

Smad7 but not Smad6 Cooperates with Oncogenic *ras* to Cause Malignant Conversion in a Mouse Model for Squamous Cell Carcinoma

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ABSTRACT

Smad7 and Smad6 are inhibitory Smads that block transforming growth factor- β (TGF- β) superfamily signal transduction. Smad7 is overexpressed in chemically induced mouse epidermal tumors, where oncogenic activation of *c-ras* is a frequent event. To test the role of Smad7 overexpression in tumor progression, we used retroviruses to transduce Smad7 or Smad6 and *v-ras*^{H_a} into primary mouse keratinocytes. By itself, Smad7 transiently enhanced keratinocyte proliferation, blocked normal differentiation, and induced keratin 8, a marker of malignant conversion, but did not cause tumor formation. Smad7 extended the *in vitro* life span, suppressed senescence, and increased transformation frequency 3-fold of primary keratinocytes coexpressing *v-ras*^{H_a}. Smad7/*v-ras*^{H_a} coinfecting keratinocytes rapidly progressed to squamous cell carcinomas *in vivo*, whereas pBabe/*v-ras*^{H_a}- or Smad6/*v-ras*^{H_a}-transduced keratinocytes formed only benign papillomas. Smad7/*v-ras*^{H_a} tumors had elevated proliferation and defective nuclear localization of Smad2, Smad3, and Smad5, whereas only Smad5 was altered in Smad6/*v-ras*^{H_a} tumors. Smad7 overexpression *in vitro* induced epidermal growth factor (EGF)-like growth factors TGF- α , heparin binding-EGF, amphiregulin, and EGF receptor tyrosine phosphorylation as well as the EGF-CFC growth factor cripto-1. TGF- α and cripto-1 were also overexpressed in Smad7/*v-ras*^{H_a} tumors. These results suggest that Smad7 overexpression accelerates tumor progression through inhibition of TGF- β superfamily signaling and up-regulation of the EGF-like superfamily of growth factors. This is the first demonstration that Smad7 overexpression can cause malignant conversion in a multistage cancer model and suggests that it may have an important role in the pathogenesis of human cancer.

INTRODUCTION

The TGF β superfamily is a large family of cytokines that includes the TGF- β s, activins, inhibins, and BMPs. Through the intracellular pathway-specific and co-Smad proteins, these growth factors regulate cell growth, differentiation, matrix production, and apoptosis in a variety of cell types (1). Smad6 and Smad7 are inhibitory Smads induced by members of the TGF- β 1 superfamily that function in a negative feedback loop to block signal transduction (2). Smad7 can block TGF- β 1, activin, and BMP signaling (2, 3), whereas Smad6 appears to be a specific inhibitor of BMP signaling (4). Both Smads inhibit signaling by blocking type I receptor phosphorylation of receptor-activated Smads (2, 4), although other mechanisms of inhibition have been described including down-regulation of the type I receptor (5–7).

Although strong evidence from human cancers and experimental models indicates that TGF- β 1 signaling acts as a tumor suppressor pathway (8–12), there is little information on the potential positive

role of the inhibitory Smads in cancer development. Smad7 is overexpressed in inflammatory bowel disease (13), chemically induced mouse epidermal papillomas, and carcinomas (14) and human pancreatic and colon cancers (15, 16). In colon cancers, there is an inverse correlation between Smad7 gene copy number and patient survival (17). Additionally, forced overexpression of Smad7 in pancreatic cell lines causes anchorage-independent growth *in vitro* and accelerated growth in nude mice (15), and mice expressing a skin-targeted Smad7 transgene display severe defects in multiple epithelial tissues including progressive epidermal hyperplasia (18). Despite these data, a causal relationship between elevated Smad7 expression and tumor progression has not been demonstrated.

In the mouse model of multistage epidermal carcinogenesis, inhibition of TGF- β signaling accelerates premalignant progression of squamous tumors (11, 12). Here, we have examined the role of Smad7 in squamous cancer development using a multistage model of skin carcinogenesis in which primary keratinocytes are initiated *in vitro* with a *v-ras*^{H_a} retrovirus. This model recapitulates the activation of *c-ras* in the epidermis by chemical carcinogens. Using high-titer retroviruses to express Smad7, Smad6, and *v-ras*^{H_a} in mouse keratinocytes, we show here that Smad7 but not Smad6 cooperates with *v-ras*^{H_a} to cause rapid *in vivo* progression of primary keratinocytes from benign papilloma to malignant squamous cell carcinoma. These results distinguish effects of the inhibitory Smads in cancer development and show for the first time that up-regulation of Smad7 can profoundly alter the tumorigenic potential of primary epithelial cells.

MATERIALS AND METHODS

Virus Production. The *v-ras*^{H_a} replication-defective retrovirus was prepared as described (19). A 1.3-kb FLAG-tagged murine Smad7 cDNA or a 1.5-kb FLAG-tagged murine Smad6 cDNA, isolated from pcDNA3 FLAG Smad7 and pcDNA FLAG Smad6, were inserted into the pBabepuro retroviral vector (20). High-titer retroviral supernatants were obtained through transient transfection of retroviral plasmid DNA into the Ecopack packaging cell line (Clontech). Virus titer was between $1-3 \times 10^6$ virus/ml, and pooled populations of infected cells were used for all experiments. The *v-ras*^{H_a} retrovirus was generated from ψ 2 producer cells as described previously (19). Titers of this virus were determined using a NIH3T3 focus-forming assay and were between $1-2 \times 10^7$ virus/ml. Cells were infected with this virus at a MOI of 1–3 to ensure infection of all cells in culture. Smad7 (21) and control empty adenoviruses were amplified using QBI 293 cells. Virus was purified over two CsCl gradients and dialyzed against a buffer containing Tris-HCl (pH 7.5), 10% glycerol, and 1 mM MgCl₂. Keratinocytes were infected with adenoviruses at a MOI of 20 on day 3 after *v-ras*^{H_a} retroviral infection in medium containing 4 μ g/ml of Polybrene or on day 3 of culture for primary keratinocytes.

Cell Culture. Primary keratinocytes from newborn BALB/c mice were prepared and cultured according to established methods (22). Keratinocytes were infected with the *v-ras*^{H_a} retrovirus on day 3 of culture and with either Smad7 or Smad6 or pBabepuro retrovirus on day 4, followed by selection with 2 μ g/ml of puromycin medium for 2 days or Smad7 adenovirus or empty virus on day 4. For growth curves, singly or doubly infected and selected keratinocytes were cultured in 12-well tissue culture trays, and cell numbers were measured on subsequent days using a Coulter counter. Flow cytometric analysis of the cell cycle was done using anti-BrdUrd-FITC and propidium iodide as described (12). SA- β -galactosidase was assayed as described (23), and positive cells were enumerated using a Nikon inverted microscope. The cal-

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¹ The abbreviations used are: TGF, transforming growth factor; BMP, bone morphogenetic protein; MOI, multiplicity of infection; SA- β -galactosidase; senescence-associated β -galactosidase; BrdUrd, 5'-bromo-2'-deoxyuridine; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF; RT-PCR, reverse transcription-PCR.

cius resistance assay to measure *in vitro* malignant transformation of primary keratinocytes infected with *v-ras*^{H4} and pBabeSmad7 or pBabepuro was done as described (24). To measure TGF- β 1 responsiveness, infected keratinocytes were cotransfected with the TGF- β 1 reporter plasmid pSBE4 and cytomegalovirus- β -galactosidase and treated with 1 ng/ml of TGF- β 1 for 24 h. Luciferase activity was determined in cell extracts using the luciferase assay system (Promega) and normalized to β -galactosidase activity.

In Vivo Grafting and Tumor Analysis. Primary keratinocytes infected with Smad7, Smad6, or pBabepuro retroviruses with or without *v-ras*^{H4} were selected in puromycin medium and then cultured in puromycin-free medium for 3 days before skin grafting. Trypsinized keratinocytes and primary dermal fibroblasts were grafted onto athymic mice as described (19). Tumor volumes were measured weekly using digital calipers. Animals were sacrificed at 4–6 weeks. Cell proliferation was measured in animals injected with BrdUrd 1 h before sacrifice using an anti-BrdUrd monoclonal antibody (Becton Dickinson) or with anti-PCNA (Oncogene Research Products). Keratins 1 and 10 were detected with polyclonal antibodies (Babco), and keratin 8 was detected with a monoclonal antibody (Department of Biological Sciences, University of Iowa). Smads and EGF-like growth factors were detected in tissue sections using the following antibodies from Santa Cruz Biotechnology: Smad6 (H-150), Smad7 (H-79), Smad3 (I-20), Smad2 (S-20), Smad1 (T-20), Smad5 (D-20), TGF- α (H-50) and cripto-1 (D-19). Blocking peptides were used to show specificity of staining.

Protein and RNA Expression. To assess differentiation marker expression, infected keratinocytes were switched from proliferation medium (0.05 mM calcium) to differentiation medium (0.12 mM calcium) for 24 h. Whole-cell extracts were made by heating cell pellets at 100°C for 5 min directly in 10 mM Tris-HCl (pH 7.5), 5% SDS, and 20% β -mercaptoethanol, and equal amounts of protein were electrophoresed through 10% polyacrylamide gels and transferred to nitrocellulose (25). Smad and EGFR proteins were isolated in a 1% Triton X-100 lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 150 mM

NaCl, 1% Triton X-100). Antibodies used were Smad7 antisera (a gift from Dr. Marene Landstrom, LICR, Uppsala, Sweden), anti-phospho-Smad2 (Upstate Biotechnology), anti-phospho-Smad1 (Upstate Biotechnology), anti-Smad2 (Transduction Laboratories), anti-FLAG M2 (Sigma Aldrich), anti-phospho-EGFR (Tyr-1068; Cell Signaling Technology), anti-EGFR (Cell Signaling Technology), and anti- β -actin (Chemicon International).

Total RNA was isolated from tumors and keratinocytes with Trizol Reagent (Invitrogen Corp., Carlsbad, CA). Smad7, Smad6, cripto-1, TGF- α , amphiregulin, HB-EGF, and betacellulin expression was detected with a semiquantitative RT-PCR assay with PCR for 35 cycles. Total RNA was treated with DNase I (Ambion), and then 500 ng were reverse transcribed by using oligo(dT) and superscript RT (Invitrogen). RT-PCR primers were obtained from published sequences (26–30): Smad7, 5'-TGGCATACTGGGAG-GAGAAGAC and 5'-CACGCTAGTCGAAACACTTCC; Smad6, 5'-TCTC-CTCCTGACCAGTACAAGC and 5'-CCGTATGACGAGTCGTTCTC; TGF- α , 5'-GCCAGATTCCACACTCAGTA and 5'-TCTGCATGCTCA-CAGCGAA; cripto-1, 5'-ATTTGGACCCGTTGCTGGGAGAGA and 5'-CAGCTAGCATAAAAAGTGGTCTGCA; amphiregulin, 5'-TCATGGC-GAATGCAGATAACA and 5'-GCTACTACTGCAATCTTGGGA; HB-EGF, 5'-TGCAGATACCTGCAGGAGTT and 5'-ACGACAGTACTGCAGCCACCA; betacellulin, 5'-CACAGCACAGTTGATGGACC and 5'-CCGTTAAG-CAATATTGGTCTC; and glyceraldehyde-3-phosphate dehydrogenase, 5'-CCCTTCATTGACCTCAACTAC and 5'-CCACCTTCTTGATGTCATCAT was used as an internal control for RT-PCR.

RESULTS

Retroviral Expression of Smad7 in Primary Mouse Keratinocytes. Smad7 is an inhibitory Smad that blocks TGF- β 1 and BMP signaling and is up-regulated in squamous tumors produced by two-

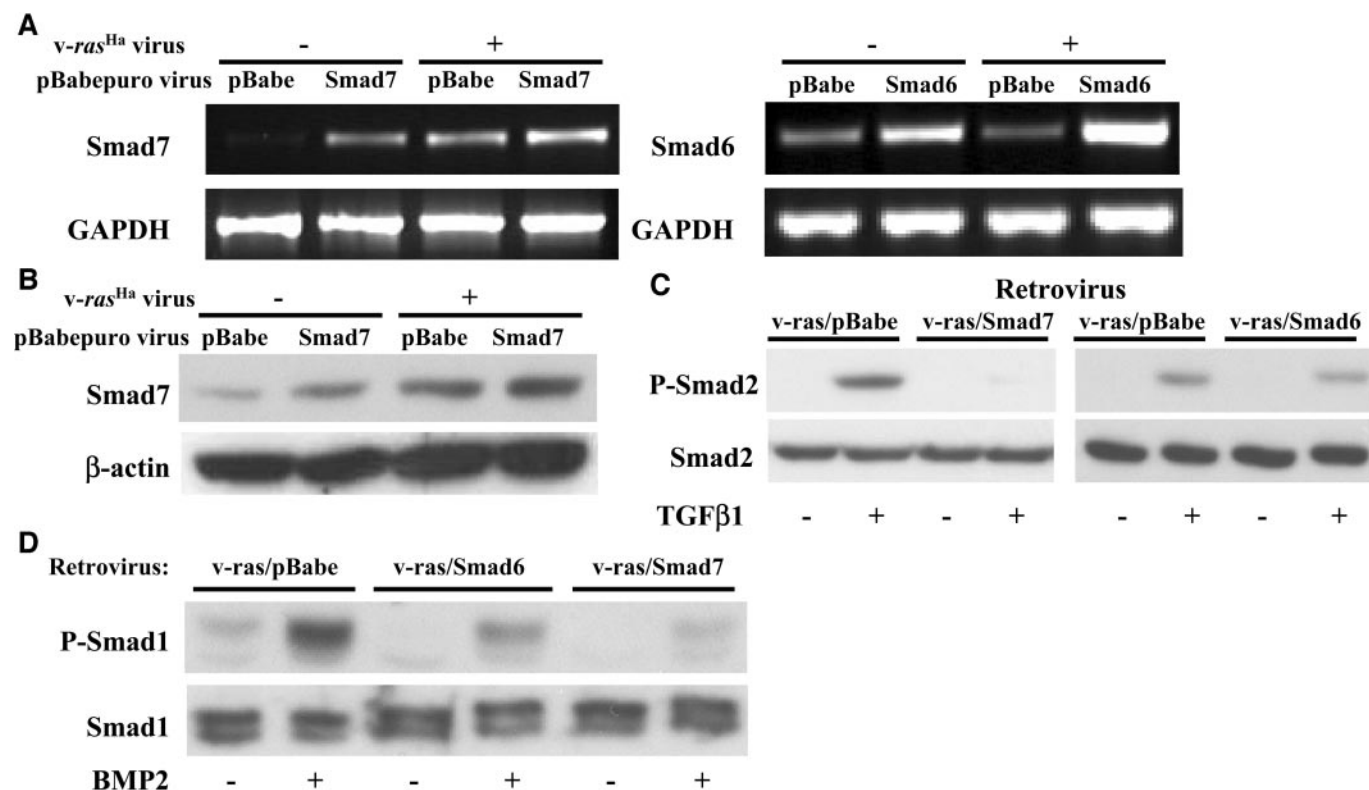


Fig. 1. Expression of retrovirally transduced Smad7 and Smad6 blocks TGF- β and BMP signaling in keratinocytes. Keratinocytes were infected with either the control pBabepuro (pBabe), pBabeSmad7 (Smad7), or pBabeSmad6 (Smad6) retroviruses or coinfecting with these viruses and a *v-ras*^{H4} retrovirus (*v-ras*) and selected with puromycin before biochemical analysis. **A**, RT-PCR analysis of Smad7 and Smad6 expression in keratinocytes with (+) or without (-) coinfection with *v-ras*^{H4} retrovirus. **B**, immunoblot analysis of Smad7 expression in keratinocytes infected with either the control or Smad7 retrovirus with (+) or without (-) coinfection with *v-ras*^{H4} retrovirus. **C**, TGF- β 1-induced Smad2 phosphorylation is blocked by Smad7 expression but not by Smad6. *v-ras*^{H4}-expressing keratinocytes were coinfecting with either pBabepuro, pBabeSmad7, or pBabeSmad6, treated with 1 ng/ml of TGF- β 1 for 10 min, and analyzed for phospho-Smad2 by immunoblotting. **D**, BMP2-induced Smad1 phosphorylation is blocked by both Smad7 and Smad6. pBabepuro-, Smad7-, and Smad6-infected *v-ras*^{H4} keratinocytes were treated with 50 ng/ml of BMP2 for 10 min and analyzed for phospho-Smad1 by Western blot.

stage chemical initiation-promotion protocols in the mouse epidermis (14). To test the consequences of elevated Smad7 expression on tumor development, we infected normal primary keratinocytes with a FLAG-tagged mouse Smad7 retrovirus or coinfecting with Smad7 and *v-ras^{Ha}* retroviruses. For comparison, we also used a Smad6-expressing retrovirus because Smad6 is an inhibitor of BMP signaling. Immunostaining showed that nearly all of the infected and selected cells expressed the transduced gene (data not shown). RT-PCR and Western blot analysis of infected cells after puromycin selection showed that Smad7 and Smad6 were expressed at levels 2–3-fold higher than cells infected with the control pBabepuro virus. Interestingly, *v-ras^{Ha}* by itself induced Smad7 mRNA and protein but not Smad6 mRNA (Fig. 1, A and B). To test the function of the retrovirally expressed Smad7 or Smad6, infected cells were treated with TGF β 1 or BMP2. Western blot analysis showed that TGF- β 1-induced Smad2 phosphorylation was blocked in the Smad7- but not Smad6-infected keratinocytes (Fig. 1C), whereas Smad7 and Smad6 overexpression in either primary or *v-ras^{Ha}* keratinocytes blocked BMP2-induced Smad1 phosphorylation (Fig. 1D). Additionally, TGF- β 1-induced expression of a pSBE-luciferase reporter was blocked in both primary keratinocytes and keratinocytes expressing *v-ras^{Ha}* by Smad7, as was the TGF- β 1-induced cell cycle arrest (data not shown).

Smad7 Enhances Proliferation and Alters Differentiation of Primary Keratinocytes But Does Not Cause Tumor Formation. Primary keratinocytes were seeded at low density and infected with retroviruses, and the growth of cells was determined after 2 days of

puromycin selection. Smad7 expression caused a reproducible 2–3-fold increase in the cell number between day 5 and day 7 after selection compared with Smad6 and pBabepuro control, but this was transient, and by day 9 the cell numbers declined to levels similar to the Smad6- and pBabepuro-infected cells (Fig. 2A). Thus, expression of Smad7 alone cannot extend the culture life span of these primary epithelial cells.

To induce squamous differentiation, infected primary cells were switched from growth media (0.05 mM calcium) to differentiation media (0.12 mM calcium), which causes expression of specific spinous and granular layer proteins (31). In Smad7-infected primary keratinocytes, the normal induction of keratin 1, an early differentiation marker, was blocked, and the expression of loricrin, a late differentiation marker, was reduced (Fig. 2B). Surprisingly, keratin 8, a marker of simple epithelia and malignant keratinocytes, was highly expressed in Smad7-infected cells at both 0.05 and 0.12 mM calcium (Fig. 2B). To determine whether this effect of Smad7 was through inhibition of TGF- β superfamily induction of differentiation, we treated keratinocytes in either growth media or differentiation media with either TGF- β 1, BMP2, BMP4, BMP6, or activin A. Although it has been reported that BMP6 can induce markers of terminal differentiation in keratinocytes (32), we reproducibly found that none of TGF- β superfamily members could induce keratin 1 expression but rather all suppressed keratin 1 induction by elevated calcium (Fig. 2C). Similarly, there was no effect of any of these growth factors on the expression of keratin 8 (data not shown). These results suggest that

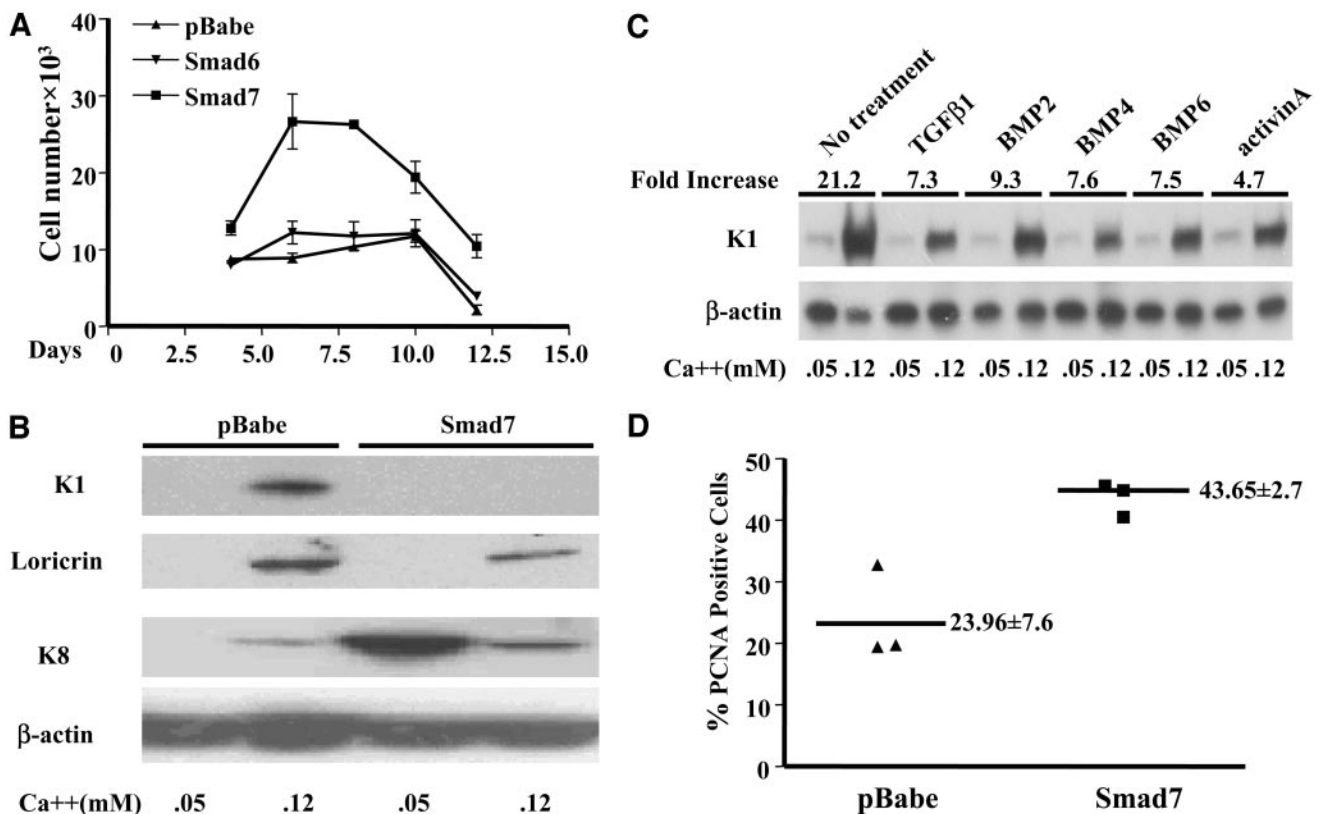


Fig. 2. Effects of Smad7 on normal keratinocytes *in vitro* and *in vivo*. **A**, Smad7 transiently enhances keratinocyte proliferation. Primary keratinocytes were infected with pBabepuro, Smad6, or Smad7 retroviruses and selected in puromycin, and cell numbers were determined in triplicate wells every 2 days. Similar results were obtained in three experiments; bars, SD. **B**, Smad7 blocks differentiation marker expression and induces a marker of malignant keratinocytes. Western blot analysis of keratin 1, loricrin, and keratin 8 expression in primary keratinocytes infected with pBabepuro or Smad7 viruses and induced to differentiate with 0.12 mM calcium chloride for 24 h. **C**, TGF- β superfamily members do not induce differentiation marker expression. Western blot analysis of keratin 1 expression in primary keratinocytes induced to differentiate with 0.12 mM calcium chloride for 24 h. Cells were treated with 1 ng/ml of TGF- β 1, 50 ng/ml of BMP2, 50 ng/ml of BMP4, 50 ng/ml of BMP6 and 10 ng/ml of activin A in media containing either 0.05 or 0.12 mM calcium chloride. The keratin 1 signal was quantitated by Image J software and was normalized to β -actin. **D**, elevated proliferation in skin grafts of Smad7-infected keratinocytes. Primary keratinocytes were infected with pBabepuro or Smad7 retrovirus, selected, and grafted onto the dorsal skin of nude mice. Skins at grafting sites were isolated 5 weeks after grafting and stained with anti-PCNA antibody. Data represent the average of the percentage of positive-staining cells counted in five different areas under the microscope, and 500 cells were counted in each area.

the mechanism through which Smad7 overexpression alters differentiation in the keratinocytes is independent of its ability to inhibit TGF- β superfamily signaling.

To determine whether Smad7 by itself could cause tumor formation *in vivo*, primary keratinocytes infected with either Smad7 or pBabepuro retroviruses were mixed with primary dermal fibroblasts and grafted onto the back of nude mice (19). Grafts of Smad7-infected keratinocytes did not develop tumors, but the epidermis was hyperplastic. The percent of PCNA-positive nuclei was 43.65 ± 2.7 in the Smad7-infected keratinocytes compared with 23.96 ± 7.6 ($P < 0.05$) in the grafted pBabepuro-infected keratinocytes (Fig. 2D).

Smad7 Blocks Senescence of Keratinocytes and Increases the Frequency of Transformation *in Vitro*. To examine the effects of Smad7 in the context of a preneoplastic epithelial cell, we sequentially infected primary keratinocytes with the Smad7 retrovirus and a retrovirus expressing *v-ras*^{H_a} (19). The titer of the *v-ras*^{H_a} virus is high enough to ensure coinfection of all cells. To determine whether Smad7 could alter the growth property of cells expressing *v-ras*^{H_a}, we initially examined changes in the cell cycle in Smad7/*v-ras*^{H_a}-infected cells by 2-color flow cytometry. This study revealed that the percentage of cells in S-phase in the *v-ras*^{H_a}/Smad7-infected keratinocytes was 2-fold greater than that of control *v-ras*^{H_a}/pBabe-infected cells (data not shown). To examine long-term effects on cell proliferation, coinfecting cells were trypsinized and seeded at low density two days after puromycin selection. Fig. 3A shows that *v-ras*^{H_a}/pBabe-infected cells proliferated for several days and then underwent an irreversible growth arrest similar to the cells infected with *v-ras*^{H_a} alone. This hyperproliferative response to oncogenic ras followed by growth arrest has been described previously (33). In striking contrast, the *v-ras*^{H_a}/Smad7-infected keratinocytes continued to proliferate, reaching a 4-fold higher cell number by 21 days, with no evidence of a growth arrest comparable with control cells. Since this G₁ arrest and senescence is mediated in part by autocrine TGF- β 1 signaling (33), we examined the senescence of keratinocytes infected with Smad7 and *v-ras*^{H_a}, using SA- β -galactosidase as a marker (23). Fig. 3B shows that 11 days after coinfection and selection, the percentage of SA- β -galactosidase-positive cells was 44.8% in the *v-ras*^{H_a}/pBabe keratinocytes but only 16.5% in the *v-ras*^{H_a}/Smad7 keratinocytes.

The ability of keratinocytes to overcome senescence is correlated with malignant transformation (33). We used a calcium resistance assay that accurately reflects malignant behavior of cells *in vivo* (24) to test whether Smad7 overexpression could transform *v-ras*^{H_a} keratinocytes. In this assay, retrovirally infected keratinocytes are cultured in elevated calcium, causing a growth arrest of nonmalignant cells. At low frequency transformed cells escape this growth arrest and form scorable foci, which share properties with cells isolated from squamous cell carcinoma (34). Primary keratinocytes were infected with *v-ras*^{H_a} and either the pBabepuro or Smad7 retroviruses, selected in puromycin, and then cultured in 0.5 mM calcium chloride media for 5 weeks. Fig. 3C shows that cells infected with *v-ras*^{H_a} and pBabepuro retroviruses formed calcium-resistant colonies at a frequency of 6/dish or 3×10^{-6} foci/cell plated, whereas the *v-ras*^{H_a}- and Smad7-infected keratinocytes formed transformed foci at a frequency of 14.6/dish or 1×10^{-5} foci/cell plated. Thus, Smad7 overexpression increases by 3-fold the frequency of *in vitro* malignant transformation of preneoplastic keratinocytes.

Smad7 Causes Malignant Conversion to Squamous Cell Carcinoma *in Vivo*. To determine whether Smad7 could accelerate malignant progression *in vivo*, primary keratinocytes coinfecting with either Smad7, Smad6, pBabepuro, and *v-ras*^{H_a} retroviruses were mixed with primary dermal fibroblasts and grafted onto the back of nude mice (19). In this model, *v-ras*^{H_a}-transduced BALB/c keratinocytes produce benign tumors similar to papillomas produced by two-stage

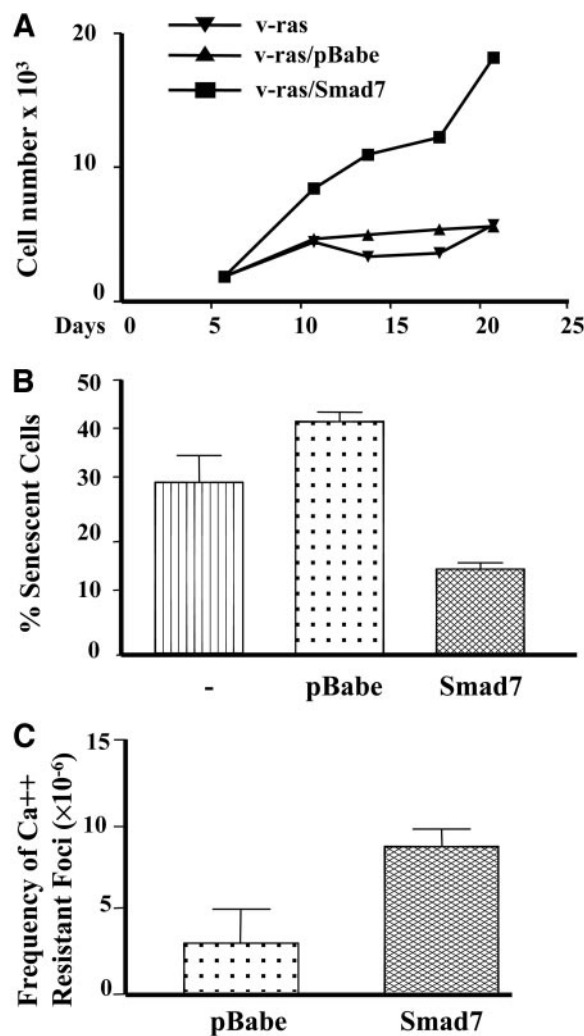


Fig. 3. Smad7 blocks a *v-ras*^{H_a}-associated growth arrest and senescence and causes increased frequency of transformation. *A*, sustained proliferation of Smad7/*v-ras*^{H_a} coinfecting keratinocytes. *v-ras*^{H_a}-expressing keratinocytes were infected with pBabe or Smad7 retroviruses, selected, and then seeded into wells for growth curves. Data represent the average of duplicate wells. *B*, decreased senescence in Smad7/*v-ras*^{H_a}-infected keratinocytes. Coinfecting cells were fixed on day 11 after *v-ras*^{H_a} infection and stained for SA- β -galactosidase. Each histogram represents the average of triplicate wells, with ~500 cells counted per well; bars, SD. *C*, elevated *in vitro* transformation in Smad7/*v-ras*^{H_a}-infected keratinocytes. Coinfecting keratinocytes were cultured in media with 0.05 mM calcium chloride growth media for 15 days and then switched to 0.5 mM calcium medium for 5 weeks. Cells were stained, and calcium-resistant foci were counted under a dissecting microscope. Each bar represents the mean of 2–7 dishes/group; bars, SD.

chemical initiation-promotion protocols (19). After 4 weeks, 7 of 7 of the *v-ras*^{H_a}/pBabe grafts and 4 of 4 of the *v-ras*^{H_a}/Smad6 grafts were papillomas (Fig. 4, *A* and *D*), whereas 10 of 12 of the *v-ras*^{H_a}/Smad7 grafts had progressed to carcinomas (Fig. 4*E*). Elevated levels of Smad7 and Smad6 mRNA were readily detected in the Smad7/*v-ras*^{H_a} or Smad6/*v-ras*^{H_a} grafts compared with endogenous Smad7 or Smad6 in the control tumors (Fig. 4, *M* and *N*).

Consistent with the squamous cell carcinoma histology, the Smad7 tumors expressed keratin 8 and had reduced or no expression of keratins 1 and 10 compared with the control tumors (data not shown). Proliferation in grafted tumors was determined by injecting mice with BrdUrd 1 h before sacrifice. The *v-ras*^{H_a}/Smad7 tumors had nearly a 2-fold higher percentage of cells in S-phase compared with control tumors (30.8 ± 3.3 versus 16.6 ± 1.4 ; $P < 0.05$). Proliferation was elevated in benign areas of the grafted tumors and in the papillomas from the Smad7/*v-ras*^{H_a} grafts, suggesting that the elevated proliferation preceded malignant conversion.

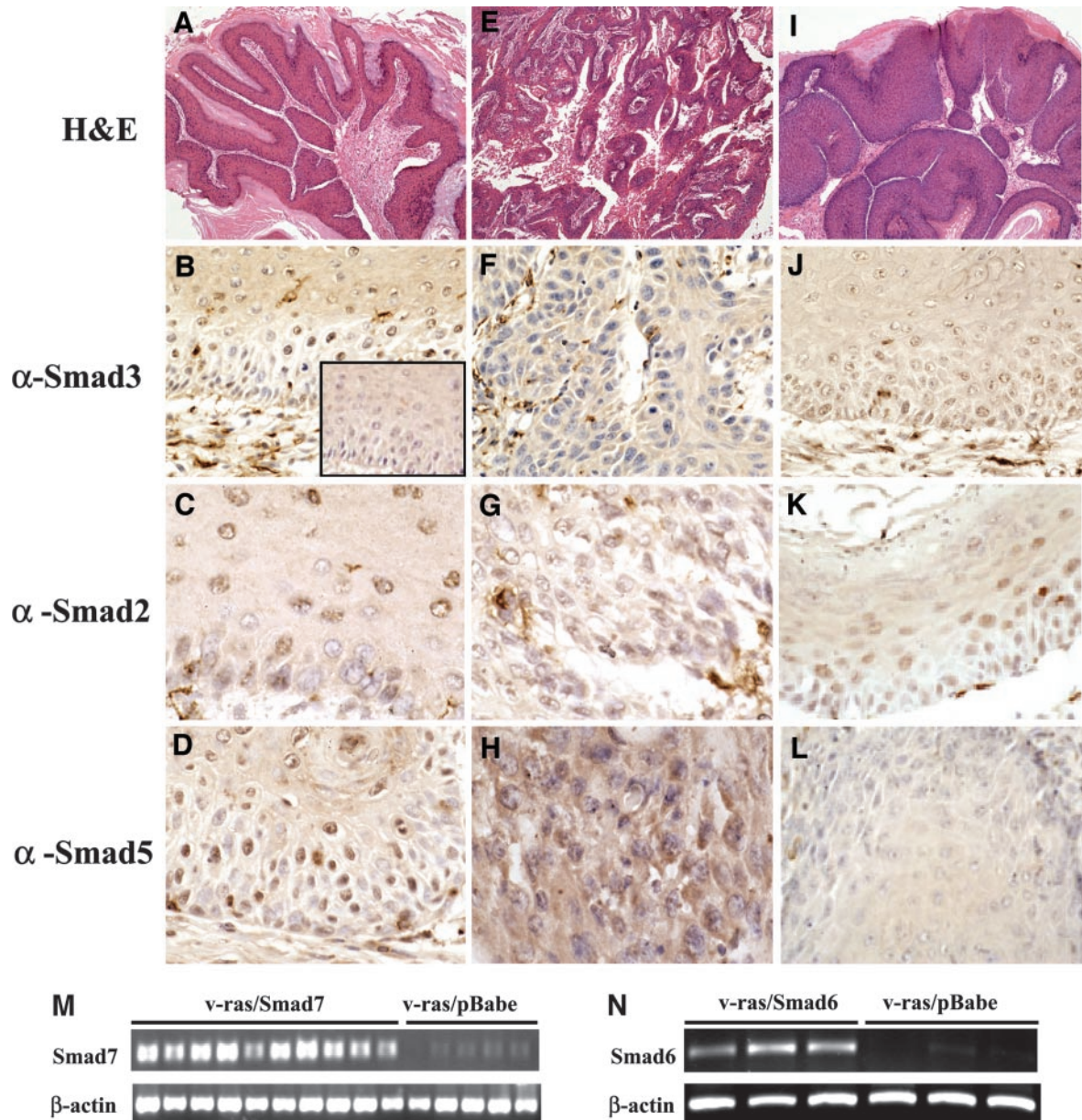


Fig. 4. Smad7 but not Smad6 causes malignant conversion of *v-ras^{Ha}* to squamous cell carcinoma *in vivo*. Retrovirally infected primary keratinocytes were mixed with primary dermal fibroblasts and grafted onto the dorsal skin of athymic mice. Tumors were isolated 5 weeks after grafting. Sections from pBabe/*v-ras^{Ha}* tumors (A–D), Smad7/*v-ras^{Ha}* tumors (E–H), and Smad6/*v-ras^{Ha}* tumors (I–L) stained with H&E or the indicated anti-Smad antibody. *B inset*, preincubation of Smad3 antibody with blocking peptide. *M*, RT-PCR analysis of Smad7 mRNA expression in Smad7/*v-ras^{Ha}* and pBabe/*v-ras^{Ha}* tumor grafts. *N*, RT-PCR analysis of Smad6 mRNA expression in Smad6/*v-ras^{Ha}* and pBabe/*v-ras^{Ha}* tumor grafts.

Altered R-Smad Localization in Smad7 Tumors. To determine whether overexpression of Smad7 disrupted TGF- β signaling in the tumors, we analyzed expression and localization of different R-Smads using specific antibodies. Strong nuclear Smad3, 2, and 5 staining was detected in the tumor cells from pBabe/*v-ras^{Ha}* grafts (Fig. 4, B–D), although not uniformly in all cells. Only Smad5 nuclear localization was disrupted in the Smad6/*v-ras^{Ha}* papillomas (Fig. 4L), but localization of Smad3 and Smad2 was not altered (Fig. 4, G and K). However, in Smad7/*v-ras^{Ha}* tumors, there was no nuclear staining for Smad3, Smad2, or Smad5 (Fig. 4, F–H), and this was evident even in benign regions within these tumors, suggesting that the change in localization preceded malignant conversion. These results suggest that in the grafted tumors, Smad7 blocks both TGF- β and BMP signaling whereas Smad6 blocks only BMP signaling.

Smad7 Induces EGF-like Superfamily Growth Factor Expression. To identify potential targets of Smad7 overexpression that could accelerate malignant conversion, we examined expression of the EGF-like growth factor family because these are frequently up-regulated in mouse squamous tumors and in human pancreatic cancers where Smad7 is overexpressed (35, 36). Initial RT-PCR analysis showed that the transcript for TGF- α transcription was elevated in keratinocytes coinfecting with the Smad7 and *v-ras^{Ha}* retroviruses relative to *v-ras^{Ha}* alone (data not shown). To determine whether this was a rapid response to Smad7 overexpression, we infected primary keratinocytes or *v-ras^{Ha}* keratinocytes with either a Smad7 or control adenovirus at a MOI of 20, and after 2 days, we measured expression of different members of the EGF-like growth factor superfamily by RT-PCR. Fig. 5A shows that infection of primary keratinocytes with the Smad7 adenovirus induced transcripts of TGF- α , HB-EGF, amphiregulin,

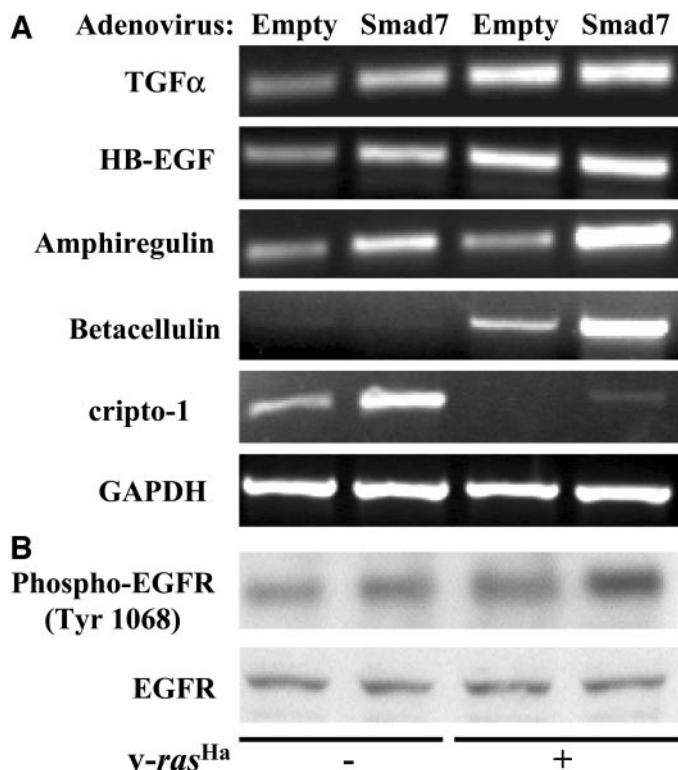


Fig. 5. Smad7 enhances mRNA levels of EGF-like growth factors in keratinocytes and tumors. **A**, Smad7 adenovirus induces TGF- α , cripto, HB-EGF, amphiregulin, and betacellulin expression in primary or v-ras^{Ha} keratinocytes. Keratinocytes with or without the v-ras^{Ha} retrovirus were infected with either the Smad7 or control empty adenovirus, and total RNA was isolated after 2 days. Adenovirus infection and RT-PCR for the indicated growth factors were done as described in "Materials and Methods." **B**, Smad7 induces phosphorylation of EGF receptor. Western blot analysis of phospho-EGFR (Tyr 1068) and EGFR expression in primary keratinocytes infected with empty adenovirus or adenovirus expressing Smad7 or coinfecting with v-ras^{Ha} retrovirus is shown.

and the EGF-CFC family member cripto-1 relative to cells infected with the control adenovirus. Betacellulin expression was not detected in these cells but was induced in cells expressing v-ras^{Ha}. In agreement with previous studies (37, 38), the transcripts for these growth factors were also induced by v-ras^{Ha} alone; however, overexpression of Smad7 significantly superinduced amphiregulin and betacellulin transcript levels and slightly increased levels for TGF- α and HB-EGF in v-ras^{Ha}-infected cells. Surprisingly, the expression of cripto-1 was much lower in the v-ras^{Ha}-expressing cells, although it was still induced by Smad7. Western blot analysis of the EGFR using phospho-specific antibodies showed an increase in phospho Tyr-1068 in Smad7-infected keratinocytes (Fig. 5B), suggesting an increased activation of the EGFR signaling pathway. RT-PCR analysis showed a significant elevation of both TGF- α and cripto-1 in tumors generated from Smad7/v-ras^{Ha} retrovirally infected keratinocytes compared with either the pBabe/v-ras^{Ha} or Smad6/v-ras^{Ha} tumors (Fig. 6A). To confirm this result, we used immunohistochemistry with anti-TGF- α and anti-cripto-1 antibodies to detect expression of these proteins in formalin-fixed sections from the different tumor types. Consistent with the RT-PCR results, we observed stronger staining for both TGF- α and cripto-1 in the tumor cells derived from grafted Smad7/v-ras^{Ha} keratinocytes compared with tumors from the Smad6/v-ras^{Ha}- or pBabe/v-ras^{Ha}-infected keratinocytes (Fig. 6B). Taken together, these results suggest that elevated EGF-like growth factor expression in these tumors is linked to Smad7 overexpression. Previous studies have shown that EGFR ligands can induce keratin 8 and block keratin 1 induction in primary keratinocytes (37). Fig. 7 shows that treatment of primary keratinocytes with cripto-1 induced keratin 8 in both

proliferation and differentiation conditions, similar to Smad7, but had no effect on keratin 1 induction. Thus, the induction of cripto-1 by Smad7 is likely to play a role in the effects of Smad7 on keratinocyte differentiation.

DISCUSSION

To examine the role of elevated Smad7 in the mouse model of multistage epidermal carcinogenesis, we have used retroviruses to introduce Smad7 or Smad6 and oncogenic ras into primary cultures of mouse epidermal keratinocytes. Keratinocytes infected with either Smad7 or Smad6 retroviruses expressed these genes at levels 2–3-fold above endogenous levels, as judged by RT-PCR and Western blot. As expected, retrovirally expressed Smad7 blocked TGF- β 1-induced phosphorylation of Smad2 and BMP2-induced phosphorylation of Smad1, whereas Smad6 overexpression only blocked Smad1 phosphorylation by BMP2. Smad7 overexpression also blocked TGF- β 1 induced gene expression from a SBE-luciferase reporter construct, and TGF- β 1 induced growth inhibition in primary keratinocytes and keratinocytes expressing v-ras^{Ha}.

Our data show that overexpression of Smad7 in the context of an activated ras oncogene can cause conversion of epithelial cells from a benign to malignant tumor phenotype. Thus, skin grafts of primary keratinocytes coinfecting with v-ras^{Ha} and pBabe retroviruses formed benign squamous papillomas, whereas nearly all grafts of v-ras^{Ha} and Smad7 converted from papillomas to squamous cell carcinomas within a 4-week period *in vivo*. The squamous cell carcinomas generated by Smad7 overexpression had elevated proliferation, invasive growth, and expressed keratin 8 and not keratin 1 or 10. Interestingly, the relative level of Smad7 overexpression in the grafted cells was significantly higher than in cultured keratinocytes after infection with the Smad7 and v-ras^{Ha} retroviruses, suggesting that positive selection for higher Smad7 levels occurred *in vivo*. However, Smad7 overexpression also caused malignant transformation of v-ras^{Ha} keratinocytes *in vitro*, indicating that the effects of Smad7 overexpression occur within the keratinocytes and do not require an *in vivo* environment. Because grafts of keratinocytes infected with Smad7 alone only formed hyperplastic epidermis, consistent with the hyperplastic epidermis of Smad7 transgenic mice (18), it is likely that Smad7 overexpression by itself is not sufficient for tumor formation but requires cooperation with an activated ras oncogene. Cooperation between TGF- β 1 signaling, through Smad2, and a ras oncogene to promote progression to spindle cell carcinoma in this model has also been reported (39, 40). Smad7 was shown to be directly induced by Smad2 overexpression in the carcinoma cells (40), suggesting that the cooperative effects of Smad7 and ras on premalignant progression reported here may also be relevant to the tumor-promoting effects of TGF- β 1 on later stages of tumorigenesis.

We observed a striking difference in the ability of Smad7 and Smad6 to accelerate tumor progression. Despite similar levels of Smad6 and Smad7 overexpression and inhibitory effects on TGF- β and BMP signaling in keratinocytes, Smad6 was unable to cause malignant progression of v-ras^{Ha} keratinocytes. In tumors derived from Smad7-infected v-ras^{Ha} keratinocytes, nuclear localization of both the TGF- β 1-specific R-Smads 2 and 3 as well as the BMP-specific R-Smad, Smad5, was disrupted whereas only nuclear localization of Smad5 was disrupted in the Smad6/v-ras^{Ha} tumors. These results are most consistent with Smad7 inhibition of both TGF- β 1 and BMP signaling at the level of R-Smad phosphorylation by activated type I receptors, because this is required for nuclear translocation. However, other mechanisms involving altered R-Smad stability, or decreased type I receptor expression as was observed in transgenic mice overexpressing Smad7 in the epidermis (18), cannot be ruled

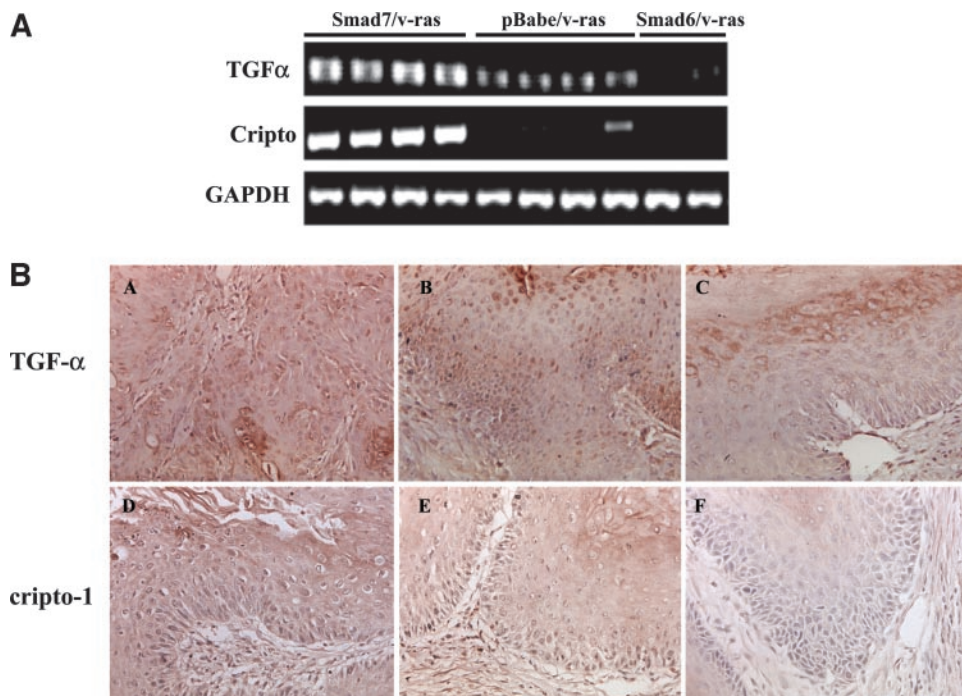


Fig. 6. Increased expression of EGF-like growth factors in Smad7 tumors. **A**, RT-PCR analysis of TGF- α and cripto-1 expression in Smad7/v-*ras*^{H_a}, pBabe/v-*ras*^{H_a}, and Smad6/v-*ras*^{H_a} tumors. RT-PCR was done using the primer sequences as described in "Materials and Methods." **B**, immunohistochemical localization of TGF- α and cripto-1 in tumors. **A–C**, TGF- α localization: **A**, Smad7/v-*ras*^{H_a}; **B**, pBabepuro/v-*ras*^{H_a}; and **C**, Smad6/v-*ras*^{H_a} tumors. **D–F**, cripto-1 localization: **D**, Smad7/v-*ras*^{H_a}; **E**, pBabepuro/v-*ras*^{H_a}; and **F**, Smad6/v-*ras*^{H_a} tumors.

out. These results support the hypothesis that the loss of R-Smad expression that occurs during progression of chemically induced mouse skin tumors (14) is a direct consequence of Smad7 overexpression and not a secondary change associated with premalignant progression. Although the relevance of Smad7 inhibition of either or both TGF- β 1 and BMP signaling pathways to malignant progression cannot be determined, the data indicate that inhibition of BMP signaling alone is not sufficient for malignant conversion.

Our *in vitro* studies suggest that one mechanism by which Smad7 acts to promote malignant conversion is by counteracting the senescence response of v-*ras*^{H_a}-transduced primary mouse keratinocytes. Senescence is thought to be an important mechanism of tumor suppression in cells harboring activated oncogenes (41). Primary keratinocytes expressing v-*ras*^{H_a} hyperproliferate and then undergo a G₁ growth arrest associated with expression of SA- β galactosidase, and the cyclin-dependent kinase inhibitors p16^{ink4a} and p15^{ink4b} (33). TGF- β 1 signaling mediates the senescence of v-*ras*^{H_a} keratinocytes because senescence is reduced in TGF- β 1 null keratinocytes (33), Smad3 null keratinocytes (42), keratinocytes expressing a dominant-negative type II receptor, or treated with neutralizing TGF- β 1 antibodies (33). The ability of Smad7 to suppress senescence and extend the life span of v-*ras*^{H_a} keratinocytes adds further support to the concept that TGF- β signaling is important for senescence.

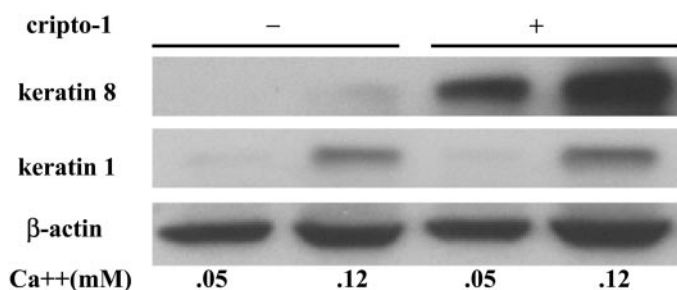


Fig. 7. cripto-1 induces keratin 8 in primary keratinocytes. Primary keratinocytes were treated with 50 ng/ml of cripto for 24 h in either 0.05 or 0.12 mM calcium chloride. Proteins were harvested and immunoblotted with anti-keratin 8 monoclonal antibody.

Smad7 may also accelerate malignant progression through induction of members of the EGF-like growth factor superfamily. TGF- α , HB-EGF, amphiregulin, and the EGF-CFC family member cripto-1 were rapidly elevated after infection of primary keratinocytes with a Smad7 adenovirus. Smad7 coexpressed with v-*ras*^{H_a} further increased expression of amphiregulin and betacellulin above that seen with v-*ras*^{H_a} alone, and in Smad7/v-*ras*^{H_a} tumors, TGF- α and cripto-1 were highly expressed compared with control and Smad6/v-*ras*^{H_a} tumors. Members of the EGF-like and EGF-CFC growth factor superfamily share varying degrees of structural homology centered on an EGF-like domain (43). There is considerable evidence linking elevated expression of these EGF-like growth factors, including cripto-1, to tumorigenesis and the transformed phenotype. Expression is elevated in many different human epithelial tumors and transformed cell lines (44–49), and forced overexpression of TGF- α in transgenic mice leads to cancer development in a number of tissues including the epidermis (50–53). The increased EGFR tyrosine phosphorylation observed in cells infected with the Smad7 adenovirus strongly suggests that the induced EGF-like growth factors are acting as autocrine activators of the EGF signaling pathway. Although cripto-1 contains an EGF-like domain, there is no evidence for direct interaction with the EGFR or other members of the ErbB receptor family (43). Rather, cripto-1 functions as a coreceptor for the TGF- β superfamily member nodal by facilitating binding to ActR1B for signaling through the Smad pathway and as a direct inhibitor of activin signaling (54, 55). Additionally, cripto-1 can stimulate mitogen-activated protein kinase and AKT phosphorylation independently of nodal and ActR1B (29). Although the exact pathway activated or inhibited by cripto-1 remains to be determined in these cells, the expression of these growth factors in the Smad7/v-*ras*^{H_a} tumors suggests that they are important targets of Smad7 and play an important role in malignant progression.

In the absence of v-*ras*^{H_a}, Smad7 but not Smad6 overexpression caused a transient enhancement of cell growth, suggesting that TGF- β signaling is the primary effector of cell cycle regulation in primary keratinocytes. Previous studies have shown that TGF- β (56) and BMP6 (57) can inhibit proliferation of primary mouse keratinocytes. A possible explanation for this difference is that proliferating kerati-

nocytes secrete TGF- β 1 (56), but BMP-6 is only produced during suspension or 12-*O*-tetradecanoylphorbol-13-acetate-induced differentiation (57, 58). Our data also show that Smad7 overexpression in normal keratinocytes suppressed induction of keratin 1 and loricrin by elevated calcium, and induced keratin 8 under conditions where it is not normally expressed. Because treatment of keratinocytes with exogenous TGF- β 1, BMP2, BMP4, BMP6, and activin A suppressed rather than induced keratin 1 expression and had no effect on keratin 8 expression, it is likely that the effects of Smad7 on keratinocyte differentiation are not simply through inhibition of TGF- β 1 or BMP signaling. Although differential regulation of keratin 1 induction and suppression by the TGF- β signaling pathway is possible, our results suggest that cripto-1 up-regulation by Smad7 may play a role in keratin 8 induction. Because TGF- α and other EGFR ligands have been shown to block the induction of keratin 1 and other differentiation markers by elevated calcium, as well as induce keratin 8, it is possible that the effects of Smad7 on reprogramming keratinocyte differentiation are mediated through up-regulation of both EGFR ligands and cripto-1.

Finally, we observed that endogenous Smad7 protein and mRNA expression were elevated 2–3-fold in primary keratinocytes infected with the *v-ras*^{Ha} retrovirus, suggesting that in keratinocytes as well as human and experimental cancers there may be a direct relationship between ras activation and Smad7 induction. The observation that EGF can induce Smad7 expression (59) supports this idea and suggests that hyperactivation of the mitogen-activated protein kinase pathway, or up-regulation of EGFR ligands by oncogenic ras, is involved in the up-regulation of Smad7 in tumors. Because even a modest 2–3-fold increase in constitutive Smad7 levels keratinocytes expressing *v-ras*^{Ha} had profound effects on growth, differentiation, and malignant progression, it is possible that slight changes in Smad7 levels will be important in the pathogenesis of human cancer, especially those in which *ras* activation plays a central role. Taken together, these results suggest that elevated Smad7 levels may accelerate malignant progression, not only through inhibition of TGF- β signaling, but also by synergistic up-regulation of EGF-like growth factors and cripto-1. This ability to block an inhibitory pathway and activate several mitogenic pathways suggests that Smad7 will play a critical role in the pathogenesis of human cancer and could be an important target for therapeutic intervention.

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