth of breast epithelial cells. We established a model cell culture system of MCF7 cells and MRC-9 fibroblasts and showed that in culture recombinant IGFBP-3 is able to block IGF-induced growth of breast cancer cells, however the effects of secreted IGFBP-3 from fibroblast media are unclear. Growth inhibition does occur, but the presence of other molecules in this system cloud IGFBP-3's contribution. In fact, we believe the secreted IGFBP-3 may be inactive, as a result of specific IGFBP proteases that could be present in the MRC9 media. The implication of this, with so many variables to take into account, is that the cell culture system becomes ineffective at defining the relative contributions of the different factors in paracrine regulation of breast cancer cell growth. As mentioned above, one would have to design a transgenic mouse model to specifically "knock-out" or constitutively overexpress IGFBP-3 in the mammary tissue of mice to determine the true significance of this molecule in regulation of epithelial cell growth.

Besides the cell culture studies, we were investigating the molecular mechanism by which the expression of IGFBP-3 is regulated by TGF- $\beta$  in MRC-9 fibroblasts. We determined that the gene is regulated by TGF- $\beta$  at the level of transcription, and not through mRNA stability. Analysis of the promoter (-1800 bp) for TGF- $\beta$  regulatory elements, however, showed that TGFb did not significantly induce IGFBP-3 promoter activity in MRC-9 fibroblasts. Additional genomic library screens also did not yield sequences containing TGF- $\beta$  regulatory elements, and the location of the TGF- $\beta$  transciptional reglulatory elements in the IGFBP-3 gene remain unknown. However, over-expression of adenoviral Smads in MRC-9 cells did determine that the molecular mechanism of IGFBP3 induction does not involve Smad2, Smad3, or Smad4.

Since all of the specific aims of the original research proposal were addressed, experiments in the final year shifted focus to elucidate the involvement of TGF-ß in later stages of breast cancer. Specifically, we are testing the hypothesis that Smad 3 is required for the ability of breast cancer cells to metastasize to bone. We have established a requirement for Smad in bone development. In addition, we have determined a method to determine the requirement for Smad3 in the ability of breast cancer cells to adhere to bone using primary osteoblasts with or without Smad3. Understanding this pathway may result in the identification of molecular targets useful for drug design in the prevention and treatment of a currently incurable disease state.

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Figure 1. IGFBP-3 Secretion Increases With TGF- $\beta$  Treatment and May Mediate TGF- $\beta$  Induced Growth Inhibition. (A) Western blot analysis of IGFBP3 secreted protein induction in conditioned media from MRC9 and MCF7 cells following 48 hrs of treatment with 100 pM TGF- $\beta$ . (B) MCF-7/PT3 cell culture paracrine test of cell growth after treatment with TGF- $\beta$ , IGF1, or conditioned media (CM) from MRC-9 fibroblasts treated with or without TGF- $\beta$ . (C) MCF-7/TEX cell culture paracrine test of cell growth after treatment media (CM) from MRC-9 fibroblasts treated with TGF- $\beta$ , IGF1, or conditioned media (CM) from MRC-9 fibroblasts treated with TGF- $\beta$ , IGF1, or conditioned media (CM) from MRC-9





# Figure 2. TGF-b Increases IGFBP-3 mRNA.

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(A) RNAse protection analysis of IGFBP-3 mRNA induction in MRC-9 fibroblasts following 26 hours of TGF- $\beta$  treatment. (B) RNAse protection analysis of IGFBP3 mRNA induction in MRC-9 cells following a time course of TGF- $\beta$  treatment and after 8 hours of TGF- $\beta$  treatment in the presence and absence of cycloheximide.





# Figure 3. TGF- $\beta$ Transcriptionally Regulates IGFBP-3.

(A) Transcription runoff assay of nuclei from MRC-9 fibroblasts treated with or without TGF- $\beta$  for the indicated times. The 32P labeled mRNA transcripts were hybridized to IGFBP-3 and GAPDH cDNAs which were immobilized to nitrocellulose. (B) RNAse protection assay of total RNA harvested from the TGF- $\beta$  treated MRC-9 cells used in Figure 3A.



# Figure 4. The Promoter of IGFBP-3 is Not Activated by TGF- $\beta$ in MRC-9 Cells.

(A) Diagram of the IGFBP-3 promoter fragment obtained from Dr. D. Powell (-1800 bps) and the deletion constructs created from the Powell promoter. All promoter constructs were subcloned in front of the luciferase reported gene. (B) Luciferase assay of MRC-9 cells transfected with the various 5' promoter deletions of the proximal IGFBP-3 promoter and treated with or without 100 pM TGF-β. As a positive control, p3TP-Lux was also included. Fold inductions are shown above each set of bar graphs.



# Figure 5. TGF- $\beta$ Effects on the IGFBP-3 Promoter are Not Smad Mediated.

(A) Overexpression of Smad2/3, Smad3/4, or the inhibitory Smad6 in HaCat cells with the -430 and -160 IGFBP-3 luciferase promoter deletion constructs. Smads were over-expressed using adenoviral constructs obtained from collaboration with Glaxo-Welcome. (B) MRC-9 cells were exposed to adenoviral Smads in the presence or absence of TGF-b and IGFBP-3 mRNA levels were assessed via RNAse protection. (C) Western blot analysis of cell lysates used in Figure 5B with antibodies from Santa Cruz.



Figure 6. TGF- $\beta$  Alters MDA-MB-231 Adhesion to ROS Cells. ROS cells plated at a density of 200,000 cells per well were grown in differentiation media for 24 hrs before the addition of TGF- $\beta$  or control buffer. for 48 hrs. Cells were fixed with paraformaldehyde and washed with PBS before the addition of 2mL of serum free media containing MDA-MB-231 cells. MDA-MB-231 cells were pre-treated for 48hrs with TGF- $\beta$  or control buffer, removed from wells with .05% EDTA in PBS, and resuspended to 100,000 cells/mL in serum free media,

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# An Essential Role for Smad3 in Bone Formation Through Regulation of Osteoblast Differentiation

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# Abstract

The role of the transcription factor, Smad3, in both TGF- $\beta$  and vitamin D receptor signaling suggests that Smad3 may function in bone formation and remodeling. Consistent with this hypothesis, we demonstrate that Smad3 null mice are osteopenic. This lower bone density is osteoblast mediated as indicated by a reduced bone formation rate, whereas osteoclast numbers and vitamin D mediated serum calcium homeostasis appear normal. Histological analysis of Smad3 null bones reveals decreased cortical width and cancellous bone area, decreased osteoid area and width, increased osteoblast and osteocyte apoptosis, and increased osteocyte density. Studies with isolated primary osteoblasts indicate that TGF- $\beta$  cannot inhibit the differentiation of osteoblasts in the absence of Smad3, but TGF- $\beta$  stimulated proliferation remains intact. Together this data suggests that a loss of Smad3 accelerates osteoblast differentiation to osteocytes and decreases osteoblast work time by shortening lifespan.

# Introduction

Bone is a highly dynamic tissue in which the degradation of old bone by osteoclasts is balanced with the formation of new bone by osteoblasts. Osteoblasts form bone through the production and deposition of extracellular matrix proteins. This matrix, or osteoid, is subsequently mineralized to provide skeletal strength. The osteoblasts entrenched in mineral differentiate to osteocytes, but alternative cell fates for the osteoblast include differentiation to bone-lining cells or apoptosis. The regulation of osteoblast proliferation, differentiation, and apoptosis by a variety of hormones and growth factors including vitamin D and Transforming Growth Factor  $\beta$  (TGF- $\beta$ )<sup>1-3</sup> is critical for bone formation and remodeling.

TGF-β produced by osteoblasts<sup>4</sup> is incorporated into bone extracellular matrix, and latent TGF-β is released and activated during resorption<sup>5</sup>. Newly released TGF-β then stimulates bone formation through several mechanisms. For example, TGF-β stimulates osteoprogenitor proliferation<sup>6-8</sup> and migration<sup>9</sup>. This proliferative capacity of TGF-β is associated with a delay in differentiation to osteocytes. Markers of differentiation, including alkaline phosphatase activity<sup>10-12</sup>, osteocalcin expression, and calcium nodule formation, are all inhibited by TGF-β<sup>12</sup>. In addition, TGF-β modulates osteoblast production of several extracellular matrix proteins, including the major organic component of bone, type I collagen<sup>13,14</sup>. Consistent with these *in vitro* effects of TGF-β on osteoblasts, injection of TGF-β results in increased osteoprogenitor proliferation and new bone formation<sup>7,8</sup>. Furthermore, deletion of the TGF-β1 gene in mouse models results in decreased bone mass<sup>15</sup>, and polymorphisms in the TGF-β1 gene are associated with an increased risk of osteoporosis in women<sup>16</sup>.

In addition to TGF- $\beta$ , normal bone formation requires regulation by the active metabolite of vitamin D, 1,25  $\alpha$  dihydroxycholecalciferol, or vitamin D3 . Vitamin D3 directly affects osteoblasts by promoting differentiation as assessed by increased alkaline phosphatase activity and enhanced osteocalcin expression<sup>17,18</sup>. Vitamin D3 also regulates bone formation by controlling serum calcium homeostasis<sup>19</sup>. When serum calcium is low, parathyroid hormone (PTH) and vitamin D3 increase calcium intestinal absorption and increase osteoclast differentiation to promote calcium release from the skeleton<sup>20</sup>. Loss of the vitamin D receptor (VDR) in mice causes rickets and osteomalacia associated with decreased serum calcium and increased PTH<sup>21,22</sup>. This bone phenotype of VDR null mice is corrected by normalization of serum ion levels<sup>23</sup>. In addition, continuous infusion of calcium and phosphorus into vitamin D-

deficient rats promotes osteoid mineralization<sup>24</sup>. Therefore, the skeletal effects of vitamin D may primarily be a consequence of its role as a regulator of serum homeostasis.

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Although vitamin D and TGF- $\beta$  have different roles in skeletal maintenance, their signaling pathways cross-talk in osteoblasts with both synergistic and antagonistic results. For example, in osteoblasts vitamin D inhibits TGF- $\beta$  mediated proliferation<sup>25,26</sup>, and TGF- $\beta$  blocks vitamin D induced osteocalcin expression<sup>26-28</sup>. Alternatively, TGF- $\beta$  and vitamin D co-treatment synergistically enhances alkaline phosphatase activity<sup>26-28</sup>. Recent evidence that TGF- $\beta$  and VDR may both rely on the transcription factor Smad3 for signaling<sup>29,30</sup> provided a potential mechanism for interaction between the pathways.

Smad3 is a member of a family of proteins, the Smads, which are well-established effector molecules in the signaling pathways of the TGF- $\beta$  superfamily ligands. The highly related Smad3 and Smad2 are specifically activated by TGF- $\beta^{31,32}$ . Upon TGF- $\beta$  treatment, the type I receptor phosphorylates the C-terminal region of Smad2 and Smad3 to promote their interaction with Smad4. Smad complexes then translocate to the nucleus where the heterodimers directly bind DNA or interact with other transcription factors to activate transcription of responsive genes<sup>33-35</sup>.

To investigate the possible requirement for Smad3 in both TGF- $\beta$  and vitamin D signaling in bone, we utilized a mouse model with targeted disruption of Smad3<sup>36</sup>. Here we demonstrate that Smad3 null mice are osteopenic with maintenance of serum calcium homeostasis and normal osteoclast numbers. Decreased bone density is due to decreased bone formation rates, indicating aberrant osteoblast performance *in vivo*. Analysis of isolated

osteoblasts reveals a requirement for Smad3 in TGF- $\beta$  mediated regulation of osteoblast differentiation.

# Results

#### Smad3 null mice are osteopenic with less cancellous and cortical bone.

To determine if Smad3 is a necessary signal transducer in bone, we used radiography to evaluate mice with targeted deletion of Smad3. Femurs and tails of Smad3 null mice consistently appear more radiolucent than those of their wild-type littermates (Figure 1a and 1b), indicating that a loss of Smad3 results in lower bone density. Densitometric quantitation of bone density reveals a 16% decrease in the total bone mineral content of Smad3 null femurs (Figure 2a). Independent analysis by atomic absorption demonstrates a 17% reduction in the percentage of calcium per femur wet weight, confirming that Smad3 null mice are osteopenic (Figure 2b). The lower bone density in Smad3 null mice reflects a decrease in the amount of both cancellous and cortical bone as determined through histomorphometric evaluation of nondecalcified sections (Figure 2d). The percentage of cancellous bone area per tissue area is 27% less in Smad3 null mice than wild-type littermates. There is also a marked decline in the amount of cortical bone, as assessed by cortical width (Figure 2d, e, f) which is reduced 37% in Smad3 deficient mice. While the amount of bone in Smad3 null mice is diminished, the mineral content of the bone which is formed appears normal. After ashing to remove all organic material, the percentage of calcium per ash weight of Smad3 null bones is similar to wild-type littermates (Figure 2*c*).

### Serum calcium homeostasis and osteoclast density is normal in Smad3 null mice.

One potential etiology of lower bone density in Smad3 null mice is loss of serum mineral homeostasis. Smad3 is a co-activator of the vitamin D receptor  $(VDR)^{29,30}$ , and targeted deletion of VDR leads to osteopenia<sup>21,22</sup>. To determine if the similarity between VDR and Smad3 null bone phenotypes resulted from analogous mechanisms, we evaluated serum homeostasis in Smad3 null mice. We find that comparable concentrations of calcium and phosphorous are present in serum isolated from wild-type and Smad3 null littermates (Figure 3*a*) whereas calcium levels are significantly reduced in VDR null animals<sup>21,22</sup>. In addition, mice deficient in Smad3 are capable of increasing serum calcium concentrations in response to Vitamin D injection (Figure 3*b*), suggesting that global Vitamin D receptor signaling remains intact in the absence of Smad3. Normal serum homeostasis is consistent with results indicating that osteoclast density in Smad3 null and wild-type littermates are similar (Figure 3*c*).

#### Decreased osteoblast function causes osteopenia in Smad3 null mice.

Since defects in serum mineral homeostasis or increased osteoclast numbers cannot explain the lower bone density of Smad3 null mice, we next evaluated osteoblast number and vigor using histomorphometry. Dual tetracycline labeling was utilized to determine that bone formation rates are reduced 40% in mice lacking Smad3 compared to wild-type littermates (Figure 4*a*). Bone formation is calculated as the product of the mineralizing surface and the rate of mineral apposition. Although there is no significant difference in mineralizing perimeter, mineral appositional rates are decreased 28% (Figure 4*b*). This reduction is indicated by the differences in the distance between dual tetracycline labels (Figure 4*c*, 4*d*) during equivalent labeling periods for Smad3 null and wild-type mice.

In addition to mineralization, bone formation by osteoblasts requires matrix deposition. The amount of extracellular matrix, or osteoid, present in femurs of Smad3 null mice is consistently reduced (Figure 4*e*). Loss of Smad3 results in a 25% decrease in the total osteoid width. The osteoid present in Smad3 null mice covers an area which is 50% lower than their wild-type littermates and a perimeter which is decreased 40%. We conclude that osteopenia in Smad3 null mice results from reduced bone formation rates due to aberrant osteoblast function. We therefore evaluated osteoblast proliferation, differentiation, and apoptosis to elucidate the molecular signaling pathways in osteoblasts that are altered upon the loss of Smad3 and result in the mouse phenotype.

### TGF- $\beta$ mediated osteoblast proliferation is Smad3 independent.

To determine if deregulation of osteoblast proliferation could result in the decreased bone formation rates in Smad3 null mice, we isolated osteoblasts from the calvaria of newborn wildtype and Smad3 null littermates. Western blotting confirms the presence of Smad3 in wild-type osteoblasts and its absence in mice with a targeted deletion of Smad3 (Figure 5*a*). Additionally, there is no compensatory increase in Smad2 in Smad3 null cells (Figure 5*a*). Using these osteoblasts, we assayed the TGF- $\beta$  induced proliferation of cell lines isolated from individual calvaria. TGF- $\beta$  treatment resulted in a dose-dependent increase in cell replication that is independent of Smad3 (Figure 5*b*). In the presence or absence of Smad3, maximal concentrations of TGF- $\beta$  cause a five-fold increase in cell growth. In addition, loss of Smad3 does not affect basal levels of osteoblast proliferation (Figure 5*b*) and total numbers of osteoblasts are not significantly decreased *in vivo* (data not shown). Together, the data indicate that Smad3 is not required for the induction of osteoblast proliferation by TGF- $\beta$ .

Another aspect of osteoblast growth that could be affected by loss of Smad3 is the ability of vitamin D to antagonize TGF- $\beta$  induced osteoblast proliferation. To determine if VDR binding to Smad3 is required for growth inhibition, wild-type and Smad3 null osteoblast cultures were treated with TGF- $\beta$  in the presence and absence of vitamin D. Addition of vitamin D to wild-type or Smad3 null osteoblasts inhibits TGF- $\beta$  induced proliferation by 40% (Figure 5*c*), demonstrating another aspect of vitamin D receptor signaling which is independent of Smad3.

# Smad3 is required for the TGF- $\beta$ mediated delay in osteoblast differentiation.

After determining that Smad3 is not essential for osteoblast proliferation, we next attempted to define a role for Smad3 in bone formation through regulation of osteoblast differentiation. To assess osteoblast differentiation in the absence of Smad3, isolated osteoblasts were treated with  $\beta$ -glycerolphosphate and ascorbic acid and allowed to differentiate for a maximum of fourteen days. Alizarin red staining demonstrates that both wild-type and Smad3 null osteoblasts are capable of forming calcium nodules by day twelve (Figure 6*a*). However, continuous treatment with TGF- $\beta$  impairs calcium deposition in wild-type osteoblasts while those lacking Smad3 are unaffected. Although Smad3 is required for TGF- $\beta$  mediated repression of calcium nodule formation, induction of nodule formation by vitamin D is Smad3 independent (Figure 6*a*).

To further evaluate the ability of TGF- $\beta$  to delay differentiation in the absence of Smad3, we utilized alkaline phosphatase activity as a marker for osteoblast differentiation. Alkaline phosphatase is produced by cultured osteoblasts in a biphasic pattern<sup>12</sup> and may increase mineralization by facilitating calcium phosphate precipitation<sup>37,38</sup>. TGF- $\beta$  treatment causes a 50% reduction in the activity of alkaline phosphatase of wild-type osteoblasts at day 4 in culture

(Figure 6*b*). However, similar treatment of Smad3 null osteoblasts does not inhibit alkaline phosphatase activity, further demonstrating the importance of Smad3 in TGF- $\beta$  mediated repression of differentiation. Late in culture, TGF- $\beta$  can repress alkaline phosphatase activity in the absence of Smad3, but still to a lesser extent than in wild-type cells (data not shown).

In addition to alkaline phosphatase activity and calcium nodule formation, osteocalcin expression was also used to assess the degree of osteoblast differentiation. Osteocalcin is an extracellular matrix protein which regulates mineralization and is a specific marker for mature osteoblasts. When RNA isolated from wild-type and Smad3 null osteoblasts at day 6 in culture is probed for osteocalcin, basal expression is upregulated in Smad3 deficient cells but repression remains intact (Figure 6*c*). Thus, Smad3 is not required for inhibition of osteocalcin expression by TGF- $\beta$ . While Smad3 may not be essential for TGF- $\beta$  mediated repression of all osteoblast differentiation markers, loss of Smad3 results in increased osteocyte cell fate *in vitro* as assessed by osteocalcin expression, alkaline phosphatase activity, and calcium nodule formation.

Further evidence also demonstrates a role for Smad3 in the regulation of differentiation *in vivo*. Histomorphometry detected an 18% increase in the density of osteocytes in cancellous bone of Smad3 null mice (Figure 6*d*), indicating that loss of Smad3 may promote the osteocyte fate of the osteoblast. This data suggests that decreased bone formation rates in Smad3 null mice result from decreased duration of osteoblast performance due to accelerated differentiation.

### Loss of Smad3 promotes osteoblast and osteocyte apoptosis.

Decreased duration of osteoblast function could result from shortened life span due to increased apoptosis as well as increased differentiation. To determine levels of apoptosis of osteoblasts and osteocytes, nondecalcified sections of femurs from wild-type and Smad3 null

mice were stained using the TUNEL reaction (Figure 7a, 7c). The amount of positive, apoptotic osteoblasts is increased 25% in mice with targeted deletion of Smad3 (Figure 7a, 7b) while osteocyte apoptosis is increased 40% (Figure 7c, 7d). Increased osteocyte apoptosis is consistent with a push toward differentiation in the absence of Smad3 since apoptosis is the ultimate fate of the osteocyte. However, the increased osteoblast apoptosis indicates that loss of Smad3 also alters osteoblast cell fate by promoting apoptosis rather than allowing osteoblasts to differentiate to osteocytes or become bone-lining cells.

# Discussion

To investigate the possible requirement for Smad3 in both TGF- $\beta$  and vitamin D signaling in bone, we utilized a mouse model with targeted disruption of Smad3<sup>36</sup>. Compared to wild-type littermates, Smad3 null mice have decreased bone density with a lower rate of bone formation, indicating aberrant osteoblast function. To determine which aspect of osteoblast regulation is altered upon loss of Smad3, we evaluated osteoblast proliferation, differentiation, and apoptosis. While proliferation responses were intact in the absence of Smad3, Smad3 is required for the regulation of osteoblast differentiation and survival.

Increased osteocyte density in Smad3 null mice is consistent with the *in vitro* findings that Smad3 is required for TGF- $\beta$  mediated inhibition of differentiation markers. Therefore, we propose that Smad3 is an essential signaling effector of TGF- $\beta$  in bone, and a primary role for Smad3 in the skeleton is to transduce TGF- $\beta$  mediated delay of osteoblast differentiation. This model is supported by previously reported evidence from primary osteoblast cultures and *in vivo* injections which demonstrate TGF- $\beta$  causes osteoprogenitor proliferation with decreased

expression of differentiation markers<sup>2,3,6-8,10-12</sup>. However, the exact function of TGF-β in bone is as complex as it is important since the cytokine may utilize several different mechanisms to regulate bone formation or maintenance. For example, results from transgenic mice overexpressing dominant-negative type II TGF-β receptor<sup>39</sup> or TGF-β2<sup>40</sup> under the control of the osteocalcin promoter indicate a role for TGF-β in resorption as well as the promotion of osteoblast differentiation. This apparent contradiction between mouse models of TGF-β in bone may be attributed to different temporal and spacial patterns of expression in the transgenic animals. Smad3 null mice have alterations in TGF-β signaling throughout osteoblast lifespan, whereas regulated expression of TGF-β ligand or dominant negative receptor by the osteocalcin promoter would primarily occur during mineralization. Phase-dependent effects of TGF-β occur in osteoblasts<sup>41</sup> and may contribute to the discrepancy in bone phenotypes of transgenic mice compared to our Smad3 null model.

In contrast to the essential role for Smad3 in TGF- $\beta$  mediated signal transduction in bone, Smad3 is not required for the VDR signaling pathways studied. Global serum calcium homeostasis remains intact in mice deficient in Smad3, indicating that the primary role of vitamin D in remodeling is Smad3 independent. Binding of VDR to Smad3 is also not required for vitamin D antagonism of TGF- $\beta$  induced proliferation, suggesting that the interaction between VDR and Smad3 is not sufficient to explain the complex relationship between the signaling pathways. However, the possibility of Smad3 dependent VDR signaling in specific cellular contexts cannot be eliminated.

Although Smad3 may not be required for VDR signaling, our experiments demonstrate an essential role for Smad3 in bone formation as a signal transducer for TGF- $\beta$  mediated

repression of differentiation. We suggest that loss of Smad3 promotes terminal differentiation to the osteocyte and promotes osteocyte and osteoblast apoptosis, resulting in decreased functional time for osteoblasts to deposit and mineralize the matrix of bone.

# Methods

# Isolation and culture of primary osteoblasts

Mice were maintained and used in accordance with protocols established by the Institutional Animal Care and Use Committee of Duke University. Mated pairs of mice heterozygous for targeted deletion of Smad3<sup>36</sup> of a mixed 129/C57B6 background produced litters used for the preparation of osteoblast cell cultures. Calvaria were dissected from all littermates, washed in PBS, and placed in osteoblast media: MEM containing 10% heat inactivated fetal bovine serum (FBS); non-essential amino acids (NEAA); and penicillinstreptomycin (PS) (Gibco BRL, Gaithersburg, Md). DNA from tails of littermates was analyzed by PCR to genotype mice as previously described<sup>36</sup>. Wild-type and Smad3 null calvaria were used for the isolation of osteoblasts by sequential collagenase digestion. Briefly, calvaria were treated with 0.4 M EDTA in phosphate-buffered saline (PBS) followed by digestion in 200 units/mL type II collagenase (Worthington Biochemical Corporation, Lakewood, NJ). Cells obtained from the first collagenase treatment were discarded while cells isolated from the following three digestions were pooled and plated in osteoblast media in 6 well tissue culture plates. Once cells were fully confluent, osteoblasts were treated with 1 mL of collagenase, counted, and plated directly for experiments in 2 mL of osteoblast media. 2 mL of 2X
differentiation media (MEM, 10% FBS, NEAA, PS, 10 mM  $\beta$ -glycerolphosphate, 50  $\mu$ g/mL ascorbic acid) was added 24 hrs after plating and this was designated as day zero.

# Radiography

Femurs and tails were cleaned of soft tissue and placed on Kodak X-OMAT TL film (Rochester, New York). The Hewlett Packard 43807 X-ray System was used to expose bones to X-rays at 30 KVP, 2.3 mA for 90 sec.

### Bone Mineral Content

Dissected femurs were removed of tissue and placed in individual pre-weighed crucibles. The mass of the bones before drying or ashing was determined as the wet weight. Femurs were dried at 100  $^{\circ}$ C overnight and dry weight was determined. Subsequently, bones were ashed at 600  $^{\circ}$ C for 24 hrs and the ash weight was recorded. Ash was re-dissolved in 1 mL of 5 N HCl. 100  $\mu$ L of the dissolved ash was diluted with 0.4N HCl, 1% lanthanum Cl (Sigma Chemical Co., St. Louis, MO) to a total volume of 100 mL. Calcium content for each sample was determined by atomic absorption using a standard curve of calcium chloride.

### Bone Densitometry

Dual-energy X-ray absorptiometry (DEXA) was used to determine the bone mineral density of the isolated femora of the wild-type and Smad3 null mice<sup>42,43</sup>. Accuracy of the DEXA measurements was demonstrated by the strong linear relationship between ash weight and bone mineral content<sup>42</sup>. Over the past 2 years, the coefficient of variation for the BMD of a plastic-embedded whole mouse skeleton was 1.85% (n = 190).

# Bone Histomorphometric Analysis

Smad3 null and wild-type littermates were injected with 30 mg/kg tetracycline on day 1 and 4 and sacrificed on day 8. The distal femora were fixed in 4 °C Millonig's phosphatebuffered 10% formalin, pH 7.4, embedded nondecalcified in methyl methacrylate and stained as previously described<sup>42-45</sup>. The histomorphometric examination was done with a computer and digitizer tablet (OsteoMetrics Inc. Version 3.00, Atlanta, GA) interfaced to a Zeiss Axioscope (Carl Zeiss, Inc., Thornwood, NY) with a drawing tube attachment. All cancellous measurements were two-dimensional, confined to the secondary spongiosa and made at X400 magnification (numerical aperture 0.75). The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research<sup>46</sup>. The trabecular width and osteoid width were measured directly<sup>44</sup>. Trabecular spacing and number were calculated as previously described<sup>47</sup>. Only TRAPasepositive cells were included in the osteoclast perimeter. The rate of bone formation  $[m^2/(m/d)]$  and turnover (%/d) were calculated as previously described<sup>42,45</sup>.

# Serum Calcium and Phosphorous

Littermates were anesthesized with Metofane (Schering-Plough Animal Health Corporation, Union, NJ) and orbitally bled. Blood was allowed to clot at room temperature and centrifuged at 4  $^{\circ}$ C to isolate serum. Levels of serum calcium and phosphorous were determined colorimetrically using a Johnson & Johnson DT6011 and DTSC II module. For the hypercalcemic response to Vitamin D injection, mice were i.p. injected with 50 µL of 1, 25 (OH)<sub>2</sub>D<sub>3</sub> (Sigma Chemical Co., St. Louis, MO) in propylene glycol (EM Science, Gibbstown,

NJ) at a final concentration of 5  $ng/g^{-}$  body weight. Serum was collected pre-injection and twenty-four hours post injection.

# Thymidine Incorporation

Primary isolated osteoblasts of indicated genotypes were plated at a density of 200,000 cells per well in 6 well tissue culture plates in differentiation media and grown to confluence. Cells were serum starved for 12 hrs before the addition of 100 pM TGF- $\beta$  or 100 pM TGF- $\beta$  with 10<sup>-8</sup> M vitamin D3 for 24 hrs. For the last 4 hrs of culture, 5 µCi of [<sup>3</sup>H]thymidine (NEN Life Science Products Incorporated, Boston, MA) was added to the culture and thymidine incorporation was assayed as previously described<sup>48</sup>.

# Alizarin Red Staining

Osteoblasts were plated at a density of 5,000 cells per well in 96 well plates. On day 12, cultures were fixed in a solution of 10% formaldehyde, methanol, and distilled water at 4 °C overnight. Cells were stained in a 2% solution of alizarin red (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature, and washed five times with distilled water.

# Alkaline Phosphatase Activity

Alkaline phosphatase activity was determined by colorimetric assay of the enzyme with the substrate p-nitrophenolphosphate (Sigma Chemical Co., St. Louis, MO). Briefly, cells were plated at 50,000 cells per well in 24 well tissue culture plates. At the indicated days, cells were washed twice with Tris-buffered saline and lysed on ice in 1 mL of 50 mM Tris, pH 8.0 with 0.2% NP40. The total volume was brought to 6 mL in lysis solution and samples were shaken for 30 min at 4 °C. Samples were centrifuged to clear debris. 75  $\mu$ L of supernatant was added to

 $150 \ \mu L$  of assay buffer containing 10 mM p-nitrophenolphosphate and absorbance was measured at 405 nm.

# Northern and Western Blotting

Northern blotting for osteocalcin was performed on RNA prepared from isolated primary osteoblast cultures using Qiagen RNA easy columns (Valencia, CA) as specified by the manufacturer. 15  $\mu$ g of total RNA was resolved on a formaldehyde-agarose gel which was subsequently transferred by capillary action to a nylon membrane (Hybond, Amersham Libe Science) and visualized by methylene blue staining to ensure RNA quality and equivalent loading. Blots were probed with a 600 base pair fragment of the mouse osteocalcin cDNA which was a gift from Dr. D. Quarles, Duke University Department of Nephrology.

Western blotting for Smad3 was performed on primary osteoblast cell culture lysates prepared in Universal Lysis Buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 50 mM NaF; 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM  $\beta$ -glycerolphosphate, 0.2 mM sodium molybdate, and protease inhibitors). Total protein content was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). 13 µg of total protein was resolved by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE), and western analysis was performed using  $\alpha$ Smad1/2/3 (Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of apoptosis in nondecalcified bone sections

Sections were mounted on silane-coated glass slides (Scientific Device Lab, Inc., Des Plains, IL) and incubated in 10 mM citrate buffer, pH 6.0, in a microwave oven at 98 °C for 5 min. Slides were then placed in 0.5% pepsin in 0.1 N HCl for 20 min at 37 °C, rinsed with TBS

and reincubated in 30% H<sub>2</sub>O<sub>2</sub> in methanol for 5 min and rinsed again. DNA fragmentation was detected by the TUNEL reaction (transferase-mediated digoxigenin-dUTP nick end-labeling) using Klenow terminal deoxynucleotidyl transferase (Oncogene Research Products, Cambridge, MA) in sections counterstained with 3% methyl green. This system allows for sensitive and specific staining of the high concentrations of 3'-OH ends that accumulate with DNA fragmentation due to apoptosis<sup>45,49</sup>. To further improve the sensitivity of the reaction, sections were subsequently incubated for 1-2 min with 0.15% CuSO<sub>4</sub> in 0.9% NaCl<sup>48</sup>. The TUNEL reaction was noted within cell nuclei, and the cells whose nuclei were clearly dark brown from the peroxidase-antidigoxigenin antibody conjugate instead of blue-green from the methyl green were interpreted as positive. Negative controls were made by omitting the transferase. Morphological changes characteristic of apoptosis were examined carefully to minimize ambiguity regarding the interpretation of results. With these precautions, TUNEL has been unequivocally associated with apoptosis<sup>45,48</sup>. In addition, TUNEL has been used with DNA fragmentation and immunohistochemical studies to demonstrate apoptosis of osteoblastic cells and osteoblasts both *in vitro* and *in vivo*<sup>45,49,50</sup>.

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# **Figure Legends**

Figure 1. Smad3 null bones are less dense than wildtype littermates. Autographic analysis of femurs (*a*) and tail vertebrae (*b*) of Smad3 null (top) and wildtype (bottom) littermates reveals Smad3 null bones are more radiolucent. These illustrations are representative of ten littermate pairs which demonstrate complete penetrance of the phenotype.

Figure 2. Osteopenia in Smad3 null mice. (a) Densitometry demonstrates a 16% decrease in the mineral content of Smad3 null femurs. Results are the average of four animals of each genotype  $\pm$  standard deviation; \*p<0.05 (b) A similar 17% decrease in the percent of calcium per wet weight of Smad3 null femurs is illustrated by atomic absorption analysis. Results are the average of seven wild-type and six null animals  $\pm$  standard deviation; \*p<0.01 (c) Atomic absorption also demonstrates that the percent of calcium per ash weight of Smad3 deficient femurs is normal. (d) Histomorphometric analysis of femurs of four animals per genotype indicates less cancellous and cortical bone in Smad3 null mice. Results are the average  $\pm$  standard deviation; \*p<0.05;  $\pm$ p<0.01 The width of cortical bone, indicated by the arrow, in Smad3 null bone (e) is reduced compared to wildtype littermates (f) in sections of decalcified femur.

Figure 3. Global vitamin D signaling is maintained in Smad3 null mice. (a) Serum phosphorous and calcium concentrations are equivalent in wild-type  $\{\Box\}$  and Smad3 null $\{\blacksquare\}$  mice. Results are the average of serum concentrations from 14 wild-type and 13 null animals ± standard deviation. (b) Smad3 mice retain the ability to elevate serum calcium in response to vitamin D injection. Serum calcium concentrations were determined in wild-type and null littermates before  $\{\Box\}$  and after  $\{\blacksquare\}$  injection with 5ng/g body weight vitamin D3 in propylene glycol. Results are the average of four animals per genotype ± standard deviation; \*p<0.001;  $\ddagger$ p<0.0025. (c). Numbers of osteoclasts per perimeter of bone are also normal in Smad3 null mice when determined via histomorphometry of four animals per genotype.

Figure 4. Decreased bone formation rate in Smad3 null mice. (*a*) Bone formation rates are decreased by 44% in Smad3 null mice. Results shown are the average of four animals of each genotype  $\pm$  standard deviation; \*p<0.05 (*b*) Mineral appositional rates are decreased 28% in mice deficient in Smad3. Results are the average of four animals of each genotype  $\pm$  standard deviation; \*p<0.05 There is greater distance between tetracycline labels, as indicted by the arrows, in wild-type mice (*c*) compared to Smad3 null littermates (*d*). The distance and time between labels are used to calculate bone formation rates and mineral appositional rates shown above. (*e*) Histomorphometric analysis of four animals per genotype also reveals decreased osteoid in Smad3 null mice although the percent of mineralizing bone is similar. Results are the average  $\pm$  standard deviation; \*p<0.05;  $\pm$ p<0.01.

Figure 5. Proliferation in response to TGF- $\beta$  is intact in Smad3 null mice.

(a) Western blot analysis demonstrates Smad3 is present in wild-type osteoblasts but is absent in osteoblasts isolated from calvaria of mice with a targeted deletion of Smad3. (b) Thymidine incorporation increases in wild-type  $\{\Box\}$  and Smad3 null cells  $\{\bullet\}$ upon treatment with increasing concentrations of TGF- $\beta$ . (C) The proliferation induced by 100pM TGF- $\beta$   $\{\blacksquare\}$  is blocked by treatment with 10<sup>-8</sup>M vitamin D3  $\{\blacksquare\}$  in the presence or absence of Smad3. The abrogation of TGF- $\beta$  induced proliferation by vitamin D does not return levels to control  $\{\Box\}$ .

Figure 6. Requirement for Smad3 in the regulation of osteoblast differentiation by TGF- $\beta$ . (*a*) Alizarin red staining of isolated osteoblasts at day 12 in culture indicates that both wild-type and Smad3 null littermates are capable of forming calcium nodules. Treatment with 100pM TGF- $\beta$  inhibits nodule formation in wild-type cells, whereas null cells are unaffected. Treatment with 10<sup>-8</sup>M vitamin D3 promotes nodule formation in the presence or absence of Smad3. (*b*) Levels of alkaline phosphatase activity {□} are not reduced in response to 100pM TGF- $\beta$  treatment **[**] in Smad3 null cells at day 4 in culture. (*c*) Although basal osteocalcin expression is elevated in Smad3 deficient osteoblasts, TGF- $\beta$  mediated downregulation of osteocalcin mRNA is intact. (*d*) Similar to results *in vitro*, histomorphometry of four mice of each genotype indicates that loss of Smad3 increases osteocyte density *in vivo*. Results are the average ± standard deviation; \*p<0.05

Figure 7. Increased osteoblast and osteocyte apoptosis in the absence of Smad3.

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Tunnel staining of nondecalcified bone sections from Smad3 null mice demonstrate apoptotic osteoblasts (*a*) and osteocytes (*b*) *in vivo* as indicated by arrows. (*c*) The percentage of apoptotic osteoblasts is increased 25% in Smad3 null cells compared to wild-type. Results are the average of four animals of each genotype  $\pm$  the standard deviation. \*p<0.05 (*d*) The percentage of apoptotic osteocytes increases 40% in Smad3 null cells. Results are the average of four animals of each genotype  $\pm$  the standard deviation. \*p<0.05 (*d*) The percentage of four animals of each genotype  $\pm$  the standard deviation. \*p<0.05 (*d*) The percentage of four animals of each genotype  $\pm$  the standard deviation. \*p<0.01





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Histomorphometric Determination	wt	ko
Cortical width (µm)	<u>211 ± 34</u>	<u> 132 ± 30‡</u>
Cancellous bone area / Tissue area(%)	$8.7 \pm 0.8$	6.3 <u>+</u> 2.1*
Trabecular width (µm)	<u>38.0 ± 3.5</u>	28.1 ± 6.4*
Trabecular spacing (um)	$407 \pm 27$	$430 \pm 63.9$

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Histomorphometric Determination	wt	ko
Osteoid area / Bone area(%)	4.1 ± 1.9	$2.0 \pm 0.8*$
Osteoid perimeter / Bone perimeter (%)	28.7 <u>+</u> 11.4	15.8 <u>+</u> 5.1*
Osteoid width (µm)	$3.1 \pm 0.3$	$2.3 \pm 0.2 \ddagger$
Mineralizing perimeter / Bone perimeter (%)	12.3 <u>+</u> 3.5	9.3 <u>+</u> 5.3















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# Targeted Disruption of Smad3 Reveals an Essential Role in Transforming Growth Factor β-Mediated Signal Transduction

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The Smads are a family of nine related proteins which function as signaling intermediates for the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily of ligands. To discern the in vivo functions of one of these Smads, Smad3, we generated mice harboring a targeted disruption of this gene. Smad3 null mice, although smaller than wild-type littermates, are viable, survive to adulthood, and exhibit an early phenotype of forelimb malformation. To study the cellular functions of Smad3, we generated Smad3 null mouse embryonic fibroblasts (MEFs) and dermal fibroblasts. We demonstrate that null MEFs have lost the ability to form Smad-containing DNA binding complexes and are unable to induce transcription from the TGF-B-responsive promoter construct, p3TP-lux. Using the primary dermal fibroblasts, we also demonstrate that Smad3 is integral for induction of endogenous plasminogen activator inhibitor 1. We subsequently demonstrate that Smad3 null MEFs are partially resistant to TGF-β's antiproliferative effect, thus firmly establishing a role for Smad3 in TGF-\beta-mediated growth inhibition. We next examined cells in which Smad3 is most highly expressed, specifically cells of immune origin. Although no specific developmental defect was detected in the immune system of the Smad3 null mice, a functional defect was observed in the ability of TGF-B to inhibit the proliferation of splenocytes activated by specific stimuli. In addition, primary splenocytes display defects in TGF- $\beta$ -mediated repression of cytokine production. These data, taken together, establish a role for Smad3 in mediating the antiproliferative effects of TGF- $\beta$  and implicate Smad3 as a potential effector for TGF- $\beta$  in modulating immune system function.

Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a multifunctional polypeptide hormone which has diverse effects on a variety of cell types to regulate many complex multicellular systems (46). The complexity and diversity of TGF- $\beta$ 's function is demonstrated through its multiple roles in immune system suppression, wound healing, fibrosis, development, and oncogenesis. Many of these global effects of TGF- $\beta$  stem from its ability to regulate cellular proliferation, differentiation, and gene expression (38). One of the most studied aspects of TGF- $\beta$  function is its ability to inhibit the proliferation of many different cell types, including cells of epithelial, endothelial, neuronal. hematopoietic, and lymphoid origins (46, 31).

These effects of TGF- $\beta$  are mediated through its interaction with cell surface receptors. By binding to its type I and type II serine/threonine kinase receptors, TGF- $\beta$  induces the phosphorylation and activation of the type I receptor by the type II receptor (57, 58). The type I receptor kinase can then phosphorylate cytoplasmic substrates, including members of the Smad family of proteins, which function as intermediates in the signaling pathways for the TGF- $\beta$  superfamily of ligands (3, 4, 12, 18, 39). Originally identified in genetic screens for TGF- $\beta$ effectors in *Drosophila* (49) and *Caenorhabditis elegans* (47), the mammalian Smad family now consists of nine structurally related proteins, Smad1 to Smad9. The identification and characterization of these proteins has provided valuable insights

\* Corresponding author. Mailing address: Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710. Phone: (919) 681-4861. Fax: (919) 681-7152. E-mail: wang @galactose.mc.duke.edu. into the early events involved in TGF- $\beta$ -mediated signal transduction.

The highly related Smad2 and Smad3 serve as substrates for the type I TGF- $\beta$  receptor kinase (14, 30, 36, 42, 53, 62, 65). Upon phosphorylation, these two Smads bind to their common partner, Smad4, to form Smad2-Smad4 and Smad3-Smad4 complexes. These complexes then translocate to the nucleus (1, 29, 33, 41, 66). Clues to the nuclear function of these Smad complexes came from studies describing an intrinsic transcriptional activity of the C-terminal domain of the Smads (29, 59). Subsequently, overexpression of particular combinations of Smads was shown to activate transcription from a number of TGF- $\beta$ -responsive promoters, including the plasminogen activator inhibitor 1 (PAI-1) promoter and the reporter construct 3TP-lux (11, 29, 65).

The role of the Smads as putative transcription factors was strengthened by the finding that Smad3-Smad4 complexes and the *Drosophila* Mad are sequence-specific DNA binding proteins which on binding DNA can activate transcription (11, 25, 63, 64). In addition to a direct DNA binding activity, the Smads can be targeted to specific promoter sequences through their interaction with other transcription factors, as demonstrated by the finding that Smad2-Smad4 complexes bind to the transcription factor FAST-1 in response to activin and TGF- $\beta$  (7, 34). In addition, recent studies have implicated a functional interaction between the Smad3-Smad4 complex and the AP1 family of transcription factors (32, 63, 67).

Apace with the rapid development of the understanding of the Smads on a biochemical level, the role of the Smads in development and diseases is beginning to be understood. Recently, mouse models for both Smad2 and Smad4 function have been described (43, 52, 55, 61). Mice with homozygous targeted disruptions of these genes are embryonic lethal at day 9.5 and days 6.5 to 8.5, respectively. Thus, these Smads play critical, nonredundant roles in early embryonic development. The early embryonic lethality of these mice, however, renders the functional analysis of these molecules in the adult animals impossible in this system and makes their study on a cellular level difficult.

In humans, the role of Smad2 and Smad4 as tumor suppressor genes is now well established (6, 14-16, 40, 44, 45, 48). Concurrent with the identification of the Smads through genetic screens, Smad4 was identified as a tumor suppressor gene, which is deleted in about 50% of pancreatic carcinomas. In addition to pancreatic cancers, Smad4 mutations have also been discovered in breast, ovary, head and neck, esophagus, colon, and lung cancers. Not only are Smad4 mutations found in spontaneous cancers, but recent reports show that inherited juvenile colon cancer can derive from the inheritance of a single mutant Smad4 allele (19). In addition, Smad2 is mutated in several types of cancers, including colon cancers and head and neck cancers (14, 44). To date, Smad3 has not been reported to be mutated in human cancers (2, 45). These data, together with their role as intermediates in the TGF-B signaling pathway, clearly implicate Smad2 and Smad4 as playing an important function in cell growth regulation.

The cellular functions of the Smads have largely been inferred from the occurrence of mutations in human diseases and from cellular studies employing the use of Smad dominant negatives and Smad overexpression in Smad-deficient cell lines which likely harbor additional genetic lesions. Thus, the physiological functions of Smad3, particularly its potential involvement in mediating the TGF- $\beta$  antiproliferative effect, remain speculative. To address the biological functions of Smad3, we generated mice harboring a targeted disruption of the Smad3 gene. Unlike the Smad2 and Smad4 null mice, Smad3 null mice are viable and survive to adulthood, demonstrating distinct roles for the three Smad proteins during mouse development. In addition, Smad3 null mice are smaller than wild-type littermates and have an incompletely penetrant joint formation abnormality. At the cellular level, we focused our study initially on defining the role of Smad3 in TGF-B signal transduction in the mouse embryonic fibroblast (MEF) and dermal fibroblast model systems. Here we show that Smad3 is required for activation of a TGF-β-responsive promoter, 3TP-lux, and the endogenous PAI-1 gene and, more importantly, acts as an integral effector of TGF-B-mediated inhibition of cellular proliferation. We next focused on the cell types with highest Smad3 expression, specifically cells of lymphoid origin, and found that under specific conditions, the antiproliferative effects of TGF-β on isolated Smad3 null splenocytes are lost. In addition, we found that the inhibition of anti-CD3 ( $\alpha$ CD3)stimulated cytokine production by TGF-B in primary splenocytes is markedly blunted due to the absence of Smad3. Taken together, these findings implicate Smad3 as a critical effector in TGF-B-mediated inhibition of cellular proliferation and a potential effector for TGF-B regulation of immune system function.

#### MATERIALS AND METHODS

Smad3 gene disruption. The Smad3 gene was isolated from a 129/sv mouse genomic library by using the 5' end of the human Smad3 cDNA as a probe. An isolated 15-kb genomic clone was used for the creation of a Smad3 targeting vector. Briefly, a 1.0-kb *EheI-Hind*III fragment was cloned into the *XhoI* site of the vector pPNT (54). A 6.0-kb *Bam*HI fragment was next cloned into the resulting construct. This produced a targeting vector which, when inserted into the genome, replaces the sequence between *EheI* and *Bam*HI with a neomycin expression cassette, as diagrammed in Fig. 1A. The resulting targeting vector was



FIG. 1. Targeted disruption of Smad3. (A) Smad3 genomic structure and targeting strategy. The Smad3 genomic clone used for the creation of the targeting vector is diagrammed. The black box denotes the first exon of Smad3. ATG denotes the initiating methionine. The first exon coding sequence was replaced by a neomycin expression cassette (NEO), creating an EcoRI digest size difference between the wild-type and targeted loci. Ep denotes the EcoRI-HindIII DNA fragment used as a probe for Southern blotting. P1, P2, and P3 denote the locations of primers used for PCR screening. Restriction sites are abbreviated as follows: R, EcoRI; H, HindIII; E, EheI; B, BamHI. HSV-TK, herpes simplex virus thymidine kinase. (B) Southern and PCR detection of the targeted allele. The targeted allele can be distinguished from the wild type (W.T.) both by Southern blotting of EcoRI-digested genomic DNA with the Ep probe and by PCR using the primers indicated in panel A. (C) Northern blot analysis of Smad3 and Smad4. Northern analyses were performed on RNA derived from multiple tissues of an adult (2-month-old) C57BL/6 mouse and a 3 untranslated sequence probe for Smad3 and a coding-sequence probe for Smad4 Organs are abbreviated as follows: B, brain; H, heart; M, skeletal muscle; I, small intestine; Lu, lung; K, kidney; T, thymus; S, spleen. (D) Western blot analysis of Smad3 expression. The top panel shows Western analysis using an antibody created against a peptide in the central linker domain of Smad3 on thymic protein extract from wild-type, heterozygous, and knockout mice. The bottom two panels demonstrate the specificity of this antibody among overexpressed Smad family members. HA-tagged Smad1, Smad2, and Smad4 and Flag-tagged Smad3 were overexpressed in COS cells from which protein extract were isolated and used for Western analysis. Identical blots were probed with the Smad3specific antibody (middle panel) and a mixture of  $\alpha HA$  and  $\alpha Flag$  (bottom panel). In all panels of all figures, +/+ denotes Smad3 wild type, +/- denotes Smad3 heterozygous, and -/- denotes Smad3 null.

linearized with *Hin*dIII and electroporated into 129/sv embryonic stem (ES) cells. Screening of neomycin-resistant clones was performed by PCR with the following primers: the common primer (P3; GTC TTT GAG GCC CGT TTT CTG C), a primer from the targeted sequence (P1; CTG GGG TGG TAA TGC ACT TGG), and a primer in the *PGK* promoter (P2; CAT GCT CCA GAC TGC CTT GGG). PCR of the wild-type allele results in a 1.2-kb product. PCR of the targeted allele results in a 1.1-kb product. Positive clones were confirmed by Southern blotting of *Eco*RI-digested genomic DNA probed with an *Eco*RI-*Hin*dIII fragment immediately adjacent to the sequences used in the targeted allele produces a 5.0-kb fragment. The targeted allele produces a 5.0-kb fragment.

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**Primary fibroblast and immune cell culture.** Primary fibroblasts were cultured from day 14 embryos. Embryos were mechanically disrupted by passage through an 18-gauge needle and plated on gelatin-coated 10-cm-diameter plates in Dubecco modified Eagle medium (DMEM) with 20% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (P-S) (Gibco BRL. Gaithersburg. Md.). Confluent cells were trypsinized and further carried in medium containing 10% FBS. In all experiments, compared wild-type and null cells represent littermate embryos at the same passage number.

Primary dermal fibroblasts were isolated from 2-day-old mice. Trunk skin was removed, washed three times in phosphate-buffered saline (PBS) containing kanamycin, amphotericin, penicillin, and streptomycin (KAPS), and incubated overnight at 4°C in 0.25% trypsin (Worthington Biochemical, Freehold, N.J.) in PBS-KAPS. The skins were then incubated at 37°C for 20 min. The trypsin was next neutralized with 20% FBS, and the skins were then washed in DMEM–10% FBS. The trypsinized skins were next placed in individual 10-cm-diameter dishes, and the epidermis was peeled off and discarded. The resulting dermis layers were mechanically dissociated, and 10 ml of DMEM–10% FBS–P-S was added to each dish. Cells were then incubated at 37°C in 5% CO<sub>2</sub> until dermal fibroblasts became confluent (3 to 4 days). The dermal fibroblasts were used in the PAI-1 assay.

Primary splenocytes, thymocytes, and purified splenic B and T cells were generated from the spleens of 2- to 4-month-old mice. Lymphocytes were isolated by mechanical dissociation in the culture medium described below. Large debris was removed, and erythrocytes were lysed by complete resuspension of pelleted cells in 145 mM ammonium chloride–17 mM Tris (pH 7.5). Cells were subsequently washed in PBS and resuspended in their final culture medium for all experiments described: RPMI with the addition of 10% heat-inactivated FBS, P-S, and 0.1 mM  $\beta$ -mercaptoethanol.

Northern and Western blotting. Northern blotting for Smad3 was performed on RNA prepared from adult C57BL/6 mouse organs by homogenizing the indicated tissues in Trizol reagent (Gibco BRL. Gaithersburg. Md.) as specified by the manufacturer. Ten micrograms of total RNA was resolved on a formaldehyde-agarose gel, which was subsequently transferred by capillary action to a nylon membrane (Hybond, Amersham Life Science) and visualized by methylene blue staining to confirm RNA loading and quality. Blots were probed with a mouse Smad3 cDNA probe created by random priming (Prime-It II, Stratagene, La Jolla, Calif.) of a *Hind*III-*Eco*RI fragment containing sequences entirely in the Smad3 3' untranslated region. The membrane was subsequently reprobed with a human Smad4 cDNA probe containing the entire coding sequence for human Smad4.

For Western blotting, all cell and organ lysates were prepared in a Nonidet P-40-based lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl. 50 mM NaF. 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.2 mM sodium molybdate, protease inhibitors). For Western blotting of thymus tissue, whole thymus was homogenized in 1 ml of lysis buffer and equal protein amounts from animals of each genotype were analyzed. Western blotting of fibroblasts was performed by lysis of  $10^6$  fibroblasts from 10-cm-diameter tissue culture plates in 200 µl of lysis buffer and analyzing equal protein amounts for each genotype. Western analyses of equal amounts of protein extracts from splenic B cells purified by using Dynabeads- Mouse pan T (Dynal, Lake Success, N.Y.) and of splenic T cells purified by using mouse T-cell enrichment columns (R&D Systems, Minneapolis, Minn.) were performed with a Smad3-specific antibody. Western analyses of aCD3-stimulated splenocytes were performed on splenocytes isolated as described above and cultured at a density of 107 cells in 2 ml of medium in six-well tissue culture plates in the presence 5 µg of αCD3 (01081D; PharMingen, San Diego, Calif.) with and without 100 pM TGF-B for 24 (cyclin E cyclindependent kinase [Cdk2]. and p27 western blots) or 48 h (retinoblastoma protein [Rb] Western blots). Cells were lysed in 200 µl of lysis buffer, and equal protein amounts were analyzed. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western analyses were performed with the following antibodies: acyclin E (M-2),  $\alpha$ Cdk2 (M-2),  $\alpha$ p27 (C-19),  $\alpha$ p21 (C-19),  $\alpha$ p15 (C-20), and  $\alpha$ CDC25A (144), all from Santa Cruz Biotechnology, Inc., and aRb (14001A; PharMingen). Smad3 Western analyses were performed with a rabbit polyclonal antibody created against the Smad3specific peptide DAGSPNLSPNPMSPAHNNLD. Crude rabbit serum was further purified on an antigen affinity column. The specificity of this antibody was assessed by Western analysis of equal protein amounts of COS cell extract in which the following tagged human Smads were individually overexpressed: Flagtagged Smad1, Flag-tagged Smad2, hemagglutin epitope (HA)-tagged Smad3. and Flag-tagged Smad4. Cytomegalovirus promoter-driven constructs encoding the various Smads were transiently transfected into COS cells by using a standard DEAE-dextran protocol as previously described (10). Expression of the various Smads was confirmed by Western analysis of equal amounts of appropriately transfected COS extract, using a mixture of aHA (Boehringer Mannheim, Indianapolis, Ind.) and aFlag (Eastman Kodak, New Haven, Conn.) antibodies.

**Cdk2 kinase assays.** Splenocytes were isolated and cultured as described above for Western blotting. Whole-cell lysates were generated after 24 h of TGF- $\beta$  treatment in the same manner as for Western analysis. Cdk2 kinase activity immunoprecipitated with a polyclonal  $\alpha$ Cdk2 antibody (M-2; Santa Cruz Biotechnology) from equal protein amounts for each condition described was measured on the substrate histone H1 as previously described (10).

**PAI-1** assay. Dermal fibroblasts were plated at a density of 10<sup>6</sup> cells per 10-cm-diameter plate in the fibroblast culture medium described above and incubated overnight at 37°C. Cells were then incubated in methionine-free DMEM–0.5% FBS–P-S for 4 h and then treated with 100 pM TGF-β for 6 h. During the last 2 h of TGF-β treatment, the fibroblasts were labeled with [<sup>35</sup>S]methionine (100 µCi/ml). The cells were then washed with PBS and removed by lysis in three washes of 10 mM Tris-HCl (pH 8.0)–0.5% sodium deoxycholate–1 mM phenylmethylsulfonyl fluoride, and the resulting platebound extracellular matrix washed a final time with PBS. The amount of matrix associated PAI-1 was assessed by scraping the plates in SDS-PAGE loading buffer containing dithiothreitol and resolving the protein on an 10% polyacryl-amide gel. Gels were subsequently dried, and autoradiography was performed.

**Thymidine incorporation assays.** Fibroblasts of the indicated genotypes were plated at a density of 20,000 cells/well in six-well tissue culture plates in DMEM-10% FBS and incubated in the presence or absence of 100 pM TGF- $\beta$  for 24 or 48 h as indicated. For the last 4 h of culture, 5 µCi of [<sup>3</sup>H]thymidine was added to the culture, and thymidine incorporation was assayed as previously described (10). For mixed wild-type and Smad3 null experiments, the indicated percentage of cach cell type was plated in six-well plates to a total cell number of 20,000/ml. Thymidine incorporation was assayed above.

Thymidine incorporation of splenocytes was performed on cells isolated as described above. Isolated splenocytes were plated at a density of  $5 \times 10^6$  cells in 200 µJ of medium in 24-well plates and stimulated with lipopolysaccharide (LPS; 10 µg/ml; Sigma, St. Louis, Mo.), anti-immunoglobulin M (αlgM; 5 µg/ml; Cappel, Durham, N.C.) and interleukin-4 (IL-4; 12.5 U/well; PharMingen) or  $\alpha$ CD3 (2.5 µg/ml; PharMingen) in the presence or absence of 100 pM TGF- $\beta$  and cultured for 48 h; 5 µCi of [<sup>3</sup>H]thymidine was added to the culture for the last 4 h. Thymidine incorporation was assayed by harvesting cells with a PHD cell harvester (Cambridge Technologies, Inc.).

Luciferase assays. Fibroblasts (200,000/well) from each genotype were seeded into six-well tissue culture plates. Cells were transfected by using a standard DEAE-dextran transfection protocol as previously described (10) with the indicated amounts of DNAs (Fig. 4B). Cells were cotransfected with 0.25  $\mu$ g of a cytomegalovirus-driven  $\beta$ -galactosidase reporter vector to normalize for transfection efficiency. Transfections with the expression and reporter plasmids used here have been previously described (63).

**EMSAs.** For electrophoretic mobility shift assays (EMSAs), nuclear extracts prepared from 10<sup>6</sup> fibroblasts of the indicated genotype either treated or untreated with 100 pM TGF- $\beta$  for 1 h were incubated with a probe derived from an *Nde1-Sph1* fragment of 3TP-lux (63). Nuclear extract preparation and gel shift conditions were exactly as previously described (62).

**RNase protection analysis of cytokine expression.** Primary splenocytes were isolated as described above. Splenocytes  $(7.5 \times 10^6)$  were plated in 2 ml of medium and stimulated with 5 µg of  $\alpha$ CD3 in the presence or absence of 100 pM TGF- $\beta$  for 48 h in individual wells of a six-well plate. The cells were harvested, and total RNA was isolated (RNeasy: Qiagen, Santa Clarita, Calif.). Cytokine RNA levels were assessed by RNase protection assays using a RiboQuant multiprobe kit (45024K/mCK-1: PharMingen) on equal amounts of RNA (7.5 µg) for each culture confirmed through the quantification of the protection fragments of two housekeeping genes provided in the multiprobe template set, L32 and *GAPDH*.

FACS analysis. Fluorescence-activated cell sorting (FACS) analysis was performed on live splenocytes and thymocytes isolated as described above. Approximately 10<sup>5</sup> pelleted cells were resuspended in 100  $\mu$ l of PBS with 5% heat-inactivated FBS with the inclusion of the antibodies indicated in each figure: phycocrythrin (PE)- $\alpha$ CD4 (01065B), fluorescein isothiocyanate (FITC)- $\alpha$ CD8 (01044D), and FITC- $\alpha$ B220 (01124D) (all from PharMingen), plus PE-algM and 7-amino actinomycin D (7AAD; Molecular Probes, Eugene, Oreg.). After 30 min, these cells were washed once in PBS-5% FBS and analyzed. The purity of the isolated splenic B and T cells used in the thymidine incorporation assays was assessed by FACS analysis with the use of PE- $\alpha$ B220 and FITC-labeled anti-T-cell receptor beta chain (01304D and 01125B, respectively; PharMingen). Viable cells were identified by exclusion of 7AAD (Molecular Probes) staining.

### RESULTS

**Smad3 null mice are viable.** To generate a targeted disruption of Smad3, we first screened a murine 129/sv genomic library with sequences in the amino terminus of Smad3 to obtain a 14-kb genomic clone. This clone contains the first exon of Smad3, including the initiating methionine and the first 69 amino acids. A targeting vector was created by replacing the first exon and part of the first intron with a *PGK*-neomycin expression cassette. Proper insertion of this targeting vector into the mouse genome removes the initiating ATG, making the production of full-length Smad3 impossible. In addition, this insertion does not disrupt any sequences 5' to the RNA transcriptional start site (Fig. 1A).



FIG. 2. Smad3 null mice are smaller than wild-type littermates and have an incompletely penetrant forepaw defect. (A) Mouse weights over time (days) in a single, representative litter. (B) Picture of the torqued-wrist defect (arrow) in a 14-day-old null mouse. (C) The skin was removed from the forelimbs of a 14-day-old Smad3 null mouse (left) and a wild-type littermate (right) to better show the severe bending of the forepaw wrist joint of the Smad3 null mouse.

Using standard ES cell technology, Smad3 mutant heterozygous 129 ES lines were generated by transfection of the described targeting vector. Initial screening for proper insertion in neomycin-resistant clones was determined by Southern blotting and PCR as indicated in Fig. 1B. Three percent of neomycin-resistant ES cell clones had a properly targeted Smad3 allele. These ES cells were then used to create 129-C57BL/6 chimeric founder mice. When bred to C57BL/6 females, mice generated from one of these lines transmitted the mutant Smad3 allele at a frequency of 50%, with 100% of offspring being derived from the 129 stem cells. Heterozygous mice from these matings were subsequently mated to produce Smad3 null mice. Smad3 null mice are born to F<sub>1</sub> heterozygotes at a frequency of 20.7%, the same frequency as for wild-type mice (297 Heterozygote, 106 wild-type, and 103 KO knockout mice). The near Mendelian inheritance of wild-type and targeted Smad3 alleles suggests no embryonic lethality of the Smad3 null mice. Thus, in sharp contrast to the Smad2 and Smad4 deficiencies, Smad3 is not essential for embryonic development. This F<sub>2</sub> generation of mice was used for the experiments described below.

To identify organs with highest Smad3 expression, we first performed multiple-tissue Northern analysis. Unlike Smad2 and Smad4, Smad3 has an expression pattern which varies with tissue types, with highest levels of expression in the spleen and thymus (Fig. 1C). Subsequently, the loss of Smad3 expression in the double-mutant animals was confirmed by Western analysis of thymus protein extracts, using a Smad3-specific antibody (Fig. 1D).

The first noticeable phenotype in these null animals is a decrease in the size and growth rate of young mice. As shown in Fig. 2A, Smad3 null mice are smaller than both wild-type and heterozygous littermates. An additional early phenotype, which occurs in approximately 31% (32 of 103) of null mice, is the presence of medially torqued forepaws (Fig. 2B and C), with a smaller percentage of mice with noticeably torqued hind limbs. Mice with this phenotype can have either one or more limbs affected. In addition, mice with severely affected limbs often develop kyphosis and display marked rib cage malformation often resulting in a concave indentation at the base of the sternum (data not shown). Interestingly, this phenotype is remarkably similar to that of mice expressing a transgenic dominant negative type II TGF- $\beta$  receptor in bone (50), suggesting that the phenotype described here is intrinsic to the bone. In addition, the similarity between these two phenotypes suggests that the previously described TGF-B effects in bone development are at least partially mediated by Smad3.



FIG. 3. Smad3 is required for TGF-β-mediated growth inhibition in MEFs. (A) Primary MEFs were created from embryonic day 14 mice. Western blotting for Smad3 was performed to determine if these MEFs express Smad3. (B) Smad3 is required for TGF-β-mediated growth inhibition in primary MEFs. MEFs were assayed for TGF-β-mediated growth inhibition after 24 and 48 h of treatment by measurement of [<sup>3</sup>H]thymidine incorporation. Bars represent the average thymidine incorporation for triplicate wells for each growth condition. (C) TGF-βmediated growth inhibition in these MEFs is cell autonomous. Various proportions of wild-type (WT) and knockout (KO) MEFs were seeded into single wells as indicated below the bars. Thymidine incorporation assays were performed as for panel B. Data are presented as percent growth inhibition or percent reduction in thymidine incorporation upon TGF-β treatment.

TGF-\beta-mediated growth inhibition and gene responses are impaired in Smad3 null fibroblasts. One functional aspect of Smad3 that we hoped to define through the generation of Smad3 null mice is its role in TGF-β-mediated inhibition of cellular proliferation. To test this, we isolated MEFs from both wild-type and Smad3 null mice. As shown in Fig. 3A, Smad3 expression can be detected in wild-type fibroblast lines but not in lines derived from Smad3 null embryos. Using these fibroblasts, we first determined the proliferative responses of these lines to TGF- $\beta$ . As shown in Fig. 3B, the proliferation of wild-type fibroblasts is inhibited approximately 50% after 24 h and 80% after 48 h of TGF-B treatment. In null fibroblasts, this growth-inhibitory effect of TGF-B is largely lost. In addition, the basal proliferation rate of the null fibroblast lines is approximately twofold higher than that of the wild type. Similar results were obtained for two additional fibroblast lines of each genotype (data not shown). Thus, these results firmly establish an essential role for Smad3 in TGF-\beta-mediated inhibition of cellular proliferation. Interestingly, none of the known mediators of the growth-inhibitory effect of TGF-B appear to be functioning in fibroblasts. In these cells TGF- $\beta$  does not alter p21, p15, or CDC25A protein levels, whereas p27 is undetectable (data not shown).

To determine if the growth-inhibitory effect of TGF- $\beta$  in these cultures is cell autonomous or due to inappropriately

regulated production of paracrine factors, growth inhibition by TGF- $\beta$  of mixed wild-type and null cultures was assayed. As shown in Fig. 3C, different percentages of wild-type and null cells were seeded into the same well, and TGF- $\beta$  mediated growth inhibition was assayed. The growth-inhibitory effect of TGF- $\beta$  in these experiments is proportional to the amount of wild-type cells. This suggests that the antiproliferative effect of TGF- $\beta$  in these cells is most likely cell autonomous and not due to a Smad3-dependent production of growth-inhibitory or inhibition of growth-stimulatory paracrine factors.

As discussed above, the Smads have been characterized as DNA binding transcription factors. To determine the requirement of Smad3 in the activation of specific promoters, we studied the regulation of the widely used TGF- $\beta$ -responsive promoter 3TP-lux in our model fibroblast system. In wild-type cells, the previously described TGF- $\beta$ -induced, Smad3-containing DNA binding complex forms on the concatemerized tetradecanoyl phorbol acetate response elements (TREs) present in this promoter. This DNA binding complex is lost in the Smad3 null fibroblasts (Fig. 4A). In addition, transcription from this promoter in wild-type cells is activated 2.4-fold upon TGF- $\beta$  treatment. This activation is lost in the null fibroblasts and can be restored by cotransfection of a Smad3 expression vector (Fig. 4B). Thus, Smad3 is necessary not only for the



FIG. 4. Smad3 is required for TGF-β-mediated Smad-containing DNA binding complex formation and activation of 3TP-Lux in primary MEFs and for TGF-B-mediated induction of the PAI-1 gene in primary dermal fibroblasts. (A) Loss of a Smad-containing DNA binding complex in the Smad3 null MEFS. EMSAs were performed with nuclear extract from MEFs of the indicated genotype, either treated with TGF-B for 30 min or untreated, and a probe derived from the TGF-β-responsive region of the promoter-reporter construct, p3TPlux. The arrow indicates the TGF-β-inducible DNA binding complex. (B) Smad3 is required for induction of the p3TP-lux reporter construct. The indicated DNAs were transfected into MEFs of the indicated genotype. Twelve hours after transfection, the cells were treated with 100 pM TGF-B for an additional 24 h, and TGF-B-induced luciferase activity (relative luciferase units [RLU]) from this reporter construct was assayed. Bars represent the average luciferase activity of duplicate transfections in a single experiment; error bars represent the standard deviation. Fold induction by TGF-B is indicated over each set of bars. (C) Smad3 is an integral component of the TGF-B-mediated induction of the endogenous PAI-1 gene. Smad3 heterozygote and null primary dermal fibroblasts were treated with TGF-β for 8 h. The arrow represents [35S]methionine-labeled, extracellular matrix-associated PAI-1, assayed as described in Materials and Methods.

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FIG. 5. Assay of TGF- $\beta$ 's effects in primary splenocytes reveals both Smad3dependent and Smad3-independent growth-inhibitory signaling pathways. (A) Smad3 is not required for TGF- $\beta$ -mediated growth inhibition in primary unstimulated splenocytes. Primary splenocytes were isolated from 8-week-old mice and cultured in the presence or absence of 100 pM TGF- $\beta$  for 48 h. Cells were incubated with [<sup>3</sup>H]thymidine for the last 4 h of culture, after which the splenocytes were harvested and <sup>3</sup>H incorporation was measured. Bars indicate the average of three identically treated wells for each growth condition; error bars represent the standard deviation. (B) Smad3 is required for TGF- $\beta$ -mediated growth inhibition of  $\alpha$ CD3-stimulated splenocytes. Primary splenocytes were isolated from 8-week-old mice and cultured in the presence of the indicated growth stimuli in the presence or absence of 100 pM TGF- $\beta$ . Cellular proliferation was assayed by [<sup>3</sup>H]thymidine incorporation as for panel A. (C) Smad3 is expressed in both B and T cells. Western blotting for Smad3 was performed on purified B and T cells from mature wild-type spleens.

growth-inhibitory effects of TGF- $\beta$  in this system but also for the induction of this specific promoter reporter construct.

To assess the effect of Smad3 loss on the induction of an endogenous gene known to be transcriptionally regulated by TGF- $\beta$ , we assayed the TGF- $\beta$ -mediated induction of PAI-1 in primary dermal fibroblasts. As shown in Fig. 4C, the induction of PAI-1 by TGF- $\beta$  seen in Smad3 heterozygote dermal fibroblasts is greatly reduced in the null cells.

Analysis of the immune cells derived from Smad3 null mice reveals a defect in TGF- $\beta$  signaling. Having defined an essential role for Smad3 in TGF- $\beta$  signaling in the fibroblast system, we next examined the cell types with highest Smad3 expression, those of lymphoid origin. We first examined the proliferation of splenocytes isolated from wild-type and null animals in the presence and absence of TGF-B. Interestingly, the proliferation of unstimulated primary splenocytes, consisting of a mixed B- and T-cell population, is inhibited by TGF- $\beta$  regardless of mouse genotype when assayed by tritiated thymidine incorporation (Fig. 5A). Thus, in contrast to the MEF data presented above, Smad3 is not required for TGF-β-mediated inhibition of cellular proliferation in unstimulated splenocytes. In addition, the antiproliferative effects of TGF- $\hat{\beta}$  in primary splenocytes stimulated by LPS or  $\alpha$ IgM plus IL-4, which specifically stimulate the proliferation of B lymphocytes through the activation of IgM receptor expressed only on the surface of B cells, is largely intact regardless of genotype. However, in primary splenocyte cultures stimulated with  $\alpha$ CD3, an activator of the T-cell receptor complex, inhibition of proliferation by TGF-β is seen only in wild-type cultures (Fig. 5B). This difference in Smad3-dependent TGF-B responsiveness of the mixed splenocytes to a specific stimulus is not due to a difference in the

expression pattern of Smad3, as demonstrated by Smad3 Western blot analysis of isolated T and B cells (Fig. 5C). Taken together, these data suggest that Smad3 plays a specific role in the inhibition of immune cell proliferation by TGF- $\beta$  dependent on the nature of stimulus.

Little is known on the molecular mechanisms through which TGF- $\beta$  inhibits proliferation of activated B and T cells, making it difficult to predict the role of Smad3 in this system. Concurrent with results obtained for a variety of cell types, TGF-B treatment of aCD3-stimulated wild-type spleen cultures leads to a decrease in Cdk2 kinase activity and a maintenance of Rb in a hypophosphorylated state. These effects occur with minimal change in the levels of Cdk2 and cyclin E and no change in the levels of p27. In contrast, TGF-B-mediated inhibition of Cdk2 kinase activity and maintained activation of Rb do not occur in the Smad3 null spleen cultures, further supporting the different growth properties of wild-type and null immune cells (Fig. 6A). Western analysis of various cell cycle components reveal no TGF-B-mediated change in the levels of the CDC25A phosphatase or the TGF-\beta-responsive Cdk inhibitors p15 and p21 (data not shown). Thus, the TGF-B growth-inhibitory pathway activated in aCD3-stimulated splenocytes represents a yet to be defined Smad3-dependent mechanism.

Subsequently, we examined the effects of TGF- $\beta$  on cytokine production in  $\alpha$ CD3-stimulated primary spleen cultures. In this system, TGF- $\beta$  prevents the  $\alpha$ CD3-mediated increase in the production of a number of different cytokines by the wildtype cells (Fig. 6B). This effect is even more dramatic than the growth-inhibitory effects of TGF- $\beta$  on these cultures. As shown in Fig. 6B, the production of several cytokines, such as IL-2, IL-4, IL-5, IL-9, IL-13, and IL-15, is more than 80% reduced by TGF- $\beta$  in wild-type splenocytes (Fig. 6C). In the Smad3 null culture, however, TGF- $\beta$  clearly does not have the same effect on the levels of these cytokines as seen in the wild-type culture. This loss of TGF- $\beta$  responsiveness is most marked in gamma interferon (IFN-y), IL-2, IL-13, and IL-15 production. In addition, the non-TGF-B-treated levels of several cytokines are elevated in the null cultures. These results strongly suggest abnormal regulation of cytokine production in the absence of Smad3-mediated TGF-β signal transduction.

Since lymphocyte proliferation abnormalities are observed in vitro in Smad3 null cells, we next determined whether any abnormalities in the profiles of lymphocyte distribution could be observed in vivo by performing FACS analysis on Smad3 null and wild-type spleens and thymuses. As shown in Fig. 7, the thymuses of Smad3 null mice contain normal proportions of CD4 and CD8 single- and double-positive T cells, suggesting that thymic T-cell maturation is normal in Smad3 null mice. Similarly, the spleens from Smad3 null mice contain normal numbers and percentages of B cells and CD4 and CD8 singlepositive T cells, suggesting that there is not an abnormal expansion of lymphocytes in the spleens of Smad3 null mice. In addition, we performed FACS analysis on bone marrow and peripheral lymph nodes for B- and T-cell populations and observed no difference between wild-type and Smad3 null mice (data not shown). Finally, we performed functional analysis of B and T cells by immunizing mice with various antigens and measuring both T-cell-dependent and T-cell-independent antibody production. Again, we did not observe any significant differences in antibody production between wild-type and Smad3 null mice (data not shown).

### DISCUSSION

In an attempt to define the roles of Smad3 in TGF- $\beta$ -mediated signal transduction, we have created mice harboring a



FIG. 6. TGF- $\beta$ -mediated growth inhibition of  $\alpha$ CD3-stimulated splenocytes is associated with a decrease in G<sub>1</sub> Cdk activity and cytokine expression. (A) Splenocytes were harvested from wild-type and knockout mice and cultured with  $\alpha$ CD3 in the presence or absence of TGF- $\beta$ . Cell lysates were prepared and subjected to Western blotting for cyclin E, Cdk2, p27, and Rb (lower panels). In addition, Cdk2 kinase activity was assayed by immunoprecipitation of Cdk2 and evaluation of its ability to phosphorylate the exogenous substrate, histone H1 (top panel). (B) Cytokine production was assayed on splenocytes from wild-type and Smad3 null mice treated as for panel A, using an RNase protection assay. The identity of each band is indicated on the right. L32 and *GAPDH* are controls for mRNA quantity and quality. (C) The intensity of the cytokine RPA bands in panel B was determined by densitometry. Plotted are the relative intensities of each band, with wild-type levels of each band, with wild-type levels

targeted disruption of Smad3. The first striking finding is that Smad3 null mice are viable and survive to adulthood. The analysis of mice deficient in other Smad genes, however, has firmly established the role of this family of proteins in embryonic development. Mice with a targeted disruption of Smad4 display an early embryonic lethal phenotype at embryonic days 6.5 to 8.5. These embryos do not undergo gastrulation or express mesodermal markers, and they show abnormal visceral endoderm development (52, 61). Smad2-deficient mice also die early in development, at embryonic day 9.5, primarily due to a loss of anterior-posterior identity within the embryo. In the absence of anterior-posterior identity, the entire epiblast develops a extraembryonic mesodermal fate, failing to give rise to the three primary germ layers (55). In a separate study, Smad2 was found to play a role in mesoderm formation, leftright patterning, and craniofacial development (43). Additional support for the critical roles of the TGF-β superfamily of ligands and the Smad family of proteins in development has been established in studies of the Xenopus oocyte developmental system (17, 24).

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In sharp contrast to mice harboring a targeted disruption of Smad2 and Smad4, the loss of Smad3 function, as we report here, has no discernible effect on embryonic development. It is conceivable that certain functions of Smad3 are redundant with, or compensated for by, that of Smad2. These two proteins are 90% identical at the amino acid level. Both proteins are inducibly phosphorylated by the TGF- $\beta$  receptors, associate with Smad4, and undergo nuclear accumulation. One main difference is that Smad2 may be expressed as two alternatively spliced variants; one contains two inserts in the MH1 domain of the protein, rendering it unable to bind to DNA (51, 60), whereas the other, without the inserts, is structurally and functionally virtually identical with Smad3 (60). Thus, the molecular functions of Smad2 and Smad3 are most likely overlapping as well as distinct, since functional differences in the DNA binding properties and promoter activation by these molecules have been reported (28, 63, 65). Although we still do not know the expression patterns of the two variants of Smad2, clearly Smad3 cannot fully compensate for the severe defect in Smad2 null mice which may have lost the expression of both forms of Smad2. On the other hand, Smad3 may play a more exclusive

role as an effector for TGF- $\beta$  and possibly activin in adult tissues, whereas Smad2 with its two forms may function more globally in development and possibly in the adult as a signaling mediator of these two ligands.

A role for Smad3 in TGF- $\beta$ -mediated growth inhibition. Our initial goal in these studies was to define the role of Smad3 in the regulation of cellular proliferation by TGF- $\beta$ . Since previous studies on this topic have involved overexpression of Smads and the use of various tumor lines which likely harbor additional mutations, a role for the Smads in the regulation of proliferation remained uncertain. To this end, we have demonstrated that Smad3 is required for TGF- $\beta$ -mediated growth inhibition in at least two cellular contexts:  $\alpha$ CD3-stimulated primary splenocytes and primary MEFs.

The results from primary splenocyte cultures are particularly interesting in that TGF-\beta-mediated growth inhibition is dependent on Smad3 only under certain stimulated growth conditions. The proliferation of unstimulated, LPS-stimulated, and aIgM-IL-4-stimulated splenocytes is inhibited in response to TGF- $\beta$  treatment in wild-type cells and to a nearly identical extent in Smad3 null cells. In contrast, a large reduction in TGF- $\beta$ -mediated growth inhibition is seen in the Smad3 null splenocytes specifically when they are stimulated by  $\alpha$ CD3. Thus, there appear to be both Smad3-dependent and Smad3independent growth-inhibitory signaling pathways for TGF-β. We have also observed a similar defect in TGF-\beta-mediated growth inhibition in MEFs derived from Smad3 null mice. In these cells, the growth-inhibitory effect of TGF-β is largely absent, and this lack of TGF-B effect is most likely cell autonomous.

The molecular nature of the growth-inhibitory effects of TGF- $\beta$  is one of its most studied properties. Through the work of a number of groups, a model has been put forward in which TGF- $\beta$  regulates proliferation by inhibiting the activity of Cdk complexes. This function of TGF- $\beta$  is likely due, in part, to its ability to increase the expression of the Cdk inhibitors p21 and p15, decrease the expression of a number of different cyclins, Cdks, the phosphatase CDC25A, and c-Myc, as well as regulate the activity of p27 (reviewed in reference 20). The signaling mechanisms of TGF- $\beta$ -mediated growth inhibition vary significantly from one cell type to another. Unfortunately,

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FIG. 7. FACs analyses of thymocytes and splenocytes isolated from wild-type and Smad3 null mice demonstrate normal T-cell and B-cell development. (A and B) Representative FACs analysis of wild-type and Smad3 null thymocytes, using  $\alpha$ CD4-PE and  $\alpha$ CD8-FITC. (C to F) Representative FACS analysis of wild-type and Smad3 null splenocytes, using the indicated conjugated antibodies. All data was gated for viable cells by the absence of 7AAD staining. Percentages represent the proportions of viable cells in each region or quadrant.

none of the previously described TGF- $\beta$ -mediated growth-inhibitory pathways appear to be functioning in wild-type MEFs or  $\alpha$ CD3-stimulated splenocytes. Specifically, MEFs and  $\alpha$ CD3stimulated splenocytes down regulate G<sub>1</sub> cyclin-Cdk complex activity without significant changes in the levels of p21, p15, p27, cyclin E, or Cdk2. Thus, Smad3 does not act through these defined downstream effectors to mediate the growth-inhibitory effects of TGF- $\beta$  in these cells. Consequently, these findings suggest a novel Smad3-dependent growth-inhibitory pathway for TGF- $\beta$ .

The work presented here is complemented by a recent report by Zhu et al., characterizing the phenotype of an independently created mouse line with a targeted insertion into the second exon of Smad3 (68). This group describes a high prevalence of colon tumor in the 129sv mouse background, and a lower prevalence of a less aggressive tumor phenotype in the 129-C57BL/6 hybrid mouse background. Although not experimentally addressed, it is an attractive hypothesis that this tumor formation occurs due to defects in TGF- $\beta$ -mediated growth inhibition of the sort that we describe here. It remains to be determined, however, if these tumors arise from some

other TGF- $\beta$ -Smad3-dependent cellular effect or through a mechanism unrelated to TGF- $\beta$  signaling. It is intriguing that we have not yet observed the 30% prevalence of colon tumors in our 129-C57BL/6 hybrid lines as in the reported study. This discrepancy may be due to differences in genetic background of the Smad3 null animals or even targeting strategies. It is also possible that a higher prevalence of tumors may still occur in our lines with longer time or when the mice with mixed genetic background are inbred into a pure 129 mouse line.

In addition to its antiproliferative role in the context of tumor suppression, TGF- $\beta$  is a well-documented global inhibitor of immune system function. This function of TGF- $\beta$  is evidenced by the phenotype of TGF- $\beta$ 1 null mice (9, 26). These mice present with a multifocal inflammatory disease, with lymphocyte infiltration into multiple organs and production of autoimmune antibodies (9, 13). The phenotype of these mice may be attributed to a loss of the antiproliferative effect of TGF- $\beta$ 1 on both B and T cells (22, 23). Given the fact that Smad3 is most highly expressed in the spleen and thymus, and the accumulating evidence that Smad3 is regulated by TGF- $\beta$ , the development of an overactive inflammatory phenotype

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similar to that of the TGF- $\beta$ 1 knockout mice may have been expected in the Smad3 null mice. This phenotype, however, is not observed.

These findings may be explained by the fact that under several conditions for assay of B- and T-cell cultures in vitro, the antiproliferative effect of TGF- $\beta$  is intact in Smad3 null cells. Thus, under in vivo conditions, the proliferation of B and T cells may be appropriately inhibited under most circumstances by endogenous TGF- $\beta$ . Since this is likely the case, a more subtle or incompletely penetrant inflammatory phenotype may still emerge in the Smad3 null mice. These findings also support a model in which although Smad3 is important in regulating the antiproliferative effects of TGF- $\beta$  under certain conditions, TGF- $\beta$  can also activate or use other Smad3-independent pathways to exert a growth-inhibitory effect.

A role for Smad3 in TGF-β-mediated gene responses. TGF- $\beta$  can affect the expression of a number of different genes of diverse functions (46). The identification of Smads as sequence-specific DNA binding transcription factors supports the notion that the regulation of specific genes by TGF- $\beta$  may be through the functions of Smad2, Smad3, and Smad4. Both 3TP-lux, a well-studied promoter reporter used for the analysis of TGF- $\beta$  signaling, and the promoter of PAI-1, a highly TGF-B inducible extracellular matrix protein, contain Smad3-Smad4 DNA binding sites (11, 21, 63). Although the Smads have been implicated in the TGF-\beta-mediated induction of 3TP-lux and PAI-1, these studies are based largely on Smad overexpression and dominant negative studies, leaving the question of the physiological role for Smad3 in TGF-B-mediated gene activation unresolved (29, 35, 65). In addition, we have shown in a previous study that the Smad-DNA interaction is dispensable for the activation of 3TP-lux by TGF-B, bringing into question the role of Smad3 in the regulation of this promoter (63). Here we demonstrate that Smad3 is integral for transactivation of 3TP-lux and PAI-1, as their induction by TGF- $\beta$  is reduced in the absence of Smad3. Interestingly, although Smad2 has been shown in the context of overexpression to activate 3TP-lux and PAI-1 (29), no compensation by Smad2 is observed in the Smad3 null fibroblasts.

In addition to the studies of 3TP-lux and PAI-1, we have investigated the role of Smad3 in the regulation of other genes by TGF-β. Specifically we provide evidence that the TGF-βmediated down regulation of aCD3-stimulated cytokine production is Smad3 dependent. This suggests that Smad3 may play an important role in both the activation as well as the repression of gene expression. We have also evaluated the role of Smad3 in the regulation of several additional promoters by TGF-β in our Smad3 null model system. Specifically, we describe the role of Smad3 in the induction of c-Jun by TGF- $\beta$  in a separate study (56). In this study we find that the c-Jun promoter contains a Smad3-Smad4 complex binding site and that Smad3 is required for the induction of c-Jun in MEFs. Thus, Smad3 may be specifically required for the activation of transcription from a subset of TGF-B-responsive promoters. In this regard, the Smad3 null MEF system provides a useful tool to define these genes which are regulated by TGF-B through Smad3. In doing so, we may be able to define novel genes or pathways which are at the root of the Smad3-dependent, TGF- $\beta$  antiproliferative effects which we have described.

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The first two authors contributed equally to this work.

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# Smad3-Smad4 and AP-1 Complexes Synergize in Transcriptional Activation of the c-Jun Promoter by Transforming Growth Factor β

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Transcriptional regulation by transforming growth factor  $\beta$  (TGF- $\beta$ ) is a complex process which is likely to involve cross talk between different DNA responsive elements and transcription factors to achieve maximal promoter activation and specificity. Here, we describe a concurrent requirement for two discrete responsive elements in the regulation of the c-Jun promoter, one a binding site for a Smad3-Smad4 complex and the other an AP-1 binding site. The two elements are located 120 bp apart in the proximal c-Jun promoter, and each was able to independently bind its corresponding transcription factor complex. The effects of independently mutating each of these elements were nonadditive; disruption of either sequence resulted in complete or severe reductions in TGF- $\beta$  responsiveness. This simultaneous requirement for two distinct and independent DNA binding elements suggests that Smad and AP-1 complexes function synergistically to mediate TGF- $\beta$ -induced transcriptional activation of the c-Jun promoter.

Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a multifunctional cytokine with a wide range of physiological as well as pathological effects (reviewed in references 31 and 42). Its physiological roles include inhibition of the proliferation of a variety of cell types, negative regulation of the immune system, and positive regulation of extracellular matrix deposition. Dysregulation of these processes can result in various fibrotic as well as malignant diseases. Indeed, many late stage cancers have lost expression of TGF- $\beta$  receptors, which renders them resistant to TGF- $\beta$ -mediated growth inhibition (19, 29, 36, 38, 50, 55); restoration of TGF- $\beta$  pathways in these cells can often restore growth inhibition and decrease the malignant phenotype. TGF- $\beta$ -mediated immune system suppression and stimulation of extracellular matrix (ECM) production may also contribute to tumor-promoting effects.

Regulation of transcription of specific sets of genes by TGF-β mediates many of these physiological roles. Upregulation of two cyclin-dependent kinase inhibitor genes, p21 and p15, has been shown to mediate TGF-\beta-induced growth arrest in certain cell types (7, 12, 41), while upregulation of ECM genes, including plasminogen activator inhibitor 1 (PAI-1), fibronectin, and collagen genes, may mediate other effects of TGF-β. However, many of the genes regulated by TGF-β are also regulated by a variety of other signals, including some signals which appear to play very distinct roles at the physiological level. Of particular note is a subset of TGF-β immediate-response target promoters, including the TGF-B1 ligand gene and most of the TGF-B-responsive extracellular matrix genes, in which AP-1 binding sites have been found to be involved in mediating the TGF- $\beta$  signal (4, 22, 51). The use of AP-1 sites in TGF-\beta-dependent transcription has been particularly puzzling, given the extensively described mitogenic signaling pathways which also activate transcription through

AP-1; the mechanism by which TGF- $\beta$  regulates these promoter sequences has not been clarified. An additional level of complexity is introduced by the regulation by TGF- $\beta$  of the expression of AP-1 family members themselves. This suggests that there can be both primary and secondary effects on transcription through AP-1 by TGF- $\beta$ .

The regulation of AP-1 transcription factors by TGF- $\beta$  varies with the specific family member and with cell type. The upregulation of c-Jun transcript occurs in a wide range of cell lines derived from both normal and transformed cells. This response to TGF- $\beta$  is early and immediate, with mRNA induced within 15 to 30 min. While cycloheximide studies have been inconclusive, due to the inducing effects of the cycloheximide itself on c-*jun* transcription, the time course of induction strongly suggests that this gene could be a primary target of TGF- $\beta$  (24, 26, 39), which is supported by the current study describing specific promoter elements capable of mediating TGF- $\beta$ 's induction of c-Jun.

The model for TGF- $\beta$  activation of transcription continues to undergo rapid development. The Smads are a recently identified family of proteins which operate downstream of various members of the TGF- $\beta$  superfamily (reviewed in references 13, 14, 23, 30, and 37). Smad2 and Smad3 are downstream effectors of the TGF-β signaling pathway. Upon ligand binding, they are phosphorylated by the TGF- $\beta$  type I receptor kinase and translocate to the nucleus in a complex with Smad4 (28, 35, 59). Recent work has identified a potential consensus Smad3-Smad4 DNA binding site, GTCTAGAC (58), by random oligonucleotide screening, as well as similar sequences in the PAI-1 promoter (9), the engineered TGF-β-responsive reporter construct, p3TP-lux promoter (57), the JunB promoter (18), and the COL7A1 collagen promoter (54). It was found that four copies of the oligonucleotide consensus site or nine copies of the PAI-1 site could confer TGF-B responsiveness on a minimal promoter. In addition, mutation of all three putative Smad3-Smad4 binding sites in the PAI-1 promoter could eliminate TGF-B responsiveness of that promoter in HepG2 cells. Although these studies demonstrate the importance of

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Smad3-Smad4 binding sites in the mediation of TGF- $\beta$  responsiveness, they do not fully address the issue of whether binding elements for other transcription factors are also required for TGF- $\beta$ -mediated transcriptional activation of target promoters. Biochemical and overexpression studies have demonstrated that Smads are capable of functional interaction with Sp1 (33) and with AP-1; in fact, direct physical interaction between Smad3 and AP-1 family members has been demonstrated in model systems (27, 60). Cooperation between Smad2-Smad4 complexes and FAST-1 has been demonstrated at an activin responsive *Xenopus* promoter (2, 3). Finally, a very recent study reports that a binding site for the transcription factor muE3 (TFE3), as well as one for Smad3 and Smad4, is required for TGF- $\beta$ -mediated transcription of a reporter controlled by a specific region of the PAI-1 promoter (16).

While the TGF- $\beta$ -responsive elements in the c-Jun promoter have not previously been characterized, extensive work has established the importance of two AP-1/CRE sequences in the c-Jun promoter in regulation by phorbol-12-myristate-13acetate (TPA), serum, UV, E1A, and interleukin 1 (IL-1) (1, 15, 34, 43, 53). Furthermore, a reporter construct controlled by the -79 to +170 sequence of the c-Jun promoter, which contains only the more proximal AP-1/CRE site (-71 to -64), has proved sufficient for a maximal response to most of these signals. Interestingly, none of these stimuli appears to change the occupancy of any identified binding sites in the c-Jun promoter. Thus, the prevailing model of activation by these other signals is thought to be through modification of a constitutively promoter-bound complex, in most cases c-Jun-ATF-2.

Here, we identify two DNA binding elements within this -79 to +170 region which are indispensable in TGF- $\beta$ -mediated induction of c-Jun: the proximal AP-1/CRE site known to be important for the response to several other signals, and a novel Smad3/Smad4 binding site. Mutation of either site alone is found to abolish or severely reduce promoter upregulation by TGF- $\beta$ , despite the presence of the remaining element. Our results suggest that the two complexes can cooperate synergistically in activating TGF- $\beta$ -mediated transcription of this c-Jun promoter region.

#### MATERIALS AND METHODS

Antibodies and reagents. Human TGF- $\beta$ 1 was from R&D Systems. Rabbit polyclonal antisera recognizing Smad3 and Smad4 were generated in this lab. Smad3 antiserum was raised against a specific Smad3 peptide (DAGSPNLSPN-PMSPAHNNLD), while Smad4 antiserum was raised against full-length human glutathione S-transferase–Smad4.

Cell culture. Mink lung epithelial cells and primary mouse embryo fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, penicillin, and streptomycin. Inmortalized human keratinocyte cells (HaCaT) were grown in MEM supplemented with 10% FBS, penicillin and streptomycin, and 20 mM L-glutamine. Primary fibroblasts were harvested from day-14 embryos. Embryos were mechanically disrupted by passage through an 18-gauge needle and plated on gelatin-coated 10-cm-diameter plates in DMEM with 20% heat-inactivated FBS, penicillin, streptomycin, and gentamicin (Gibco BRL, Gaithersburg, Md.). When confluent, cells were trypsinized and further maintained in DMEM with 10% FBS. The targeted disruption of the Smad3 allele in these mice and the characterization of their phenotype are described elsewhere (6).

(6). **Plasmid constructs.** Flag-tagged human Smad4 was a generous gift from Rik Derynck. Human pCGN Smad3 was described previously (57). The c-Jun luciferase reporter containing the -79 to +170 sequence of the human c-Jun promoter was generously provided by Bin Su (48). The rest of the promoter mutants and 3' deletion constructs were made by PCR mutagenesis using the following primer sets: as 5' primers, wild type, 5'CCC AAG CTT GGC CTT GGG GTG ACA TCA TGG GC3'; AP-1/CRE mutant, 5'CCC AAG CTT GGC CTT GGG GAT CCA CCA TGG GCT ATT TTT AGG GG3'; and as 3' primers, wild type, 5'AAA CTG CAG GCC GAC CTG GCT GGC TGG CTG TCT GTC3'; mutant, 5'AAA CTG CAG GCC GAC CTG GCT GGC TGG CTG TTC CAA GCT CCT TGC CTG ACT CCG3'. A *Hind*III site was engineered into the 5' end of each PCR product, and a *Pst*I site was engineered into the 3' end of each PCR product. PCR products were subcloned into pGEMT (Promega, Madison, Wis.), and then the *HindIII/PstI* fragments were purified on an agarose gel, extracted with a QIAEX II gel extraction kit (Qiagen Inc., Santa Clarita, Calif.), and subcloned back into the *HindIII* and *PstI* sites flanking the 5' and 3' ends, respectively, of the -79 to +170 sequence insert in the -79 to +170 luciferase reporter construct. Constructs were verified by restriction digestion with *HindIII/PstI* and by sequencing.

**Transfection and luciferase assays.** Transient transfections were performed with the standard DEAE-dextran method and the luciferase activity was measured 24 h after the addition of 100 pM human TGF- $\beta$ 1 as described previously (8). For all experiments, 3 µg of the indicated luciferase reporter and, when indicated, 1 µg of Smad3 expression vector were used (57). Total DNA was kept constant by using empty pCGN vector. All transfections were normalized to  $\beta$ -galactosidase activity by cotransfection of 0.5 µg of a  $\beta$ -galactosidase (pCMV  $\beta$ -Gal) expression vector. The luciferase data shown are representative of experiments performed in duplicate in at least three independent experiments.

Nuclear extracts. Nuclear lysates were prepared from control and TGF- $\beta$ I-treated cells. Briefly, confluent cells from 10-cm-diameter dishes were washed twice with phosphate-buffered saline. After washing, 5 ml of ice-cold hypotonic lysis buffer was added (20 mM HEPES [pH 7.6], 20% glycerol, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM NaF, 25 mM  $\beta$ -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovandate, 1 mM dithiothreitol, and protease inhibitors). The cells were allowed to swell on ice for 5 min before they were scraped and collected. Nuclei were pelleted by centrifugation at 500 pm in a Beckman swinging-bucket tabletop centrifuge for 5 min and resuspended in 100 to 200  $\mu$ l of nuclear extraction buffer (hypotonic buffer plus 500 mM NaCl). After incubation and rocking at 4°C, the lysates were cleared of debris by centrifugation.

Western blot analysis. Western blot analysis for c-Jun was performed on nuclear lysates prepared from MEFs. Prior to treatment with TGF- $\beta$ 1 for the indicated times, cells were serum starved for 12 h in DMEM-0.2% FBS. Equal protein amounts were resolved by sodium dodecyl sulfate-10% polyacylamide gel electrophoresis, and Western blotting was performed with a 1:1,000 dilution of the rabbit polyclonal antibody  $\alpha$ -c-Jun (9162) from New England Biolabs, Inc. (Beverly, Mass.).

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed by using 1 to 3  $\mu g$  of nuclear extracts prepared from untreated cells or cells treated with 100 pM TGF-B1 for 1 h and probes derived from a SacI/BamHI fragment of luciferase construct containing the c-Jun sequence from -79 to +170. The digest produced two fragments of the c-Jun promoter that consist of the sequences from -79 to -19 and -18 to +170. Gel shift conditions were exactly as previously described (57). For supershift analysis of Smads, 2  $\mu$ l of Smads 3 and 4 immune-phase and preimmune-phase antisera and 2 µg of Smad2 (S-20-X) or Smad4 (C-20-X) antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.), were used. For other supershifts, 2 µg of anti-c-Jun (KM-1-X), ATF-2 (FRBR-1-X and C-19-X), and CREB (C21-X and 24H4B-X) from Santa Cruz Biotechnology, Inc., were used. The sequences of the competitor oligonucleotides used to identify the Smad binding site are shown in Fig. 3A. The sequence of the competitor oligonucleotide containing a CREB/ATF binding site (CRE) was 5'-AGA GAT TGC CTG ACG TCA GGA GCT AG-3' and its complementary strand. The sequence of the mutated CRE site was 5'-AGA GAT TGC CTG TGG TCA GAG AGC TAG-3'. Where results for only one lysate are shown, similar results were obtained for both HaCaT lysates and mink lung lvsates

### RESULTS

TGF-B treatment induces DNA binding of a Smad3- and Smad4-containing complex to a sequence in the 3' region of the c-Jun promoter. An increase in c-Jun mRNA level has been previously observed within 15 to 30 min of TGF-B treatment in a variety of cell types (24, 39, 49). In order to confirm the induction of endogenous c-Jun by TGF- $\beta$ , we performed Northern analysis of RNA and Western analysis of nuclear extracts isolated from similarly treated cells. In both mink lung epithelial cells (Mv1Lu) and HaCaT cells, the level of c-Jun transcript increased within 1 h of TGF-B treatment and protein levels were dramatically increased within 2 h of TGF-β treatment (data not shown), confirming that the induction of c-Jun by TGF- $\beta$  occurs in these cells and is likely to be an early response. The induction by TGF-β was most evident in Mv1Lu cells if the cells were serum starved overnight before addition of TGF- $\beta$ , since the c-Jun transcript is upregulated by serum.

To aid in defining TGF- $\beta$  responsive elements in the human c-Jun promoter, we next obtained a luciferase reporter construct under control of the sequence from -79 to +170 of the c-Jun promoter (48). This region, diagrammed in Fig. 1A,



API/CRE RESE TATA -79 CCTT GGGGT GACAT CHTGG GCTAT TITTA GGGGT TGACT GGTAG CAGAT ANGTG TTGAG CTCGG GCTGG +1 ATAAG GGCTC AGAGT TGCAC TGAGT GTGGC TGAAG CAGCG AGGCG GGAGT GGAGG TGCGC GGAGT CAGGC AGACA GACAG ACACA GCCAG CCAGC CAGGT CGGCA GTATA GTCCG AACTG CAAAT CTTAT TTTCT TTTCA CCTTC TCTCT AACTG CCCAG AGCTA GCGCC TGTGG CTCCC +170



FIG. 1. The -79 to +170 region of the human c-Jun promoter is sufficient to convey TGF- $\beta$  and Smad3 responsiveness to a luciferase reporter. (A) Schematic representation of the -79 to +170 luciferase reporter. (B) The reporter was transiently transfected into Mv1Lu or HaCaT cells, and TGF- $\beta$ -induced luciferase activity was measured in relative light units (luciferase units). Fold inductions are indicated above the bars and were calculated by comparing the luciferase activities of cells treated with TGF- $\beta$  and those of untreated controls.

contains the proximal AP-1/CRE site and the adjacent AT-rich sequence (a putative RSRF [related to serum response factor] site) which is important in epidermal growth factor (EGF) induction of c-Jun, as well as the native TATA box and approximately 170 bp of the sequence 3' of the start site. As mentioned above, this region was sufficient to convey maximal responsiveness to UV, TPA, EGF, and serum. We transiently transfected this construct into Mv1Lu and HaCaT cells, and measured luciferase activity after TGF- $\beta$  treatment. As shown in Fig. 1B, the construct was highly responsive to TGF- $\beta$ , giving 4.6-fold induction in Mv1Lu cells and 14.8-fold induction in HaCaT cells.

Having determined that the -79 to +170 portion of the c-Jun promoter was sufficient to convey TGF-β responsiveness, we next examined whether the mechanism of activation might involve induction of Smad DNA binding to a site in this region. We performed an EMSA by using a 5' portion or a 3' portion of the -79 to +170 region as a probe (Fig. 2A) and nuclear extracts from HaCaT cells treated for 1 h with TGF-B. The -79 to -19 probe bound two complexes (small arrows), and no change was observed upon TGF- $\beta$  treatment (Fig. 2B). On the other hand, the -18 to +170 probe bound a complex that was strongly induced by TGF- $\beta$  treatment (Fig. 2C). This induced complex appeared within 30 min of TGF-B treatment and was still present at 2 h (data not shown). Using an antiserum specific to Smad3 as well as an antiserum and commercial antibody specific to Smad4, we were able to supershift the induced complex, indicating the presence of both Smad3 and Smad4 in the complex. No supershift was seen with the corresponding preimmune-phase antisera, and a commercially available Smad2 antibody also failed to cause a supershift (Fig. 2C). Similar results were obtained with nuclear extracts from Mv1Lu cells (data not shown).

These results establish the existence of a Smad3-Smad4 binding site contained within the -18 to +170 region of the c-Jun promoter. The binding of Smad3-Smad4 is rapidly induced upon TGF- $\beta$  treatment, with a time course consistent with that of Smad phosphorylation and subsequent translocation to the nucleus (see references 14 and 23 for reviews). In contrast, the pattern of binding to the -79 to -19 region of the promoter is unchanged upon TGF- $\beta$  treatment.

The Smad3-Smad4 binding site in the c-Jun promoter is a CAGA triplet located 3' of the TATA box. In order to identify the Smad3-Smad4 binding site within the -18 to +170 region, four oligonucleotides scanning this sequence (Fig. 3A) were used as cold competitors in the EMSA. Only the +35 to +83region was found to compete with the binding of the induced complex (Fig. 3B). When the oligonucleotide for this region was cut at a convenient HinfI site and the two halves were compared, binding could be further localized to the +53 to +83 region. Three mutant competitor oligonucleotides of the +53 to +83 region were then designed. We had noted a sequence in the middle of this region, ACAGACAGACAGAC ACAG, which bore great similarity to repeats of the Smad box as identified by previous studies (9, 57, 58) and was recently confirmed by the crystal structure of MH1-Smad3 bound to the CAGA box (47). Therefore, we made mutations to disrupt either this potential Smad binding site or the sequence 5' or 3' of it within the +53 to +83 region. Of the three, only the CAGA mutant oligonucleotide had lost its ability to compete


FIG. 2. EMSAs showing induced binding of a Smad3- and Smad4-containing complex to the 3' region of the human c-Jun promoter. (A) Schematic representation of the -79 to +170 region of the c-Jun promoter showing the probes used for EMSAs. (B) EMSA was performed by using a radiolabeled restriction fragment spanning the -79 to -19 region of the c-Jun promoter and nuclear lysates from either untreated HaCaT cells or HaCaT cells treated with TGF- $\beta$ 1 for 1 h. Two constitutively binding complexes are indicated with arrows. (C) EMSA was performed by using a radiolabeled restriction fragment spanning the -18 to +170 region of the c-Jun promoter and the same HaCaT lysates. A complex that shows binding induced by TGF- $\beta$  treatment is indicated with an arrow. Supershifts were performed using antiserum against Smad3 or Smad4, shown with their corresponding preimmune-phase antiserum (Pre $\alpha$ 3 and Pre $\alpha$ 4) or with commercial antibodies against Smad4 [ $\alpha$ Smad4(C)] and Smad2 [ $\alpha$ Smad2(C)].

with binding of the induced complex (Fig. 3B and data not shown), indicating that this mutation had disrupted the Smad binding site. Confirming this, a -18 to +170 probe containing mutated CAGA sequence was shown to no longer bind the induced Smad3-Smad4 complex (Fig. 3C). From these experiments we concluded that the Smad3-Smad4 binding site was located at the CAGA repeats within the +62 to +73 region of the c-Jun promoter. These results also established that no other sequences in the -18 to +170 region are absolutely required for DNA binding of the induced complex containing Smad3-Smad4.

Mutation of the Smad3-Smad4 binding site in the c-Jun promoter abrogates responsiveness to TGF-β. Having identified the Smad3-Smad4 binding site in the c-Jun promoter, we set out to determine its importance in mediating the TGF-β response. Using PCR mutagenesis, we created a -79 to +94wild-type reporter and corresponding -79 to +94 mutant reporters (Fig. 4A). We found that the wild-type -79 to +94reporter was induced by TGF-β 11-fold and 5.1-fold in HaCaT cells and Mv1Lu cells, respectively. Mutation of the CAGA sequences reduced the response to 2.7-fold and 2.3-fold, respectively (Fig. 4B and C). This demonstrates that the Smad3-Smad4 binding site is important for response to TGF-β in the context of this c-Jun promoter construct.

Although these findings indicate that the identified Smad3-Smad4 binding site is critical in conferring a complete response to TGF- $\beta$ , there is a small degree of responsiveness which remains after mutation of the Smad3-Smad4 binding site. It is possible that the remaining TGF- $\beta$  responsiveness is mediated

through the AP-1/CRE site at the -71 to -64 region of the c-Jun promoter in a manner similar to that observed in our previous study of the 4XTRE reporter (57).

TGF-β induction and induced complex binding are lost in Smad3-deficient MEFs. The recent creation of Smad3-deficient mice (6) has introduced a powerful new tool for studying the functional importance of Smad3 in isolation. We first compared induction by TGF-β of endogenous c-Jun in primary MEFs established from Smad3<sup>+/+</sup> and Smad3<sup>-/-</sup> mice. Primary MEFs were serum starved for 12 h and treated with TGF-β for 4 h, and then nuclear lysates were prepared. As shown by Western blot analysis, induction by TGF-β of total c-Jun protein levels is lost in Smad3<sup>-/-</sup> MEFs whereas that in Smad3<sup>+/+</sup> MEFs is intact (Fig. 5A).

We next investigated the ability of exogenous Smad3 expression to rescue c-Jun reporter induction in Smad3 null fibroblasts. Smad3<sup>+/-</sup> and Smad3<sup>-/-</sup> MEFs were transfected with -79 to +170 reporter with empty vector or with a Smad3 expression vector. Note that Smad3<sup>+/-</sup> MEFs express Smad3 and that they activate representative responses to TGF- $\beta$  to an extent similar to Smad3<sup>+/+</sup> MEFs (6). The c-Jun promoter was induced approximately threefold by TGF- $\beta$  treatment in Smad3<sup>+/-</sup> fibroblasts (Fig. 5B), which is comparable to the fold induction of other TGF- $\beta$ -responsive promoters examined in these cells (6). However, in Smad3<sup>-/-</sup> MEFs, TGF- $\beta$  failed to induce reporter activity (Fig. 5B). Although the uninduced overall activity is lower in the null cells, the full threefold induction by TGF- $\beta$  was restored upon cotransfection with Smad3. This establishes the absence of Smad3 as the defect



FIG. 3. The Smad3-Smad4 binding site in the human c-Jun promoter is identified as a CAGA triplet located 3' of the TATA box. (A) Schematic diagram of the -79 to +170 region of the c-Jun promoter. Four oligonucleotide sequences, named A through D, were designed to span the -18 to +170 region of the promoter. An additional oligonucleotide bearing a mutation in a CAGA triplet from +62 to +73 (BMUT) is also diagrammed (see text for additional discussion). The mutation changed the sequence from GACAGACAGACA to AGGAGCTTGCAA. (B) EMSA was performed by using the same -18 to +170 probe and HaCaT lysates as described for Fig. 2. A 100-fold molar excess of unlabeled oligonucleotides was incubated with the nuclear lysates before addition of radiolabeled probe, in order to compete with binding. The induced Smad3-Smad4 binding complex is indicated with an arrow. (C) EMSA was performed by using nuclear lysates from untreated mink lung cells treated with TGF- $\beta$ 1 for 1 h and the same -18 to +170 probe was either the wild-type sequence from -18 to +170 or the mutated sequence from +62 to +73 (the CAGA triplet).

responsible for loss of c-Jun promoter activation in these cells, and this result demonstrates that Smad3 is absolutely and specifically required for c-Jun promoter regulation by TGF- $\beta$ .

Finally, we looked at DNA binding to the Smad3-Smad4 site in the absence of Smad3, to determine whether Smad3 was indeed required for binding of the TGF-\beta-induced complex. An EMSA was performed by using the wild-type -18 to +170probe containing the Smad3-Smad4 binding site (see Fig. 2A). Induced complex binding was observed in Smad3+/ fibroblasts, but no induced complex was seen in Smad3-/- fibroblasts (Fig. 5C). This suggests that Smad3 is not only present in but also critical to the formation of the DNA binding complex which is induced upon TGF-B treatment. The correlation between loss of the induced complex and loss of endogenous c-Jun induction and c-Jun reporter activation further supports the importance of the induced Smad3-Smad4 binding complex to TGF-B regulation of c-Jun transcription, as well as firmly establishing the requirement for Smad3 in this process.

Mutation of an AP-1/CRE site can independently abrogate TGF- $\beta$  responsiveness of the c-Jun promoter. The AP-1/CRE site at -71 to -64 has previously been shown to be important for induction of c-Jun by other signals (1, 15, 34, 53). A consensus AP-1 site was also shown to be not only necessary but also sufficient for TGF- $\beta$  and Smad responsiveness in the context of a multimerized TRE reporter (57), and mutation of the

Smad3-Smad4 binding site in the c-Jun promoter eliminated nearly all but not all TGF-B responsiveness (Fig. 4). While a recent study by Dennler et al. (9) established the importance of three Smad3-Smad4 binding sites in TGF-β regulation of the PAI-1 promoter, it did not address whether the AP-1-like sites present in the promoter (21) may also be important for TGF- $\beta$ regulation in that context. In order to investigate the importance of the AP-1/CRE site in induction by TGF-β of this c-Jun promoter region, we used PCR mutagenesis to mutate this site in the -79 to +170 reporter. Mutating the AP-1/CRE site abrogated all transcriptional induction of the reporter by TGF- $\beta$  (Fig. 6A), despite the fact that the Smad3-Smad4 site identified as described above (Fig. 4) remained intact. This suggests that a synergistic functional cooperation exists between Smads and AP-1/CRE complexes in the context of TGFβ-induced transcriptional activation of this c-Jun promoter region.

We next sought to identify which proteins bind to this AP-1/CRE site in our system. We performed an EMSA using the -79 to -19 probe diagrammed in Fig. 2, where we had observed that there was no change in the pattern of binding to this sequence upon TGF- $\beta$  addition. Since previous studies (15) had identified c-Jun and ATF-2 as the components constitutively bound to this site, we attempted to supershift the bound complexes with antibodies against these two transcrip-



FIG. 4. Mutation of the Smad3-Smad4 binding site abrogates responsiveness to TGF- $\beta$ . (A) Diagram of new reporter constructs created by PCR mutagenesis. Two reporters for the region from -79 to +94 of the c-Jun promoter were created, i.e., one with wild-type sequence and the other mutated at the Smad3-Smad4 binding site (CAGA triplet) from +62 to +73. (B) The -79 to +94 wild-type and -79 to +94 mutant reporters were transfected into HaCaT cells. Cells were treated with TGF- $\beta$ 1 for 24 h before harvesting for luciferase assays. Fold inductions were calculated by comparing the luciferase activities of TGF- $\beta$ -treated cells and untreated control cells. (C) The procedures used were the

same as described for panel B except that Mv1Lu cells were used instead of

HaCaT cells.

tion factors. An antibody specific to c-Jun caused a supershift of the slower-migrating complex, confirming the presence of c-Jun (Fig. 6B). However, we did not see a supershift on this probe when we used two commercially available antibodies specific to ATF-2 (Fig. 6B), which had been successfully used to supershift ATF-2-containing complexes in a previous study (11). Additionally, several commercial antibodies against CREB were unable to supershift this complex (data not shown). Nonetheless, we were able to compete away binding of the faster- and slower-migrating complexes using unlabeled consensus CRE site oligonucleotide in 200× molar excess, whereas the same molar excess of unlabeled mutant CRE oligonucleotides did not compete with the binding. This suggests that a component of the bound complexes is a CRE binding protein. These results demonstrate that a constitutively bound complex containing c-Jun, either as a homodimer or in combination with a yet unknown CRE binding partner, is re-



-18/+170 Probe

FIG. 5. Induction of c-Jun by TGF-β is lost in Smad3 null fibroblasts. (A) Western blotting was performed by using nuclear lysates from Smad3<sup>+/+</sup> or Smad3<sup>-/-</sup> primary MEFs treated with TGF-β1 for 0, 1, 2, or 4 h. MEFs were serum starved for 12 h in 0.2% serum before treatment. (B) The -79 to +170 reporter was transfected into Smad3<sup>+/-</sup> or Smad3<sup>-/-</sup> MEFs with empty expression vector (mock) or Smad3 expression vector (Smad3). Cells were treated with TGF-β1 for 24 h before harvesting for luciferase assays. Fold induction by TGF-β1 is indicated over the bars. (C) EMSA was performed by using the -18 to +170 probe and nuclear lysates from untreated Smad3<sup>-/-</sup> MEFs or Smad3<sup>-/-</sup> MEFs or cells of the same types treated with TGF-β1 for 1 h. The induced Smad3-/- Smad4 complex is indicated with an arrow.

quired in conjunction with the Smad complex in mediating the TGF- $\beta$  activation of this promoter region.

#### DISCUSSION

We identify here a novel Smad3-Smad4 binding site in the 5' untranslated region (UTR) of the c-Jun promoter and introduce evidence for the simultaneous requirement for two different responsive elements in mediating TGF- $\beta$ -induced c-Jun transcription. The first is a Smad3-Smad4 binding site, and the second is a spatially distinct AP-1/CRE binding site. The two elements are capable of binding their corresponding transcrip-



FIG. 6. An AP-1/CRE site is also required for TGF- $\beta$  and Smad responsiveness of the c-Jun promoter. (A) A -79 to +170 luciferase reporter carrying a mutation in the AP-1/CRE site induced by PCR mutagenesis was transfected into HaCaT cells alongside the wild type -79 to +170 reporter. The mutation changed the sequence from TGACATCA to ATCCACCA. Fold induction was calculated by comparing TGF- $\beta$ -treated cells to untreated control cells. Cells were treated with TGF- $\beta$ 1 for 24 h before harvesting for luciferase assays. (B) EMSA was performed as described in the legend for Fig. 2B. Attempts to perform supershifts were made using a monoclonal antibody against c-Jun ( $\alpha$ -CJun) and polyclonal [ $\alpha$ -ATF2(P)] and monoclonal [ $\alpha$ -ATF2(M)] antibodies against ATF-2 (third through eighth lanes). Competition with 200-fold molar excess of wild-type CRE consensus site oligonucleotide (200× CRE) or 200-fold molar excess of mutated CRE site oligonucleotide (200× Mut CRE) is illustrated in the last four lanes.

tion factor complexes independently. Importantly, mutation of either element alone severely diminishes TGF- $\beta$  responsiveness, suggesting that the two elements have a functionally synergistic relationship. This notion is supported by the fact that, in an additive system, mutation of either element would result in only a partial loss of TGF- $\beta$  response; a complete loss of TGF- $\beta$  response would require the simultaneous mutation of all contributing elements because each element could function alone to mediate a partial response. On the other hand, in a synergistic relationship such as the one we have identified, neither element is capable of mediating a vigorous transcriptional response in the absence of the other, so the effect of the two elements acting together is greater than the sum of the effects of each element alone.

These findings introduce important nuances into the developing model of Smad-mediated transcriptional regulation and offer an illustration to support aspects of Smad function predicted by biochemical and structural observations. They suggest that synergy between Smads and other transcription factors could be an important mechanism for mediating both the specificity and the responsiveness to cross talk of the TGF- $\beta$  transcriptional activation signal.

Sequence comparison of Smad3-Smad4 binding sites. Numerous studies have identified Smad3-Smad4 DNA binding sites using various approaches. As seen in Table 1, the sequences found by various groups are essentially identical; regardless of whether one defines a Smad3-Smad4 binding site as the palindrome AGACGTCT, as the CAGA box, or as repeats of GACA, all of the identified sites contain the Smad box, 5'-GTCT-3', or its reverse complement, 5'-AGAC-3' (9, 57, 58). Most recently, another Smad3-Smad4-responsive site, CAGACAGtCTGTCTG in the *junB* promoter, was identified (18). Only the COL7A1 promoter presents a discrepancy, in that the deletions which abrogate Smad binding do not directly

Reference	Location	Sequence	
This study	c-Jun, +62 to +73	CAGACAGACAGACACA	
9	PAI-1 -730 -580 -280	AGCCAGACA AGACAGACA AGACAGACA	
58	Oligo screen	GTCTAGAC	
57	2X TRE (from p3TP-lux)	TGAGTCAGACA (21 bp) TGAGTCAGACA	

 TABLE 1. Comparison of Smad3-Smad4 binding sites

disturb the Smad box-like sequences (54). It may be, as the authors suggest, that the small deletions at the ends of their binding element disrupted binding in a non-sequence-specific manner.

In agreement with these other studies, the novel Smad3-Smad4 binding site identified in the c-Jun 5' UTR consists of three Smad boxes in a row. Although it is unusual to find enhancing elements in the 5' UTR, it is not unprecedented. Transcriptional activators with binding sites in the 5' UTR of promoters or in intronic sequences are hypothesized to function transiently, i.e., during the establishment of the initiation of transcription (5, 32, 44). The molecular mechanism for Smad-mediated activation of transcription is not yet well defined, but a transient role of Smads in transcriptional initiation, through their binding to the sequence in the 5' UTR of the c-Jun gene, would be consistent with the transient presence of Smads in the nucleus after TGF- $\beta$  stimulation.

The Smad consensus binding site, or Smad box, has now been confirmed by the elucidation of the crystal structure of Smad3 bound to DNA (46). A single Smad3 MH1 binds asymmetrically through a novel DNA binding  $\beta$ -hairpin structure to a 4-bp Smad box (CAGA) with sequence-specific interactions (9, 58). Note that in vivo Smads exist as homo- and heterooligomers (20, 25, 46, 56, 61), which would explain why more than one 4-bp repeat has been found to be required for binding of natural Smad complexes in the studies discussed above.

Synergy between Smads and AP-1 family members. Our results further demonstrate that while the Smad3-Smad4 site is important for TGF- $\beta$  induction of c-Jun, an AP-1/CRE site is also required for TGF- $\beta$  regulation of the c-Jun promoter. Mutation of either site in the context of the -79 to +170 region of this promoter eliminated the ability of TGF- $\beta$  to elicit maximal induction of the c-Jun promoter. There are several possible mechanisms by which such synergy may be achieved, and elucidating the mechanism for this synergistic cooperation is an important area for future investigations.

The first possibility is that direct physical interaction between Smads and AP-1 family members is responsible for mediating the functional cooperation. Recent studies have described an interaction between Jun family members and Smad3 (27, 60). In fact, Smad3 and Smad4 have both been found to interact with all members of the Jun family to varying degrees. The Jun family members interact with Smads at a small Cterminal domain which is highly conserved among Jun proteins. While the interaction between Jun and Smads is direct, the involvement of this protein-protein interaction in transcriptional activation of the c-Jun promoter is unclear. Although it is a strong possibility, direct protein interaction is certainly not the only possible explanation for the observed functional cooperation seen in the c-Jun promoter between Smads and AP-1. Another possible mechanism for functional synergy is cooperative DNA binding. We do not know whether AP-1/CRE complexes and Smad3-Smad4 complexes may cooperatively bind their corresponding sites in vivo, even though they clearly can strongly bind their corresponding c-Jun promoter sites independently in vitro. It is possible that the interactions of each complex with DNA in vivo may be enhanced by cooperative recruitment and stabilization or by an alteration in local DNA structure which is fostered by the binding of both complexes at once.

Synergy is a functional cooperation that can also be independent of any physical interaction. It is possible that Smads and AP-1 may cooperate by contributing complementary but necessary subfunctions of transcriptional activation, for instance by recruiting different required members of the basal transcriptional machinery. The location of the Smad binding site 3' in relation to the TATA box in the c-Jun promoter strongly suggests that the role of Smads is transient and limited to the start of transcription, perhaps involving the establishment of the transcription initiation complex. AP-1 may contribute complementary functions to promote transcription.

Finally, it is possible that TGF- $\beta$  signal transduction can directly affect the activity of AP-1 complex bound to the promoter element. It has been postulated that TGF- $\beta$  may signal through the mitogen-activated protein kinase pathway and activate AP-1 through phosphorylation. This remains to be clearly shown and is currently under investigation. Such an activity would add yet another dimension to the cooperativity in the c-Jun promoter region demonstrated here.

Further implications. The model of required synergistic cooperation may explain some discrepancies in our understanding of Smad function to date. A number of recent studies have established the abilities of Smad3 and Smad4 to interact and function synergistically with the transcriptional coactivator CREB binding protein/p300 (10, 17, 40, 45, 52). Although these findings suggest that a DNA-bound Smad3-Smad4 complex is able to independently recruit CREB binding protein/ p300 and hence possibly initiate transcription on its own, it does not appear to do so. A close examination of studies on Smad3-Smad4 binding sites reveals that no single Smad3-Smad4 site has been found to be sufficient for TGF-B responsiveness. In all of these studies, multiple copies of the Smad binding site were found to be required to confer TGF-B responsiveness (9, 58). Perhaps a single Smad3-Smad4 complex is unable to successfully recruit the factors necessary to accomplish transcriptional activation on its own. Cooperation with another transcription factor, such as AP-1, Sp1 (33), or TFE3 (16), or collaboration between a number of Smad3-Smad4 binding sites is required to build strong enough interactions to activate transcription.

It is worth noting that while we have examined responsive

elements and Smad binding in the -79 to +170 region of the c-Jun promoter, there may be additional Smad3-Smad4 binding sites, or other TGF-B responsive elements, elsewhere in the native c-Jun promoter sequence. These could in fact cooperate further with the Smad3-Smad4 binding site identified in this study to mediate c-Jun regulation in vivo.

Finally, an investigation into other examples of cooperating responsive elements could yield critical insight into TGF-B signaling specificity and cross talk with other signaling pathways. Given the description in the present study of a joint requirement for Smad3-Smad4 binding and an AP-1/CRE site, it may be interesting to look for additional required elements in other TGF-B-responsive promoters. The recent work by Hua et al. (16) revealed another important example of such cooperativity and lends further support to the possibility that similar modes of synergistic transcriptional activation may exist in the context of many Smad-responsive promoters.

We have identified in these studies a functional cooperation between a novel Smad3-Smad4 site and an AP-1/CRE binding site within the -79 to +170 region of the c-Jun promoter, which functions in transcriptional activation by TGF-B. These findings not only solidify the role of Smad3 as an intracellular effector for the TGF-B signal but also support a new and more complex model of Smad3-Smad4 transcriptional regulation, i.e., one which involves cooperation with neighboring response elements and may allow coordination of other interacting pathways with the TGF-B signal. The synergistic interaction between TGF-β-specific effectors and other transcription factors proposed in this model could mediate the activation of different subsets of target genes in different cell types and physiological states, translating into the diversity of physiological and pathological roles played by TGF- $\beta$  in different tissue types, stages of development, and disease states.

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C.W. and E.M.R.-C. contributed equally to this work.

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# Smads bind directly to the Jun family of AP-1 transcription factors

(Smad3/Smad4/cJun/JunB/transforming growth factor)

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Smad3 and Smad4 are sequence-specific ABSTRACT DNA-binding factors that bind to their consensus DNAbinding sites in response to transforming growth factor  $\beta$  $(TGF\beta)$  and activate transcription. Recent evidence implicates Smad3 and Smad4 in the transcriptional activation of consensus AP-1 DNA-binding sites that do not interact with Smads directly. Here, we report that Smad3 and Smad4 can physically interact with AP-1 family members. In vitro binding studies demonstrate that both Smad3 and Smad4 bind all three Jun family members: JunB, cJun, and JunD. The Smad interacting region of JunB maps to a C-terminal 20-amino acid sequence that is partially conserved in cJun and JunD. We show that Smad3 and Smad4 also associate with an endogenous form of cJun that is rapidly phosphorylated in response to TGFB. Providing evidence for the importance of this interaction between Smad and Jun proteins, we demonstrate that Smad3 is required for the activation of concatamerized AP-1 sites in a reporter construct that has previously been characterized as unable to bind Smad proteins directly. Together, these data suggest that TGF<sub>β</sub>-mediated transcriptional activation through AP-1 sites may involve a regulated interaction between Smads and AP-1 transcription factors.

Transforming growth factor  $\beta$  (TGF $\beta$ ) is a multipotent cytokine that regulates a variety of cellular activities, such as cell proliferation, differentiation, and extracellular matrix (ECM) formation. The combined actions of these cellular responses are likely to mediate more global effects of TGF $\beta$  including its role in development, wound healing, immune responses, and the pathogenesis of cancer (1-3). The identification of genes transcriptionally regulated by TGF $\beta$  and the elucidation of the molecular mechanisms responsible for this transcriptional regulation will help define how TGF $\beta$  exerts its cellular effects and its role in resulting physiological processes. Although progress has been made in the identification of TGF $\beta$  target genes, including the cyclin-dependent kinase inhibitors p21 and p15 (1, 2) and the ECM component plasminogen activator inhibitor-1 (PAI-1) (3), which has subsequently contributed toward our understanding of TGF<sub>B</sub>-mediated growth inhibition and ECM deposition, the mechanisms by which  $TGF\beta$ controls gene expression remain largely unknown.

Numerous studies have characterized the differential expression of specific genes in response to TGF $\beta$ , revealing a common link in the ability of TGF $\beta$  to regulate many of these genes through the functions of the AP-1 family of transcription factors. This protein family, which includes the Fos and Jun proteins, binds a specific DNA sequence and facilitates transcriptional regulation (4). The ability of TGF $\beta$  to induce the expression of several genes, including PAI-1, clusterin, mono-

cyte chemoattractant protein-1 (JE/MCP-1), type I collagen, and TGF<sup>β1</sup> itself depends on specific AP-1 DNA-binding sites in the promoter regions of these genes (3, 5-10). Furthermore, TGF $\beta$ -mediated transcriptional activation of several of these genes requires AP-1 proteins (5, 8-10). Intriguingly, the expression of many AP-1 proteins themselves is induced as an early response to TGF $\beta$  in a cell type-specific manner (11–14). It has been demonstrated that this induced expression of particular AP-1 family members is involved in TGFB-mediated regulation of subsequent target genes (10). In addition, genetic studies of TGF $\beta$  signaling in *Drosophila melanogaster* reveal a direct overlap between AP-1 and TGF $\beta$  signaling and suggest an evolutionarily conserved convergence of these pathways (15). Together, these studies demonstrate a link between TGF $\beta$  signaling and AP-1 in the TGF $\beta$ -regulated expression of various genes. The molecular mechanisms responsible for the TGF $\beta$ -mediated transcriptional activation of these genes are just beginning to be elucidated.

Insight into the mechanism of TGF $\beta$ -regulated gene expression has come about with the discovery of the Smad family of proteins. The Smads are phosphorylated by the activated type I receptor in response to ligand (16). Specifically, Smad2 and Smad3 were shown to be inducibly phosphorylated in response to TGF $\beta$  (17–19). Smad phosphorylation results in heteromerization of either Smad2 or Smad3 with Smad4 (20–23). Smad4-containing heteromers then enter the nucleus where they can activate transcription of specific genes (24, 25). Current research is focused on elucidating the role of Smads in TGF $\beta$ -induced transcriptional activation.

Through attempts made at understanding the mechanism of Smad-mediated transcriptional activation, two distinct roles for Smads have emerged: Smads as DNA-binding factors and Smads as transcription factor-binding proteins. Several lines of evidence suggest that Smads activate transcription by binding directly to DNA. For instance, transcription of a reporter plasmid containing the concatamerized consensus Smadbinding site is induced by TGF $\beta$  in a Smad4-dependent manner (26). Smad3 and Smad4 were recently shown to form a complex on similar DNA sequences derived from the PAI-1 promoter (27). Mutation of these sequences in the PAI-1 promoter reduced TGF $\beta$  responsiveness. Furthermore, Gal4 fusions with the C-terminal domains of Smad1 and Smad4 activate transcription from concatamerized Gal4 DNAbinding sites (28).

Other evidence suggests that Smads can activate transcription by binding to other transcription factors. For example, the interaction between Smad2/Smad4 heteromers and the transcription factor FAST-1 is critical for the formation of the

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Abbreviations: TGF $\beta$ , transforming growth factor  $\beta$ ; HaCaT, human keratinocyte cells; JNK, cJun N-terminal kinase; CMV, cytomegalovirus; GST, glutathione S-transferase; TNT, transcription and translation.

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activin responsive factor (ARF), an activin-inducible DNAbinding complex in *Xenopus* (24, 29). Overexpression of the Smad-binding domain of FAST-1 blocked ARF formation and transcriptional induction of an activin-inducible early response gene. Together, these data indicate that although Smads bind DNA directly, association with other transcription factors may play a crucial role in Smad-mediated transcriptional activation.

In an attempt to identify transcription factors involved in Smad-mediated transcriptional activation, we performed a yeast two-hybrid screen using Smad3 as a bait. Two interacting cDNAs encoding two different clones of the AP-1 family member, JunB, were isolated, indicating that Smads may bind to AP-1 members directly. Supporting a direct interaction between Smads and AP-1, we show that Smad3 and Smad4 bind all known members of the Jun family of proteins *in vitro*. Furthermore, we demonstrate that Smad3 is critical for the ability of TGF $\beta$  to activate AP-1 sites independent of Smad DNA binding. These data, therefore, provide insight into a possible mechanism by which TGF $\beta$  activates AP-1-mediated transcription through the induction of Smad/AP-1 complex formation.

#### **MATERIALS AND METHODS**

**Materials.** TGF $\beta$ 1 was a generous gift of Amgen Biologicals. Human keratinocyte cells (HaCaT) were the generous gift of P. Baukamp and N. Fusenig. A HaCaT cDNA library in the pACT2 expression vector was the generous gift of Y. Xiong. The full length cDNAs for murine Jun family members, FosB, cFos, Fra2, and human Fra1, were the generous gifts of R. Wisdom. Smad3 polyclonal antibody was generated against amino acids 200–219 of Smad3 and affinity purified in this laboratory. Antibodies used included JunB polyclonal N-17 (Santa Cruz Biotechnology), cJun monoclonal KM-1 (Santa Cruz Biotechnology), cJun polyclonal no. 9162 (NEB, Beverly, MA), cJun polyclonal no. 06–828 (Upstate Biotechnology, Lake Placid, NY) and JunD-329 polyclonal antibody (Santa Cruz Biotechnology).

**Cell Culture.** COS cells were maintained in DMEM with 10% FBS. HaCaT cells were maintained in MEM with 10% FBS. Primary fibroblasts were prepared from day 14 embryos by mechanical dissociation of whole embryos by passage through an 18-gauge needle and plating onto gelatin-coated 10-cm tissue culture plates in DMEM with the inclusion of 20% FBS. Cells were grown to confluence and carried in DMEM with 10% FBS. All experiments were performed on littermate fibroblasts at the same passage number.

**Plasmid Construction.** The *Bam*HI fragment containing full length human Smad3 cDNA was subcloned from pGEX-3X into pGBT9 (CLONTECH) (30). cDNAs encoding each Jun and Fos family member were subcloned into pCMV5 and pCMV6 expression vectors (CMV, cytomegalovirus). Full length JunB was PCR amplified with the following primers: CGGGATCCCGATGTGCACGAAAATGG (5' primer) and GGATCCTCAGAAGGCGTGTCC (3' primer). Full length cJun was PCR amplified with the following primers: CGG-GATCCCGATGACTGCAAAAGATGGAAACG (5' primer) and CGGGATCCCGTCAAAAGATGGAAACG (5' primer) and CGGGATCCCGTCAAAACGTTTGCAACTGC (3' primer). The cDNAs were completely sequenced and subcloned into pACT2 (CLONTECH). Construction of 4xSBSMT and 4xAP1MT reporter plasmids was previously described (30).

Yeast Two-Hybrid Assay. The yeast strain Hf7c was transformed with Smad3/pGBT9, and expression of the appropriate-size fusion protein was confirmed by Western blotting by using GAL4 DBD monoclonal antibody (Santa Cruz Biotechnology). Bait-expressing yeast were transformed with a HaCaT cDNA library in the pACT2 expression vector. Individual cDNAs ( $5 \times 10^6$ ) were screened. Transformants (484) grew on media lacking tryptophan, leucine, and histidine that contained 5 mM 3-aminotriazole. Transformants (242) were positive for  $\beta$ -galactosidase activity, which was measured by the appearance of blue color on colony filter lifts incubated in the presence of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactosidase (X-Gal). Bait dependence for each positive transformant was established similarly.

Binding Studies. Full length JunB in pGEM4 was digested with BspHI, BssHII, or DraI (NEB). The full length construct and the digested DNAs were used as templates for in vitro transcription and translation (TNT) with [<sup>35</sup>S]methionine in rabbit reticulocyte lysates (Promega). The TNT-JunB lysates were incubated with an equal amount of bacterially purified glutathione S-transferase (GST), or GST-Smad3 or GST-Smad4 (30) in B/P (150 mM NaCl/50 mM Tris, pH 7.5/0.1% Tween/1 mM DTT) for 2.5 hours at 4°C. The GST reactions were washed three times in TBS (500 mM NaCl/25 mM Tris, pH 7.5/0.1% Tween-20/1 mM DTT). Samples were resolved by SDS/PAGE. The gels were treated with 10% sodium salicylate, dried, and exposed to film. Whole-cell COS lysates overexpressing each AP-1 member were lysed as described (30) and incubated with the GST fusions as described above. The binding reactions were washed three times with B/P and separated by SDS/PAGE.

For endogenous protein interactions, HaCaT cells were treated with 100 pM TGF $\beta$ 1 in DMEM/10% FBS for 15, 30, or 60 min. Cells were then lysed and either whole-cell or nuclear extracts were prepared (30, 31). Four hundred fifty  $\mu g$ of each whole-cell lysate was incubated with an equal amount of bacterially purified GST-Smad3 or GST-Smad4 normalized for protein by Coomassie blue and for volume of glutathione-Sepharose added to each binding reaction. After 2 hr at 4°C, the reactions were washed three times and separated by SDS/PAGE. One hundred  $\mu g$  of each nuclear lysate was diluted to 150 mM NaCl with buffer A and incubated with GST, GST-Smad3, or GST-Smad4, as above. For phosphatase treatment, HaCaT cells were treated for 15 min with 100 pM TGFB1 or DMEM containing 10% FBS, penicillin, streptomycin, and 0.5 M sorbitol and whole-cell extracts were prepared as described (30). Briefly, the lysates were treated with potato acid phosphatase (0.034 units) and calf intestinal phosphatase (2 units) for 30 min at 37°C.

Western Blot Analysis. Electrophoresed proteins were transferred to Immobilon (Millipore) and treated as previously described, except that the blots were blocked and blotted in PBS/0.1% Tween-20/5% milk (30).

Luciferase Assays. Transfections were performed by using a standard DEAE-Dextran protocol (32). Primary fibroblasts were allowed to recover from glycerol shock for 20 hr before treating with 100 pM TGF $\beta$ 1 in DMEM/0.2% FBS. Luciferase assays were performed as previously described (33). All transfections were normalized to  $\beta$ -galactosidase activity by co-transfection of 0.5  $\mu$ g of CMV- $\beta$ -galactosidase expression vector.

#### RESULTS

**Smad3 and JunB** Associate in Yeast. To identify Smad3binding proteins, we performed a yeast two-hybrid screen using the Gal4 DNA-binding domain fused to Smad3 as a bait. Of the five million yeast transformants screened for Smad3 binding, 242 transformants were positive for growth in the absence of histidine and for the appearance of blue color on staining with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactosidase (X-Gal). Two of the clones sequenced contained two different cDNA fragments encoding the AP-1 member, JunB (Fig. 1). Clone 44 lacked the N-terminal 126 amino acids of JunB indicating that these residues are not required for binding in yeast. Fusions of the Gal4 activation domain with cDNAs encoding JunB and cJun also tested positive for interaction with the Smad3 bait protein (data not shown). 4846



FIG. 1. Isolation of JunB from a Gal4-Smad3 screen of a human keratinocyte library (HaCaT) in yeast. Schematic diagram of two Smad3-interacting clones encoding JunB. Amino acids associated with particular functional domains of JunB are shown.

In Vitro Binding of Smads and AP-1. To determine whether this interaction occurs in solution with recombinant proteins, GST pulldown experiments were performed. Bacterially produced GST-Smad3, but not GST alone, bound TNT-JunB (Fig. 2B). In addition, GST-Smad4-bound TNT-JunB, indicating that AP-1 binding is not exclusive to Smad3. Similar studies with GST-Smad1, GST-Smad2, and GST-Smad5 showed that these proteins also bind JunB TNT products but with lower affinity than that observed with GST-Smad3 and GST-Smad4 (data not shown). To map the Smad interaction domain, GST-Smad fusion proteins were used to pull down various TNT-JunB deletion products (Fig. 2A and B). Deletion of only 20 amino acids from the C terminus of JunB abrogated the interaction between GST-Smad3 and GST-Smad4 and JunB (Fig. 2). Thirteen of these 20 amino acids are conserved among Jun family members, including cJun and JunD (Fig. 3A). To



FIG. 2. In vitro association of Smad3 and Smad4 with JunB. (A) Schematic diagram of JunB deletion mutants used in the binding study shown in B. (B) In vitro association of JunB and JunB deletion mutant TNT products with GST, GST-Smad3, or GST-Smad4. Bacterially produced GST, GST-Smad3, and GST-Smad4 proteins were coupled to glutathione-Sepharose, incubated with the indicated TNT product, centrifuged, and the Sepharose-bound proteins visualized by Coomassic stained SDS/PAGE (data not shown). The amount of each GST protein and the volume of glutathione-Sepharose used in each binding reaction were normalized. Each reticulocyte lysate was produced as described in Materials and Methods. Domains depicted in A refer to the same domains depicted in Fig. 1. Five percent of each reticulocyte lysate input was run in lanes 1–4: lane 1, JunB full length; lane 2, 1–324; lane 3, 1–272; lane 4, 1–80.

determine whether these proteins also interact with Smads, we tested whether GST-Smad3 and GST-Smad4 could associate with AP-1 members from transfected COS cell lysates overexpressing each Jun member. As shown in Fig. 3B, all three Jun family members associated with both GST-Smad3 and GST-Smad4 to a similar extent. Furthermore, GST-Smad3 and GST-Smad4 bound full length TNT-cJun and deletion of 20 amino acids from the C terminus of TNT-cJun also reduced association with GST-Smad3 and GST-Smad4 (data not shown). Conversely, in studies using lysates from transfected cells overexpressing each Fos family member, no association with GST-Smad3 or GST-Smad4 was observed (data not shown). Consistent with these findings, the amino acids required for Jun binding to GST-Smads *in vitro* are not conserved among Fos family members.

Smad3 and Smad4 Associate with An Inducibly Phosphorylated Form of Endogenous cJun. To determine whether GST-Smad3 and GST-Smad4 can associate with endogenous cJun, we incubated GST-Smad3 and GST-Smad4 with TGFβtreated HaCaT whole-cell extracts. Western blot analysis of these binding reactions with a cJun-specific antibody raised against a phosphopeptide containing phosphorylated Ser-63 of cJun (KM-1 from Santa Cruz Biotechnology) showed that GST-Smad3 and GST-Smad4 bind this form of cJun (Fig. 4A). We noticed that the level of cJun recognized by the KM-1 antibody appeared to increase with  $TGF\beta$  treatment. To more clearly determine whether the level of cJun was induced by TGF $\beta$ , nuclear lysates were analyzed by using the KM-1 antibody and a different cJun antibody raised against a nonphosphorylated N-terminal portion of cJun (no. 9162, NEB). As shown in Fig. 4B, the form of cJun recognized by the KM-1 antibody is clearly induced by TGF $\beta$  treatment, whereas the levels of total cJun recognized by the NEB antibody remain relatively unchanged. This result suggests that cJun is rapidly phosphorylated, at least on the residue of Ser-63, in response to TGF $\beta$ . Subsequent binding studies with these nuclear lysates and GST. GST-Smad3, and GST-Smad4 revealed that GST-Smad3 and GST-Smad4 bound equally well to cJun recognized by either antibody (data not shown). To confirm that the TGF $\beta$ -induced increase in the cJun species detected by the KM-1 antibody was indeed the phosphorylated form of cJun, we performed a phosphatase assay using HaCaT lysates treated with TGF $\beta$  (Fig. 4C). As a positive control, HaCaT cells were treated with 0.5M sorbitol, which has been shown to induce cJun N-terminal kinase (JNK) kinase activity (34), and similar results were obtained with lysates treated with 50  $\mu$ g/ml anisomycin. Under phosphatase treatment conditions, the TGF<sub>β</sub>-induced form of cJun recognized by the KM-1 antibody was lost, whereas total cJun detected by the no. 9162 antibody did not change. The specificity of the KM-1 antibody for the phosphorylated form of cJun was further confirmed by using another antibody (no. 06-828, Upstate Biotechnology) raised against a cJun peptide also containing phosphorylated Ser-63.

Smad3 Is Required for TGF<sub>B</sub>-Mediated Activation of AP-1 Sites Independent of Smad DNA-Binding Activity. In an attempt to determine whether the interaction between AP-1 and Smads contributes to the ability of TGF $\beta$  to activate the transcription of AP-1 sites, we transfected Smad3 heterozygous and Smad3 homozygous null primary mouse embryonic fibroblasts with AP-1 site-containing reporter constructs previously described (Fig. 5) (30). Intriguingly, 4xSBSMT, which contains consensus AP-1 sites adjacent to mutated Smad DNA-binding sites incapable of Smad protein binding, requires Smad3 for transcriptional activation by TGF $\beta$ . TGF $\beta$  is unable to activate transcription, however, in the analogous reporter, 4xAP1MT, in which the Smad-binding sites are intact and the AP-1 sites are mutated. Thus, within the context of the 4xSBSMT reporter, the AP-1 sites are required for transcriptional activation by TGF $\beta$ . Activation of these AP-1 sites



B



FIG. 3. Association of Smad3 and Smad4 with Jun family members. (*A*) The Smad-binding site on JunB is conserved among Jun family members. The very C-terminal amino acids of the Jun proteins are aligned. The leucine at the beginning of the JunB and cJun sequences is the most C-terminal leucine of the leucine zipper domain. Amino acids that were deleted in the JunB 1–324 mutant are underlined. Conserved amino acids that may play a role in Smad binding are shaded. (*B*) *In vitro* association of overexpressed JunB, cJun, and JunD from COS cell lysates with GST, GST-Smad3, or GST-Smad4. COS cells transfected with JunB/pCMV, cJun/pCMV, or JunD/pCMV were lysed, and these extracts were treated with GST fusion proteins prepared as described in *Materials and Methods*.

requires Smad3 but is independent of Smad DNA binding, suggesting that the ability of Smad3 to act through the AP-1 sites in this reporter is required for transcriptional activation by TGF $\beta$ .

#### DISCUSSION

Over the past year, a model for the functional role of Smads in TGF $\beta$ -mediated transcriptional regulation has emerged. Here, we provide evidence supporting a role for Smads as transcriptional coactivators, in addition to their role as DNA binding-dependent activators of transcription. Smads may thus transduce the TGF $\beta$  signal to the promoter level and activate transcription through direct physical interaction with DNA-bound AP-1 proteins.

The potential role of Smads as transcriptional coactivators of AP-1 is supported by a previous study in which we reported that TGF $\beta$  as well as Smad3/Smad4 cooverexpression could activate transcription of 4xWT, a luciferase reporter containing a concatamerized TGF $\beta$ -responsive element derived from



FIG. 4. GST-Smad3 and GST-Smad4 associate with an inducibly phosphorylated form of endogenous cJun. (A) HaCaT cells were treated with 100 pM TGF $\beta$ 1 for 15, 30, or 60 min and lysed. GST-Smad3 and GST-Smad4 were incubated with 450  $\mu$ g of whole-cell HaCaT lysates, and the binding reactions were analyzed by Western blot by using a cJun specific monoclonal antibody raised against a phosphopeptide containing phosphorylated Ser-63 of cJun, KM-1 from Santa Cruz. The lower row shows 60  $\mu$ g total lysate blotted with the same antibody as the upper rows. (B) Nuclear lysates (60  $\mu$ g) from HaCaT cells treated as in A were analyzed with the KM-1 antibody. This blot was stripped and reprobed with antibody no. 9162 from NEB raised against a fusion protein containing the unphosphorylated N-terminal portion of cJun. (C) HaCaT cells were treated with 100 pM TGF $\beta$ 1 or 0.5 M sorbitol for 15 min, lysed in the absence or presence of phosphatase inhibitors, and treated with or without potato acid phosphatase and calf intestinal phosphatase. The reactions were analyzed by SDS/PAGE and Western blotting. The upper row shows 60  $\mu$ g of each lysate blotted with the KM-1 antibody. This blot was stripped again and reprobed with no. 06–828 antibody (Upstate Biotechnology). The bands in the KM-1 blot align with the upper bands in the no. 9162 antibody and the no. 06–828 antibody blots.

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FIG. 5. Smad3 is required for TGF $\beta$ -mediated transcriptional activation of 4xSBSMT. Smad3 homozygous null or Smad3 heterozygous mouse primary fibroblasts were transfected with 3  $\mu$ g of 3TP-Lux, 4xSBSMT, or 4xAP1MT reporter constructs. Twenty hours after transfection, vehicle or 100 pM TGF $\beta$ 1 was added. Twenty-four hours after treatment, luciferase activity was quantified. The averages of duplicate transfections were used to determine the fold induction by TGF $\beta$  over the vehicle control. The 3TP-Lux data is shown as a positive control for the experiment (35).

3TP-Lux that contains both consensus AP-1 binding sites and Smad4-binding sites (30). Interestingly, a mutation in this sequence that inhibited Smad association had no effect on the ability of TGF $\beta$  or Smad3/Smad4 cooverexpression to induce transcription of this reporter. In contrast, a mutation in the adjacent AP-1 site that inhibited the association of a constitutively bound, AP-1-containing complex, abrogated the ability of TGF $\beta$  or Smad overexpression to activate transcription of the 4xWT reporter. These data suggest that within the context of this reporter, TGF\beta-mediated transcriptional activation does not depend on the DNA- binding function of Smads but rather the ability of Smads to act through the AP-1 sites. In light of the interaction studies described here, Smads may act through these AP-1 sites by binding directly to AP-1 proteins. In studies of Smad3 wild-type and homozygous null fibroblasts, it was demonstrated that  $TGF\beta$ -mediated transcriptional activation of 3TP-Lux (35) and the cJun promoter (14), which contain both Smad and AP-1 DNA-binding sites, was lost in fibroblasts lacking Smad3. Transfection of Smad3 into the null fibroblasts rescued transcriptional activation by TGFB. Here, we further show that activation of the AP-1 sites in a reporter containing mutant Smad-binding sites similarly requires Smad3. Taken together, the interaction and transcriptional activation data suggest that the regulated association between Smads and AP-1 may be necessary for the TGFβmediated transcriptional activation of AP-1 sites. Consistent with these findings, recently published data demonstrate that cotransfection of Smad3 and Smad4 and cJun facilitates mild synergistic transcriptional activation of AP-1-site containing reporters (36).

We demonstrate that Smad3 and Smad4 bind JunB directly in vitro, and that the interaction involves a stretch of 20 C-terminal amino acids of JunB. It is possible that deletion of these 20 amino acids that abrogated Smad binding may alter the conformation of JunB, rendering it incapable of binding Smads. However, we do show that both cJun and JunD, which contain conserved identity in 13 of these 20 C-terminal amino acids, also bind GST-Smad3 and GST-Smad4, whereas Fos family members that lack this conserved amino acid sequence do not bind GST-Smad3 or GST-Smad4. Therefore, we postulate that Smad3 and Smad4 binding requires specific residues within the C-terminal 20 amino acids of JunB. Furthermore, direct association with Smads may be a conserved function of the Jun family of transcription factors.

Because Smads translocate to the nucleus in response to TGF $\beta$  (20–23), the interaction between Smads and Jun family members may be regulated largely by TGFB-induced alteration of Smad subcellular localization. Intriguingly, we found that endogenous cJun was rapidly phosphorylated in response to TGF $\beta$ , most likely at Ser-63. Phosphorylation at this site peaks by 15 min and is maintained over the course of an hour of TGF $\beta$  treatment, a time course that overlaps TGF $\beta$ -induced Smad entry into the nucleus (17, 21, 22, 25). This inducibly phosphorylated form of cJun binds both GST-Smad3 and GST-Smad4. Although this phosphorylation does not appear to alter the association of Smads and AP-1 in vitro, we suspect this phosphorylation may contribute to TGFβ-mediated transcription in vivo. This notion is supported by evidence showing that JNK is activated in response to TGF $\beta$  (37, 38), and that this kinase is known to phosphorylate cJun at Ser-63 and -73, thereby enhancing the ability of cJun to activate transcription (39). These data, in combination with previously discussed results, suggest that TGF $\beta$  treatment may initiate two simultaneous signaling pathways that converge on AP-1 complexes in the nucleus: a Smad-mediated pathway and a JNK-mediated pathway. The combined result of these pathways may be stronger interactions between Smads and AP-1 and as a result, a more robust induction of transcription. Although cJun phosphorylation may indeed enhance association with Smads under physiological conditions, we do not have evidence to suggest that Jun phosphorylation is required for the Smad/ AP-1 interaction, since we observe the interaction under in vitro conditions in which JNK-mediated phosphorylation would not occur and also detect a constitutive interaction of GST-Smads with endogenous cJun in the absence of  $TGF\beta$ treatment. Furthermore, JunB, which is not a JNK substrate, also binds to Smads (40). Future work is necessary to define the role of Jun phosphorylation in TGF<sub>β</sub>-mediated transcription in vivo.

The data presented here may provide a plausible explanation for the specificity of TGF $\beta$ -mediated induction of specific responsive promoters that contain AP-1 DNA-binding sites. On TGF $\beta$  treatment, Smad3 and Smad4 heteromerize and enter the nucleus, where they can associate with TGFβresponsive promoters by binding a discreet DNA sequence and/or AP-1 members bound to AP-1 sites on the same promoter. Thus, Smads in response to TGF $\beta$  act as the signaling intermediates to initiate transcription from specific promoters by recruiting required factors to form an active transcriptional complex. The transcriptional adapter molecule p300/CBP, which binds directly to AP-1 and serves as a coactivator of AP-1-mediated transcription, has recently been shown to associate directly with Smads in response to  $TGF\beta$ (41–44). TGF $\beta$ -induced Jun modification may promote the stability of these interactions, thereby facilitating complex formation. Given that AP-1 complex composition depends on differential expression of specific family members, the distinct constitution of AP-1 complexes in different cell types may contribute to promoter targeting specificity by TGF $\beta$ . This possibility is supported by the observation that under conditions where Smads bind Jun proteins, Smads are unable to associate with Fos family members. Thus, it is possible that AP-1 complexes containing Fos members may have a lower affinity for Smads than AP-1 complexes containing Jun-Jun dimers, and that promoters associated with these specific AP-1 complexes would be favored for Smad binding and TGFβmediated activation. The induced interaction of Smads with particular AP-1 complexes in vivo may determine the ability of

TGFB to initiate transcription from specific AP-1 sitecontaining promoters.

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