# Cripto-1 Alters Keratinocyte Differentiation via Blockade of Transforming Growth Factor-β1 Signaling: Role in Skin Carcinogenesis

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## Abstract

**Cripto-1 is an epidermal growth factor-Cripto/FRL1/Cryptic family member that plays a role in early embryogenesis as a coreceptor for Nodal and is overexpressed in human tumors. Here we report that in the two-stage mouse skin carcinogenesis model, Cripto-1 is highly up-regulated in tumor promoter–treated normal skin and in benign papillomas. Treatment of primary mouse keratinocytes with Cripto-1 stimulated proliferation and induced expression of keratin 8 but blocked induction of the normal epidermal differentiation marker keratin 1, changes that are hallmarks of tumor progression in squamous cancer. Chemical or genetic blockade of the transforming growth factor (TGF)-β1 signaling pathway using the ALK5 kinase inhibitor SB431542 and dominant negative TGF-β type II receptor, respectively, had similar effects on keratinocyte differentiation. Our results show that Cripto-1 could block TGF-β1 receptor binding, phosphorylation of Smad2 and Smad3, TGF-β–responsive luciferase reporter activity, and TGF-β1–mediated senescence of keratinocytes. We suggest that inhibition of TGF-β1 by Cripto-1 may play an important role in altering the differentiation state of keratinocytes and promoting outgrowth of squamous tumors in the mouse epidermis.**
**(Mol Cancer Res 2008;6(3):509–16)**

## Introduction

Mouse Cripto-1 is a member of the epidermal growth factor-Cripto/FRL1/Cryptic (EGF-CFC) protein family that includes human Cripto-1 and cryptic, mouse cryptic, chicken Cripto-1, *Xenopus* FRL1, and zebrafish one-eyed pinhead (oep; refs. 1, 2). These proteins are glycosylphosphatidylinositol linked and contain an EGF-like domain and a cysteine-rich CFC domain. A short COOH terminus contains consensus sequences for glycosylphosphatidylinositol linkage to the cell membrane (3). EGF-CFC proteins have activity both when expressed as soluble proteins and when secreted from the cell surface following enzymatic cleavage of their glycosylphosphatidylinositol anchor (4, 5).

Cripto-1 is expressed in early development and plays an important role in differentiation of heart muscle cells, formation of germ layers, formation of the organizer, and specification of the anterior-posterior and left-right axes and of the embryonic midline (6-10). During embryonic development, Cripto-1 functions as a coreceptor for Nodal, a transforming growth factor β (TGF-β) family ligand, to initiate mesoderm formation. Nodal signals through the activin receptor ALK4 but requires Cripto-1 to bind to ALK4 and signal (11). On the other hand, Cripto forms a complex with activin and a type II receptor, ActRII or ActRIIB, and prevents association of activin with ALK4 (12). Activin, in the absence of Cripto-1, binds and strongly activates ALK4. Thus, Cripto-1 inhibits activin signaling but potentiates Nodal signaling. Apart from activin signaling, in a recent report, Cripto was also shown to suppress TGF-β signaling by reducing association of TGF-β with its receptor in 293T cells (13).

Cripto-1 is overexpressed in a wide variety of human carcinomas such as gastric, pancreatic, colorectal, gall bladder, breast, bladder, ovarian, and basal cell carcinomas, and may play a role in cancer pathogenesis (14). Overexpression of Cripto-1 increases cell proliferation, anchorage-independent growth, and transformation of mammary epithelial cells *in vitro* and causes mammary gland hyperplasia and tumor formation in transgenic MMTV-Cripto-1 mice (15, 16).

We previously showed that mouse skin tumors caused by overexpression of Smad7 had elevated expression of Cripto-1 (17). Here we show that Cripto-1 expression is elevated in chemically induced mouse skin tumors compared with normal skin. Cripto-1 causes impaired TGF-β1 signaling in primary mouse keratinocytes, resulting in modification of their differentiation status and a phenotype associated with tumor progression. Our results suggest a mechanism by which Cripto-1 may enhance skin tumor progression.

## Results

### *Cripto-1 Expression Is Up-Regulated in Multistage Skin Carcinogenesis*

A number of human cancers are associated with enhanced Cripto-1 expression. We wanted to determine if Cripto-1 expression was altered during chemically induced skin tumor

Received 8/22/07; revised 10/22/07; accepted 11/30/07.
**Grant support:** NIH grant CA122109 (A.B.Glick) and the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH.
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doi:10.1158/1541-7786.MCR-07-0396

formation and if there was a tumor stage–specific response. We examined Cripto-1 expression by quantitative reverse transcription-PCR in RNA isolated from samples of normal skin, skin treated chronically with the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), benign papillomas, and squamous cell carcinomas generated by a 7,12-dimethylbenz(*a*)-anthracene-TPA chemical skin carcinogenesis protocol. Interestingly, relative to normal skin where it was nearly undetectable, Cripto-1 mRNA was elevated in TPA-treated normal skin (an average expression value of 2.7). Cripto-1 expression was further enhanced in papillomas (an average expression value of 4), but with further progression in tumor stage, the levels decreased in carcinomas (an average expression value of 0.9; Fig. 1A). To validate these results and to determine the skin compartment where Cripto-1 is expressed, tissue sections from papillomas, carcinomas, and normal skin were immunostained using an anti–Cripto-1 antibody. Figure 1B shows that, similar to mRNA expression results, Cripto-1 protein expression is higher in squamous papillomas compared with normal skin, whereas the squamous cell carcinoma tissue is completely devoid of Cripto-1. Figure 1B (*middle*), depicting a papilloma section, shows that the protein is localized to the differentiating suprabasal layers of the papilloma.

### *Cripto-1 Stimulates Keratinocyte Proliferation and Blocks Differentiation*

To determine the significance of the tumor stage–specific and suprabasal expression pattern of Cripto-1, we analyzed the effect of Cripto-1 on keratinocyte proliferation and differentiation *in vitro*. Primary mouse keratinocytes were treated with varying concentrations of recombinant Cripto-1 for 24 hours and the effect on keratinocyte proliferation was measured by a radioactive thymidine incorporation assay. Figure 2 shows that Cripto-1 treatment caused a 2-fold increase in DNA synthesis at 400 ng/mL, indicating that Cripto-1 can enhance keratinocyte proliferation.

High-calcium media has been shown to induce differentiation of keratinocytes (18). To examine the effects of Cripto-1 on differentiation, we pretreated primary keratinocytes in growth medium with 200 ng/mL Cripto-1 for 24 hours before switching to high-calcium media (0.12 mmol/L $Ca^{2+}$) to induce differentiation. Whole-cell protein lysates were analyzed for keratin 1, an early differentiation marker that is induced in 0.12 mmol/L $Ca^{2+}$ media (18). Pretreatment with Cripto-1 before switching to 0.12 mmol/L $Ca^{2+}$ media blocked the induction of keratin 1, whereas treatment with Cripto-1 after the cells had been switched to the 0.12 mmol/L $Ca^{2+}$ media reduced, but did not completely block, the expression of keratin 1 (Fig. 3A). Keratin 8, a marker of simple epithelium and malignant conversion, is not expressed in normal mouse keratinocytes. Interestingly, Cripto-1 strongly induced expression of keratin 8 in these cells, suggesting that it was altering the differentiation state of normal keratinocytes (Fig. 3B).

### *TGF-β Superfamily Signaling Blockade Alters Keratinocyte Differentiation*

Because previous studies have shown that Cripto-1 inhibits activin signaling, we tested if activin signaling induced or inhibited keratin 8 expression in keratinocytes. We determined that although primary mouse keratinocytes do not express detectable levels of nodal, they do express activin A, activin B, ActRI, ActRIB, and ActRII/ALK4 (data not shown), suggesting that Cripto-1 was acting to inhibit activin signaling. When preneoplastic keratinocytes were treated with activin A or

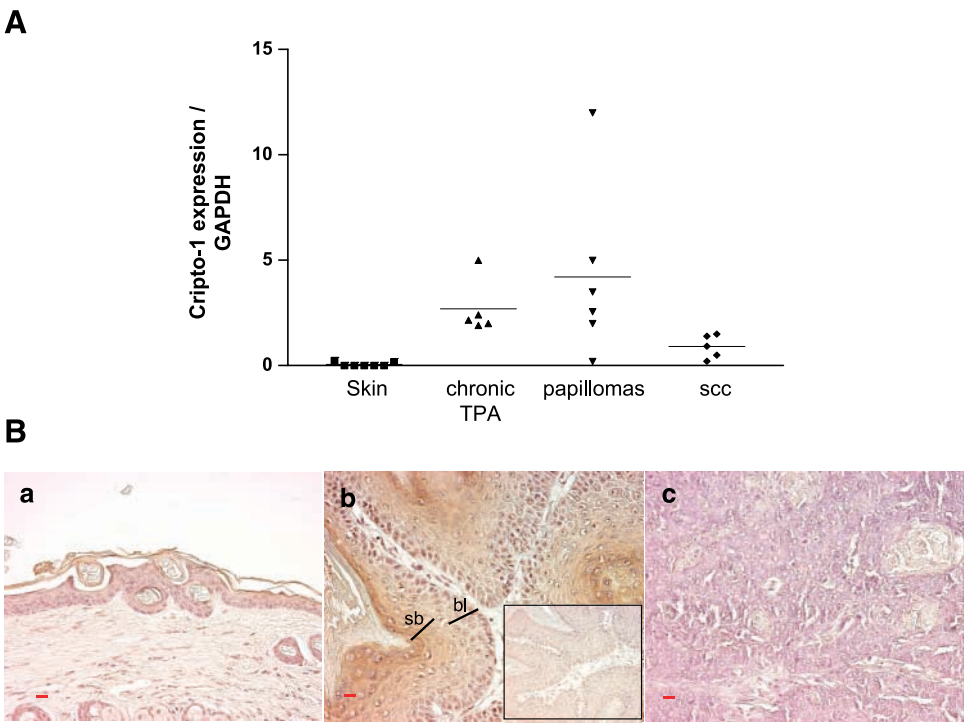


**FIGURE 1.** Cripto-1 expression is up-regulated in chemically induced skin tumors. **A.** cDNAs from multiple sections of normal skin, papillomas, squamous cell carcinomas, and skin chronically treated with TPA were analyzed for Cripto-1 expression by quantitative real-time PCR. Data are presented as Cripto-1 expression normalized to respective GAPDH levels used as an internal control ($2^{-(\Delta DCt)}$). Normal skin (control) samples had a $2^{-(\Delta DCt)}$ of 0.0000. The values are statistically different from control; chronic TPA-treated skin, $P = 0.0003$; papillomas, $P = 0.02$; and squamous cell carcinoma (*SCC*), $P = 0.003$. **B.** Formalin-fixed tissue sections from normal skin (**a**), papillomas (**b**), and squamous cell carcinomas (**c**) were immunostained for Cripto-1 as described in Materials and Methods. bl, basal layer; sb, suprabasal layer. **b,** inset, section of papilloma stained with secondary antibody alone. Bar, 15 μm.

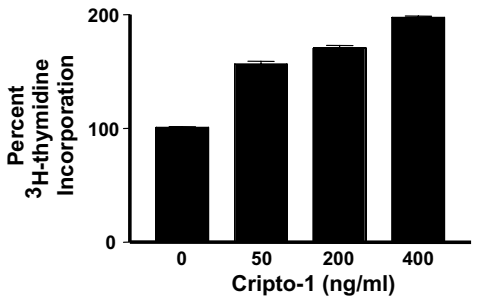


**FIGURE 2.** Cripto-1 stimulates keratinocyte proliferation. Primary BALB/c keratinocytes were treated with Cripto-1 for 24 h. DNA synthesis was measured in quadruplicate wells using a [$^3$H]thymidine incorporation assay. Columns, percentage of the control; bars, SD.

activin B in culture conditions that normally induce keratin 8, the expression of this keratin was blocked (Fig. 4A). To test the hypothesis that TGF-β1 superfamily members regulated keratinocytes differentiation more directly, we treated primary mouse keratinocytes with the ALK5 inhibitor SB431542. Figure 4B shows that treatment with 5 μmol/L SB431542 induced expression of keratin 8 in keratinocytes, whereas induction of keratin 1 by elevated calcium was suppressed by pretreatment with SB431542 (Fig. 4C). Blocking TGF-β signaling with an adenovirus expressing a dominant negative TGF-β type II receptor also induced keratin 8 expression, suggesting the importance of TGF-β1 signaling for this pathway (Fig. 4D). We isolated RNA from keratinocytes under basal or elevated calcium conditions in the presence or absence of SB431542 and examined changes in keratin 1 and keratin 8 expression by quantitative PCR. As expected, keratin 1 mRNA levels were induced by 0.12 mmol/L $Ca^{2+}$ (Fig. 4E). Inclusion of SB431542 at the same time as 0.12 mmol/L $Ca^{2+}$ completely blocked the calcium-dependent induction of keratin 1 transcript, whereas pretreatment with SB431542 and its inclusion during the calcium switch significantly suppressed expression. In contrast, treatment of keratinocytes in 0.05 mmol/L $Ca^{2+}$ media with SB431542 caused a 50% increase in keratin 8 expression compared with untreated control. Thus, the changes in keratin 1 and keratin 8 protein levels caused by SB431542 are, at least in part, due to changes in mRNA expression.

### *Cripto-1 Inhibits TGF-β1 Signaling in Keratinocytes*

The similar effects of inhibition of TGF-β1 signaling and Cripto-1 treatment on keratinocyte differentiation markers suggested that Cripto-1 may be acting through inhibition of TGF-β1 signaling. To test this, we pretreated mouse keratinocytes with Cripto-1 for 30 minutes before addition of TGF-β1 for 1 hour, and protein lysates were analyzed for phospho-Smad2 and phospho-Smad3. Under these conditions, Cripto-1 abolished the rapid induction of phospho-Smad2 and phospho-Smad3 by TGF-β1, although this was more apparent at lower concentrations of TGF-β1 (Fig. 5A). As expected, activin A– and activin B–induced phosphorylation of Smad2 was also blocked (data not shown). Cripto-1 doses as low as 25 to 50 ng/mL showed inhibition of Smad2 and Smad3 phosphorylation with greater suppression occurring in a dose-dependent manner (Fig. 5B). The effect of Cripto-1 on TGF-β–dependent gene transcription was analyzed using pGL-SBE4-luc, a Smad binding element–containing luciferase reporter (19). Activation of SBE4-luciferase reporter construct with 200 pg/mL TGF-β1 was reduced by 50% following pretreatment with 200 ng/mL Cripto-1 (Fig. 5C).

To test if Cripto-1 blocked Smad phosphorylation by interfering with TGF-β1 interaction with its receptor, we cross-linked $^{125}$I-TGF-β1 to keratinocyte surface TGF-β1 receptors in the presence or absence of Cripto-1. Figure 5D shows that in the absence of Cripto-1, $^{125}$I-TGF-β1 bound the expected ~70-kDa and ~280-kDa proteins representing the type II and type III TGF-β1 receptors (*lane 4*). In this case, the type I receptor, although present on keratinocytes (20), was not efficiently cross-linked. A 30-minute pretreatment with Cripto-1 abolished detectable cross-linking of $^{125}$I-TGF-β1 to TGF-β1 receptors on the primary keratinocytes similar to competition with unlabeled TGF-β1. These data suggest that Cripto-1 interferes with $^{125}$I-TGF-β1 binding to its receptor, although it cannot be determined if this occurs through Cripto-1 binding to TGF-β1 or through blocking a binding site on the receptor.

### *Cripto-1 Blocks TGF-β1–Induced Senescence of Ras-Expressing Keratinocytes*

One potential mechanism of tumor suppression by TGF-β1 is induction of senescence in premalignant keratinocytes (21). We examined the effect of Cripto-1 on TGF-β1–mediated senescence using senescence-associated β-galactosidase as a marker, which is shown to be strongly correlated with an irreversible $G_1$ arrest in keratinocytes (22). Primary FVB/n keratinocytes were infected with v-*ras*$^{Ha}$ retrovirus treated with

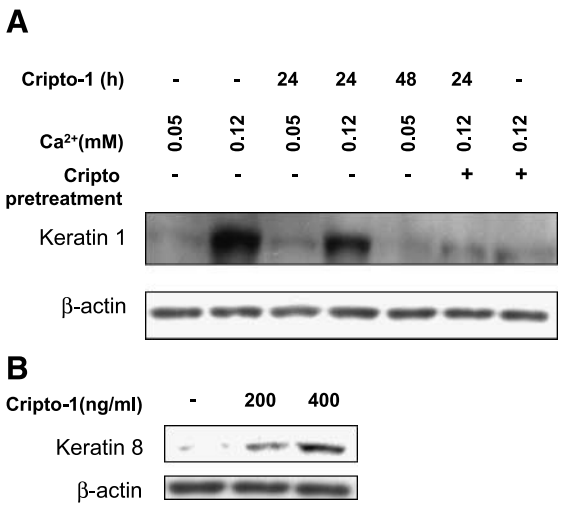


**FIGURE 3.** Cripto-1 alters keratinocyte differentiation. Primary BALB/c keratinocytes were treated as indicated and total protein extracts isolated for detection of keratin 1 or keratin 8 expression by immunoblotting, with β-actin as a loading control. **A.** Cripto-1 suppresses induction of keratin 1. Keratin 1 expression in keratinocytes cultured in media containing 0.05 mmol/L $Ca^{2+}$ (*lane 1*), switched to 0.12 mmol/L $Ca^{2+}$ media for 24 h (*lanes 2* and *4*) to induce differentiation, treated with Cripto-1 (200 ng/mL) in either 0.05 or 0.12 mmol/L $Ca^{2+}$ media (*lanes 3-5*), or pretreated with Cripto-1 (200 ng/mL) in 0.05 mmol/L $Ca^{2+}$ for 24 h before being switched to 0.12 mmol/L $Ca^{2+}$ ± Cripto-1 (*lanes 6* and *7*). **B.** Cripto-1 induces keratin 8 expression. Keratin 8 expression in keratinocytes treated with indicated concentrations of Cripto-1 for 24 h.

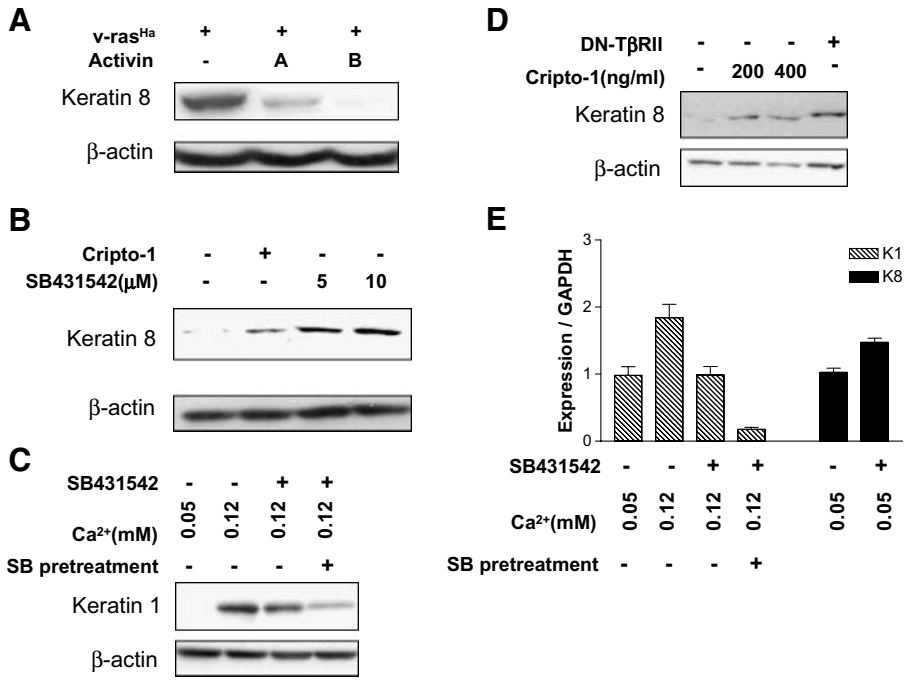


**FIGURE 4.** TGF-β signaling blockade alters keratinocyte differentiation. **A.** Activin A and activin B inhibit keratin 8 expression in preneoplastic keratinocytes. Primary BALB/c keratinocytes were transduced with v-*ras*$^{Ha}$ and treated with activin A or activin B (5 ng/mL). Protein lysates were immunoblotted for keratin 8. **B**. Inhibition of ALK5 kinase induces keratin 8. Keratinocytes were treated with indicated concentrations of TGF-β1 signaling inhibitor SB431542 for 24 h, and lysates analyzed for keratin 8 expression. **C.** Inhibition of ALK5 kinase blocks keratin 1 induction. Keratinocytes were cultured in 0.05 mmol/L $Ca^{2+}$ (*lane 1*) and switched to 0.12 mmol/L $Ca^{2+}$ to induce differentiation (*lane 2*). Cells were treated with SB431542 (5 μmol/L) after switching to 0.12 mmol/L calcium (*lane 3*) or were pretreated for 24 h with SB431542 (5 μmol/L) before switching to high calcium + SB431542 (*lane 4*), and protein lysates immunoblotted for keratin 1 expression. **D.** Keratin 8 is induced by expression of a dominant negative TGF-β type II receptor. Keratinocytes were infected with an adenovirus expressing a dominant negative TGF-β type II receptor (*DN-TβRII*) or treated with indicated concentrations of Cripto-1, and lysates were analyzed for keratin 8. **E.** Inhibition of ALK5 kinase regulates keratin 1 and keratin 8 expression at the transcriptional level. cDNAs from primary keratinocytes treated as indicated were analyzed for keratin 1 and keratin 8 expression by quantitative real-time PCR. Data are presented as fold change in gene expression normalized to GAPDH and to respective keratin expression of the control 0.05 mmol/L $Ca^{2+}$ sample. The relative level of expression of the keratin 8 transcript was ~25-fold lower than that of keratin 1.

recombinant Cripto-1 and TGF-β1, and senescent cells were identified using senescence-associated β-galactosidase as a marker as described in Materials and Methods. Figure 6 shows that 11 days after ras infection, 28% of TGF-β1–treated cells, compared with 15% of the control, expressed senescence-associated β-galactosidase. Cripto-1 (400 ng/mL) treatment of ras-infected keratinocytes suppressed the TGF-β1–induced senescence by 40%. Reduction in senescence would potentiate the tumor-promoting ability of Cripto-1 and could be one of the mechanisms used by Cripto-1 to enhance tumor progression.

## Discussion

Normal epidermal keratinocytes express keratin 1 when induced to differentiate *in vitro* with elevated calcium, and this parallels the expression of keratin 1 in the first suprabasal layer of the epidermis (23). Expression of normal differentiation markers such as keratin 1 is lost during progression of mouse squamous tumors (24). In contrast, keratin 8 is not expressed in normal squamous epithelia but is a marker for simple epithelia that is reexpressed on malignant conversion of keratinocytes (25, 26). Our results show that Cripto-1 blocks normal differentiation marker expression and induces aberrant differentiation marker expression in mouse keratinocytes through its ability to block TGF-β binding to its receptor, phosphorylation of Smad2 and Smad3, and downstream signaling. This effect on keratinocyte differentiation seems to be a general consequence of the inhibition of TGF-β1 signaling because treatment of keratinocytes with the ALK5 small-molecule inhibitor SB431542 or a dominant negative type II receptor also suppressed calcium-mediated induction of keratin 1 and induced keratin 8. We have previously shown that TGF-β2 is expressed in the differentiating layers of the epidermis and is induced during calcium-mediated differentiation of keratinocytes *in vitro* (27). Because activin itself blocked expression of keratin 8 in preneoplastic keratinocytes, these data do not rule out effects of Cripto-1 on endogenous activin signaling. Although other known oncogenes/tumor promoters, including an activated ras oncogene and EGF receptor ligands, also suppress keratin 1 and induce keratin 8 expression (28, 29), our results suggest that activation of TGF-β signaling may be important for the normal expression of the squamous differentiation phenotype and suppression of a simple epithelial phenotype. The enhanced expression of Cripto-1 in benign papillomas suggests that it may function to block normal differentiation in these benign tumors. Thus, the loss of TGF-β1 and TGF-β2 protein expression, which occurs in benign skin tumors that rapidly progress to squamous cell

carcinoma (30), may be causally linked to the altered differentiation profile of these tumors.

Our *in vitro* data also suggest that Cripto-1 could play a role in malignant conversion as well through its ability to block TGF-β1 signaling. We have shown that inhibition of TGF-β signaling by genetic or biochemical means blocks senescence of v-*ras*$^{Ha}$ oncogene–expressing mouse keratinocytes *in vitro* and causes rapid malignant progression *in vivo* (17, 21, 31). Our results here show that Cripto-1, by virtue of its inhibition of TGF-β1 signaling, also blocks senescence of v-*ras*$^{Ha}$ keratinocytes and thus could be an additional mechanism through which Cripto-1 elevation could affect tumor progression.

Cripto-1 is highly expressed in human gastric, breast, ovarian, pancreatic, colorectal, and other carcinomas (14). The overexpression of Cripto-1 induces transformation and enhances cell motility and branching morphogenesis in mouse mammary epithelial cells *in vitro* (15, 16). Our data show for the first time that Cripto-1 expression is elevated in tumor promoter–treated skin and chemically induced mouse skin papillomas. The higher Cripto-1 level in TPA-treated normal skin and papillomas compared with squamous cell carcinomas, its expression in the differentiating layer of the tumor, and its ability to block keratinocyte differentiation *in vitro*, along with similar effects of inhibition of TGF-β1 signaling, suggest that Cripto-1 may act to promote tumor formation by blocking the normal function of TGF-β signaling as a regulator of normal squamous differentiation and cell cycle control, and promote malignant conversion by interfering with TGF-β1–regulated senescence of *ras* oncogene expressing keratinocytes.

## Materials and Methods

### *Cell Culture*

Primary keratinocytes from newborn BALB/c or FVB/n mice were prepared and cultured according to established methods (32) in Eagle's MEM supplemented with 0.05 mmol/L $CaCl_2$ and 8% chelexed fetal bovine serum. Soluble recombinant

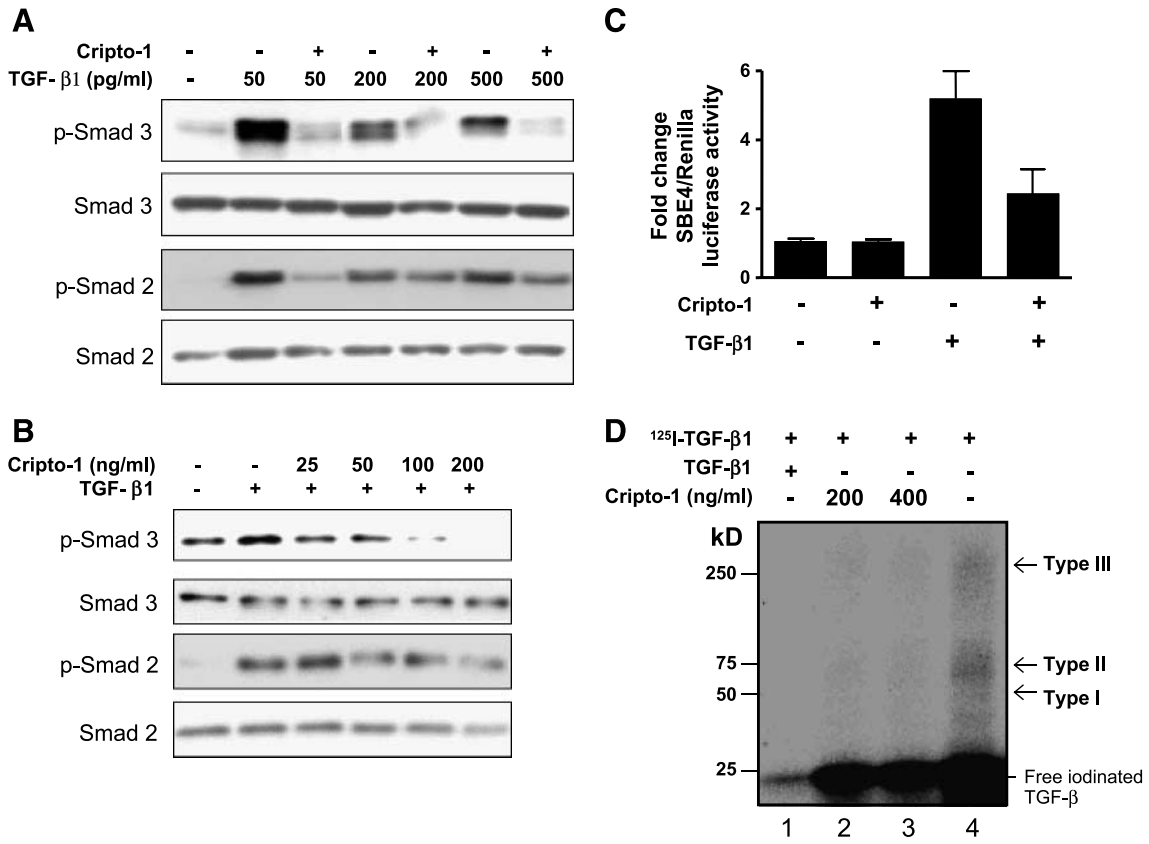


**FIGURE 5.** Cripto-1 inhibits TGF-β1 signaling. **A.** Cripto-1 blocks TGF-β1–induced phosphorylation of Smad2 and Smad3. Primary BALB/c keratinocytes were treated with indicated concentrations of TGF-β1 for 1 h, with (+) or without (–) a 30-min pretreatment with Cripto-1 (200 ng/mL). Protein lysates were analyzed for phospho and total Smad2 and Smad3 by Western blot. **B**. Inhibition of TGF-β1–induced phosphorylation of Smad2 and Smad3 by Cripto-1 is Cripto-1 dose dependent. Primary BALB/c keratinocytes were pretreated for 30 min with various doses of Cripto-1 (25-200 ng/mL) followed by treatment with TGF-β1 (50 pg/mL) for 1 h. Protein lysates were analyzed for phospho and total Smad2 and Smad3 by Western blot. **C**. Cripto-1 blocks TGF-β1–induced SBE4-luciferase reporter activity. Keratinocytes were transfected with the TGF-β reporter plasmid pGL-SBE4-luc and control plasmid pRL-TK. Cells were pretreated with Cripto-1 (200 ng/mL) for 8 h before addition of TGF-β1 (200 pg/mL) for 16 h. Luciferase activity was determined in cell extracts using dual luciferase reporter assay system (Promega) and normalized to Renilla luciferase activity. Columns, fold change of control activity. **D**. Cripto-1 blocks binding of TGF-β1 to surface receptors. BALB/c keratinocytes were pretreated with Cripto-1 for 30 min (200 and 400 ng/mL; *lanes 2* and *3*), labeled with $^{125}$I-TGF-β1, and subjected to chemical cross-linking as described in Materials and Methods. A 100-fold molar excess of unlabeled TGF-β1 was used to compete with the radiolabeled TGF-β1 (*lane 1*) to determine specificity of receptor binding. Equal amounts of protein were electrophoresed on SDS-PAGE and visualized by autoradiography. Arrows, bands corresponding to $^{125}$I-TGF-β1 cross-linked complexes.

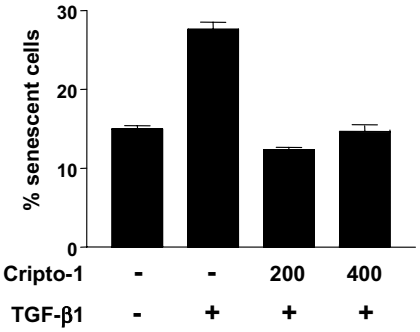


**FIGURE 6.** Cripto-1 inhibits TGF-β1–mediated senescence in v-*ras*$^{Ha}$–expressing primary keratinocytes. Primary FVB/n keratinocytes were transduced with v-*ras*$^{Ha}$ and treated with TGF-β1 ± Cripto-1 4 d after viral infection. Triplicate wells were fixed 11 d after ras transduction and assayed for senescence by staining for senescence-associated β-galactosidase as described in Materials and Methods. Columns, percentage of the total cell number; bars, SD.

Cripto-1 has previously been shown to have similar biological activities as the membrane-bound form (33) and was therefore used for *in vitro* assays in this study. Keratinocytes were treated with indicated concentrations of recombinant mouse Cripto-1 (R&D Systems) and/or TGF-β1 (R&D Systems) and SB431542 (5 μmol/L) for indicated periods of time. To induce differentiation, keratinocytes were treated with SB431542 (5 μmol/L) and/or Cripto-1 (200 ng/mL) for 24 h before or after switching cells from growth media containing 0.05 mmol/L $Ca^{2+}$ to differentiation media containing 0.12 mmol/L $Ca^{2+}$. For genetic blockade of TGF-β signaling, keratinocytes were transduced with 20 multiplicity of infection of a dominant negative TGF-β type II receptor expressing adenovirus for 16 h before cell lysates were prepared. Adenoviruses were amplified using QBI 293 cells and purified over two CsCl gradients.

### *Protein Extraction, Western Blotting, and Antibodies*

Protein extracts for detection of keratins were prepared by scraping the cells in cell lysis buffer containing 0.25 mol/L Tris, 5% SDS, 20% β-mercaptoethanol, 10 μg/mL leupeptin and pepstatin, 0.2 units/mL aprotinin, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium orthovanadate, and 1 mmol/L sodium fluoride, followed by heating the lysates at 98°C for 5 min. Smad proteins were isolated in Cell Lysis Buffer (Cell Signaling Technologies) supplemented with protease and phosphatase inhibitors. Equal amounts of total cell protein were separated by electrophoresis on SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were probed with anti–β-actin antibody to ensure even loading and transfer. The immunoblots were visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc.). The antibodies used were phospho-Smad2 (Cell Signaling Technology), phospho-Smad3 (Cell Signaling Technology), β-actin (Bio-Rad Laboratories), keratin 8 (University of Iowa), Smad2, and Smad3 (Zymed Laboratories, Inc.).

### *Skin Tumor Generation and TPA Treatment*

Tumors were induced in 7- to 8-wk-old SENCAR mice using a one-time 7,12-dimethylbenz(*a*)-anthracene treatment (5 μg) and repeated TPA (2 μg) treatments for 14 to 15 wk as described (34). Papillomas were collected after 14 to 15 wk of promotion whereas squamous cell carcinomas were harvested as they appeared. To obtain samples of TPA-treated skin, 7- to 8-wk-old SENCAR mice were treated topically 1×/wk with 2 μg of TPA for 5 wk and skins were harvested 3 d after last TPA treatment.

### *Reverse Transcription-PCR*

RNA was isolated from primary mouse keratinocytes and tissue samples of murine skin, papillomas, and carcinomas using Trizol following the manufacturer's protocol (Invitrogen). RNA was subjected to DNase I (Ambion) treatment. For cDNA synthesis, 500 ng of total RNA were reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). For real-time PCR analysis, the expression levels of Cripto-1, keratin 8, keratin 1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined with a Bio-Rad iCycler iQ and Gene Expression Macro (version 1.1; Bio-Rad). iQ SYBR Green Supermix (Bio-Rad) was used for PCR amplification. The primer sequences were as follows: Cripto-1, TTTGGACCCGTTGCTGGGAGAGA and AGCTAGCATAAAAGTGGTCGTCA; keratin 8, TGCAGAACATGAGCATTC and CAGAGGATTAGGGCTGAT. Keratin 1 primers were from Superarray. GAPDH primers were from Gene Link. Relative standard curves were generated from log input (serial dilutions of cDNA) versus the cycle threshold (Ct). The slope of the standard curve was used to determine the efficiency of target amplification (35). Relative quantitation was used to calculate the $2^{-(\Delta DCt)}$ formula, where ΔDCt represents the cycle difference corrected for GAPDH, used as an internal control.

### *Immunohistochemistry*

Cripto-1 was detected in formalin-fixed tissue sections of normal mouse skin, papillomas, and carcinomas collected as described in "Skin Tumor Generation and TPA Treatment." Sections were immunostained with rabbit polyclonal antibody to Cripto-1 (Abcam). Bound antibody was detected with biotinylated antirabbit secondary antibody and the Vectastain Elite kit (Vector Laboratories). Parallel tissue sections incubated with secondary antibody alone, followed by treatment with Vectastain Elite for color development, were used as negative control. The tissue sections depicted in Fig. 1B are representative of at least six samples stained similarly.

### *[$^3$H]Thymidine Incorporation Assay*

To assay for DNA synthesis, primary mouse keratinocytes were plated in 24-well tissue culture plates and treated with Cripto-1 in quadruplicate for 24 h. [$^3$H]Thymidine (1 μCi/well; GE Healthcare) was added to the wells 21 h after addition of Cripto-1 and incubated for 3 h. Cells were fixed with methanol and acetic acid (in a 3:1 ratio) and solubilized in 5 N NaOH. Incorporated counts were measured with a scintillation counter.

### *Luciferase Assay*

Luciferase assay was carried out using TGF-β1 responsive Smad binding element containing luciferase reporter pGL-SBE4-luc (19). Primary BALB/c keratinocytes, plated in

12-well culture plates, were transfected with pGL-SBE4-luc (2.0 μg/well). To control for transfection efficiency, 0.2 μg/well of the Renilla luciferase reporter plasmid pRL-TK was cotransfected. Cells were pretreated in triplicate with Cripto-1 for 8 h before addition of TGF-β1 at 30 h after transfection. Cells were harvested 16 h after TGF-β1 treatment and luciferase activity was determined in cell extracts using the Dual Luciferase Reporter assay system (Promega) and normalized to Renilla luciferase activity.

### *TGF-β1 Receptor Cross-Linking*

Primary mouse keratinocytes were plated in six-well tissue culture plates and pretreated with or without 200 and 400 ng/mL Cripto-1 for 30 min before addition of $^{125}$I-labeled TGF-β1 (GE Healthcare). TGF-β1 was cross-linked to its receptors using the chemical cross-linker disuccinimidyl suberate according to previously described methods (36). A 100-fold molar excess of unlabeled TGF-β1 was used to compete with the radiolabeled TGF-β1 to determine the specificity of receptor binding. Equal amounts of protein were electrophoresed on SDS-PAGE and visualized by autoradiography.

### *β-Galactosidase Senescence Assay*

The v-*ras*$^{Ha}$ retrovirus was generated from ψ2 producer cells as previously described (37). Primary mouse keratinocytes were plated in 12-well tissue culture plates and infected with ras virus on day 3 of culture. TGF-β1 (200 pg/mL) and different concentrations of Cripto-1 were added on day 4 after ras infection to triplicate wells. Cells were fixed 11 days after ras infection in 0.5% glutaraldehye at pH 6.0 and stained using a solution of 500 mmol/L $K_3Fe[CN]_6$, 500 mmol/L $K_4Fe[CN]_6$, and 50 mg/mL X-gal. Senescence-associated β-galactosidase–positive cells were quantitated using a Nikon inverted microscope (31).

## Acknowledgments

We thank Christina Cheng for providing tumor and normal skin RNA; Dr. David Salomon for his expert advice; Dr. Stuart Yuspa for his guidance, support, and criticism; and Dr. Luowei Li for critical reading of the manuscript and valuable suggestions.

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*Mol Cancer Res* 2008;6:509-516.