Cancer Research

The Oncoprotein c-Ski Functions as a Direct Antagonist of the Transforming Growth Factor-β Type I Receptor

Nathalie Ferrand[1], Azeddine Atfi[1,2], and Céline Prunier[1]

Abstract

The oncoprotein c-Ski has been implicated in the negative regulation of transforming growth factor-β (TGF-β) signaling owing to its ability to repress Smad transcriptional activity via recruitment of a transcriptional corepressor complex containing histone deacetylases. However, c-Ski has also been shown to localize to the cytoplasm, raising the interesting possibility that it might disable TGF-β signaling through alternative mechanisms. Here, we provide evidence that c-Ski can restrict TGF-β signaling by interacting directly with the activated TGF-β type I receptor (TβRI). We explored the physiologic relevance of the c-Ski/TβRI interaction and found that it can culminate in a constitutive association of TβRI with a nonfunctional R-Smad/Smad4 complex. Based on these findings, we hypothesize that the interaction between c-Ski and TβRI might interfere with nuclear translocation of the R-Smad/Smad4 complex, thereby attenuating TGF-β signaling. Such a mechanism may play a crucial role in tumor progression, because many tumors that express high levels of c-Ski also display impaired nuclear accumulation of Smads. *Cancer Res; 70(21); 8457–66. ©2010 AACR.*

Introduction

Because of its critical role in key biological processes such as embryogenesis, differentiation, and proliferation, the transforming growth factor β (TGF-β) signaling pathway is subjected to many levels of regulation (1). Among the negative regulators of this signaling, c-Ski plays an important role in carcinogenesis. Indeed, overexpression of c-Ski induces morphologic transformation in chicken and quail embryo fibroblasts, and elevated levels of c-Ski have been detected in human tumors (2–7).

TGF-β signaling through Smads is initiated when the TGF-β type I receptor (TβRI) kinase, activated by the type II receptor (TβRII) kinase, directly phosphorylates the R-Smads, Smad2 and Smad3, which then form complexes with Smad4 and move into the nucleus to regulate transcription (1). c-Ski has been shown to associate with Smad2, Smad3, and Smad4 (8–11). As c-Ski also associates with the corepressors N-CoR and Sin3A (12), it has been postulated that the mechanism through which c-Ski suppresses TGF-β signaling relies on repression of Smad transcriptional activity (13). However, several recent studies have shown that c-Ski also resides in the cytoplasm, suggesting that alternative mechanisms of repression of TGF-β signaling by c-Ski might also exist (6, 7, 14–17). Consistent with this, previous data from our laboratory and others have indicated that expression of c-Ski can induce a constitutive association of Smad4 with Smad2 or Smad3 (15, 18). Moreover, Wu and colleagues reported that c-Ski can prevent nuclear accumulation of a functional Smad2/Smad4 complex (19). Such evidence that c-Ski may operate via suppression of Smad signaling at early steps upstream of transcriptional regulation raises fundamental questions about the regulatory mechanisms whereby c-Ski fulfills its inhibitory function in the TGF-β signaling pathway.

We show here that c-Ski, in addition to interacting with Smads, interacts with TβRI and suppresses nuclear accumulation of the R-Smad/Smad4 complex, which occurs as a consequence of retention of this complex by TβRI. The ability of c-Ski to induce accumulation of a nonproductive R-Smad/Smad4 complex through its association with TβRI provides new insights suggesting that c-Ski might act by two independent mechanisms to suppress TGF-β signaling.

Materials and Methods

Cell culture

Normal human mammary HMT3522-S1 cells were generous gifts of M.J. Bissell (Lawrence Berkeley National Laboratory, Berkeley, CA), A427 cells were obtained from Deutsche Sammlung Von Mikroorganismen und Zellkulturen and all other cell lines were obtained from the American Type Culture Collection. All cell lines were maintained in DMEM supplemented with 10% FCS except HMT3522-S1 cells that were maintained as previously described (20). To establish the doxycycline-inducible 293T cell line, cells were transfected with pcDNA6/TR encoding the doxycycline transactivator and selected with blasticidin. Cells that expressed high level

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of the transactivator were transfected with pcDNA5/TO-Flag-c-Ski or pcDNA5/TO-LacZ and selected with hygromycin. To establish 293T cells stably expressing short hairprin RNA (ShRNA)–c-Ski, cells were transfected with pBLOCKiT-shRNA-c-Ski and selected with neomycin.

Immunoprecipitation and immunoblotting

Cells were transfected by Lipofectamine (Invitrogen) and lysed at 4°C in Tris-NaCl-MgCl2-glycerol buffer (21). Cell lysates were subjected to immunoprecipitation with the appropriate antibody for 2 hours, followed by adsorption to Sepharose-coupled protein G for 1 hour. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

Cytosolic and nuclear fractionation

Cells were lysed in hypotonic buffer (21) for 15 minutes at 4°C and centrifuged at 1,000 rpm for 10 minutes. The supernatant was collected and designated as the cytosolic fraction. The pellet was resuspended in nuclear buffer (21), incubated for 20 minutes at 4°C, and centrifuged at 13,000 rpm for 5 minutes. The supernatant was collected and designated as the nuclear fraction.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 30 minutes and permeabilized in 0.1% Saponin. They were incubated overnight at 4°C with the indicated antibodies, followed by the secondary AlexaFluor antibodies (594 nm and 488 nm; Invitrogen) and examined on a fluorescence microscope.

Immunohistochemistry

One hundred sections from skin tissue array (US Biomax) were studied. Briefly deparaffinized, sections were boiled 20 minutes in 10 mmol/L citrate buffer (pH6) 0.05% Tween 20 and blocked in 5% normal goat serum for 20 minutes. The sections were incubated with a mix of primary antibodies overnight at 4°C followed by a mix of secondary AlexaFluor antibodies. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole. The sections were washed and were cover-slipped with Fluorsave mounting medium (EMD Chemicals).

In vitro translation

In vitro translation of 6xMyc-Ski, 6xMyc-Smad1, and TβRI was performed with the TNT Coupled-Reticulocyte Lysate System (Promega) in the presence of [^{35}S]methionine. Samples were then immunoprecipitated with anti-Myc, subjected to SDS-PAGE, and analyzed by autoradiography.

Results

c-Ski interacts with TβRI

The mechanism of c-Ski–mediated suppression of TGF-β was initially attributed to its ability to recruit to Smad proteins a transcriptional corepressor containing histone deacetylases (HDAC) (12, 13). However, there is now overwhelming evidence that c-Ski also accumulates in the cytoplasm (14, 16, 21–23), raising the attractive possibility that c-Ski might suppress TGF-β signaling by an alternative mechanism that is distinct from transcriptional repression. To probe this possibility, we investigated whether c-Ski could interact with other cytoplasmic components of the TGF-β signaling pathway, in addition to Smads. To our surprise, we observed that c-Ski can interact with TβRI (Fig. 1A). Comparative studies using wild-type and a constitutively activated TβRI (TβRI.act) indicated that c-Ski displayed enhanced affinity for the activated TβRI (Fig. 1A). Similar results were obtained with SnoN, which is closely related to c-Ski and also functions as an inhibitor of TGF-β signaling (Fig. 1A). The interaction between TβRI and c-Ski or SnoN seems to be specific for members of the Ski family, as TβRI failed to interact with TG-interacting factor (TGIF), another unrelated negative regulator of TGF-β signaling (ref. 1; Fig. 1A). Furthermore, the association of c-Ski with TβRI is likely to be direct, as *in vitro* translated TβRI could coprecipitate *in vitro* translated c-Ski (Fig. 1B). In a control experiment, Smad1 was unable to interact with TβRI, showing the specificity of the association of c-Ski with TβRI (Fig. 1B).

To analyze the interaction of c-Ski with TβRI in more detail, various c-Ski deletion mutants were tested for interaction with TβRI. As shown in Fig. 1C, loss of the first 337 amino acids did not interfere with c-Ski binding to TβRI, whereas a further deletion that removes the first 490 amino acids completely abolished the interaction. A similar analysis of COOH-terminal truncations revealed that a fragment containing amino acids 1 to 309 was unable to associate with TβRI, whereas a fragment containing amino acids 1 to 490 retained the capacity to interact with TβRI (Fig. 1C). Taken together, these results suggest that the TβRI-interacting region of c-Ski maps to the middle domain of c-Ski between residues 338 and 490 (Fig. 1C).

To ascertain the physiologic relevance of the c-Ski/TβRI interaction, we examined the endogenous interaction between c-Ski and TβRI. In anti-TβRI immunoprecipitates from Mv1Lu cells, we could detect c-Ski coprecipitating with TβRI and this interaction was increased by TGF-β (Fig. 2A). Similar results were obtained with HepG2 and 293 cells (Fig. 2A), further supporting the hypothesis that c-Ski and TβRI may form a physiologic complex whose level is increased by TGF-β. Consistent with this, we observed that activation of TGF-β signaling induced translocation of c-Ski from the nucleus to the cytoplasm, where the association of c-Ski and TβRI takes place (Fig. 2B).

c-Ski interacts with TβRI in a manner independent of Smad2 and Smad4

Because Smad2 and Smad4 directly interact with c-Ski (8–11), we examined the possibility that they may facilitate the association of c-Ski with TβRI. Remarkably, neither Smad2 nor Smad4 overexpression increased the abundance of the c-Ski/TβRI complex (Fig. 2C). In an alternative experimental approach, we compared the association of c-Ski deletion mutants with TβRI versus Smad2 or Smad4. As shown in Fig. 2C, the c-Ski fragment 1–309, which is defective in its ability to interact with TβRI (Fig. 1C), associates with Smad2 and Smad4. Conversely, we were unable to detect an interaction of

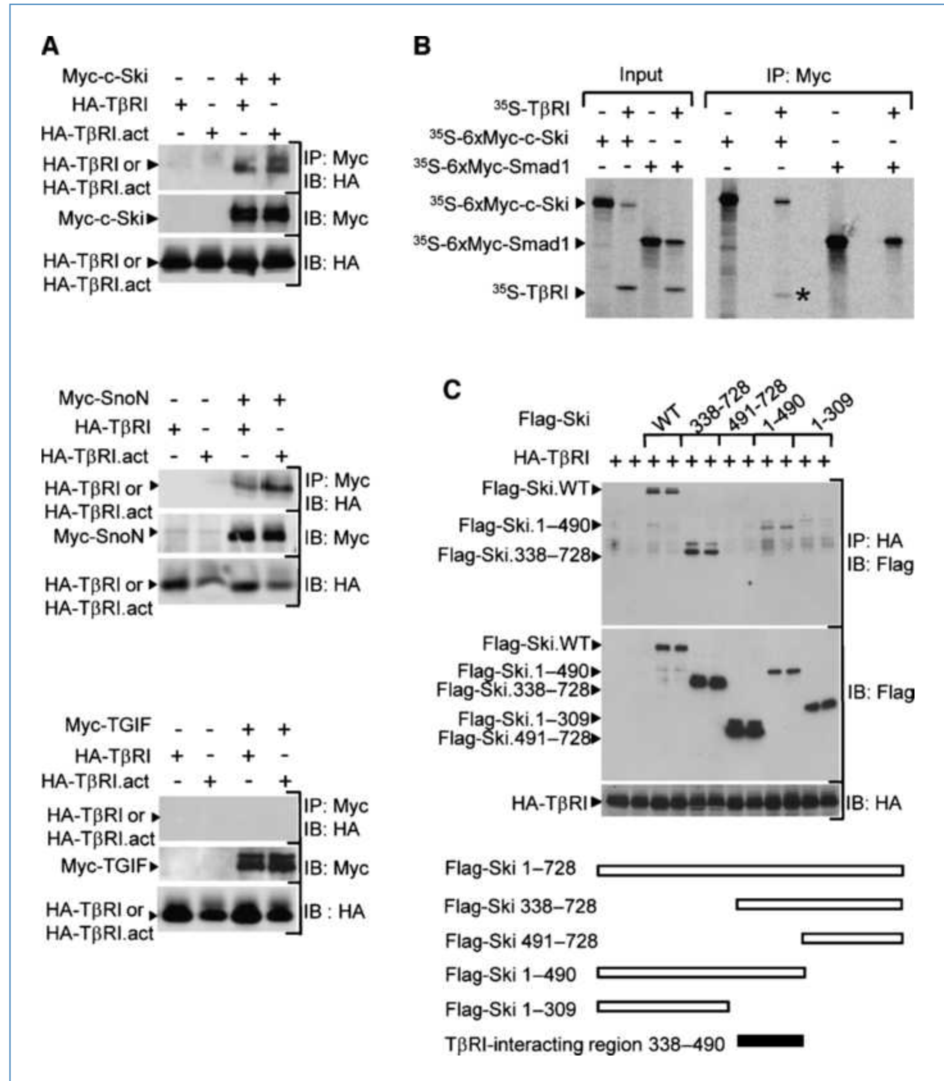

Figure 1. Association of c-Ski with TβRI. A, lysates from transfected COS-7 cells were immunoprecipitated (IP) with anti-Myc directed towards c-Ski (top), SnoN (middle), or TGIF (bottom) and immunoblotted (IB) with anti-hemagglutinin (HA) for the presence of TβRI or TβRI.act. B, *in vitro* interaction of TβRI with 6xMyc-c-Ski or 6xMyc-Smad1 (negative control) was performed by immunoprecipitating [^{35}S]methionine-labeled proteins with anti-Myc. Samples were resolved by SDS-PAGE and bound proteins were visualized by autoradiography. C, lysates from transfected COS-7 cells were immunoprecipitated with anti-HA directed towards TβRI and immunoblotted with anti-Flag that recognizes various Ski fragments. WT, wild-type.

Smad2 or Smad4 with the c-Ski fragment 338–728, which strongly interacts with TβRI (Figs. 1C and 2C). We concluded from these experiments that c-Ski contains discrete binding sites for TβRI and Smad2 or Smad4, which would explain why c-Ski binds to TβRI independently of Smad2 or Smad4.

To further corroborate these findings, we investigated whether disruption of the association of TβRI with Smad2 could disable assembly of the c-Ski/TβRI complex. To test this possibility, we took advantage of the availability of a TβRI mutant, TβRI.mL45.act, which has a constitutive active kinase activity, but no longer associates with Smad2 (24). We found that c-Ski interacts with TβRI.mL45.act with efficiency similar to that of wild-type TβRI.act, providing further evidence that the association of c-Ski with TβRI does not depend on the interaction of TβRI with Smad2 (Fig. 2D). We also investigated whether disruption of the c-Ski/Smad4 interaction could compromise the c-Ski/TβRI interaction by employing c-Ski.H262A, a c-Ski mutant previously shown to be defective in its ability to interact with Smad4 (19). As may be seen in Fig. 2D, TβRI strongly interacts with c-Ski.H262A, showing that the c-Ski/TβRI interaction occurs in the absence of the c-Ski/Smad4 interaction.

The association of c-Ski with TβRI promotes the accumulation of a nonproductive R-Smad/Smad4 complex

Previous findings indicated that R-Smads transiently associate with TβRI because their phosphorylation provokes their dissociation from this receptor (25). In light of our precedent data highlighting a direct interaction between c-Ski and TβRI, we wondered whether expression of c-Ski could affect the transient association of R-Smads with TβRI. In agreement with a published observation (25), we were unable to detect an interaction between Smad2 or Smad3 and TβRI (Fig. 3A). Under the same experimental conditions, we observed that enforced expression of c-Ski resulted in a constitutive association of Smad2 or Smad3 and TβRI (Fig. 3A). Collectively, these results suggest that c-Ski might prevent

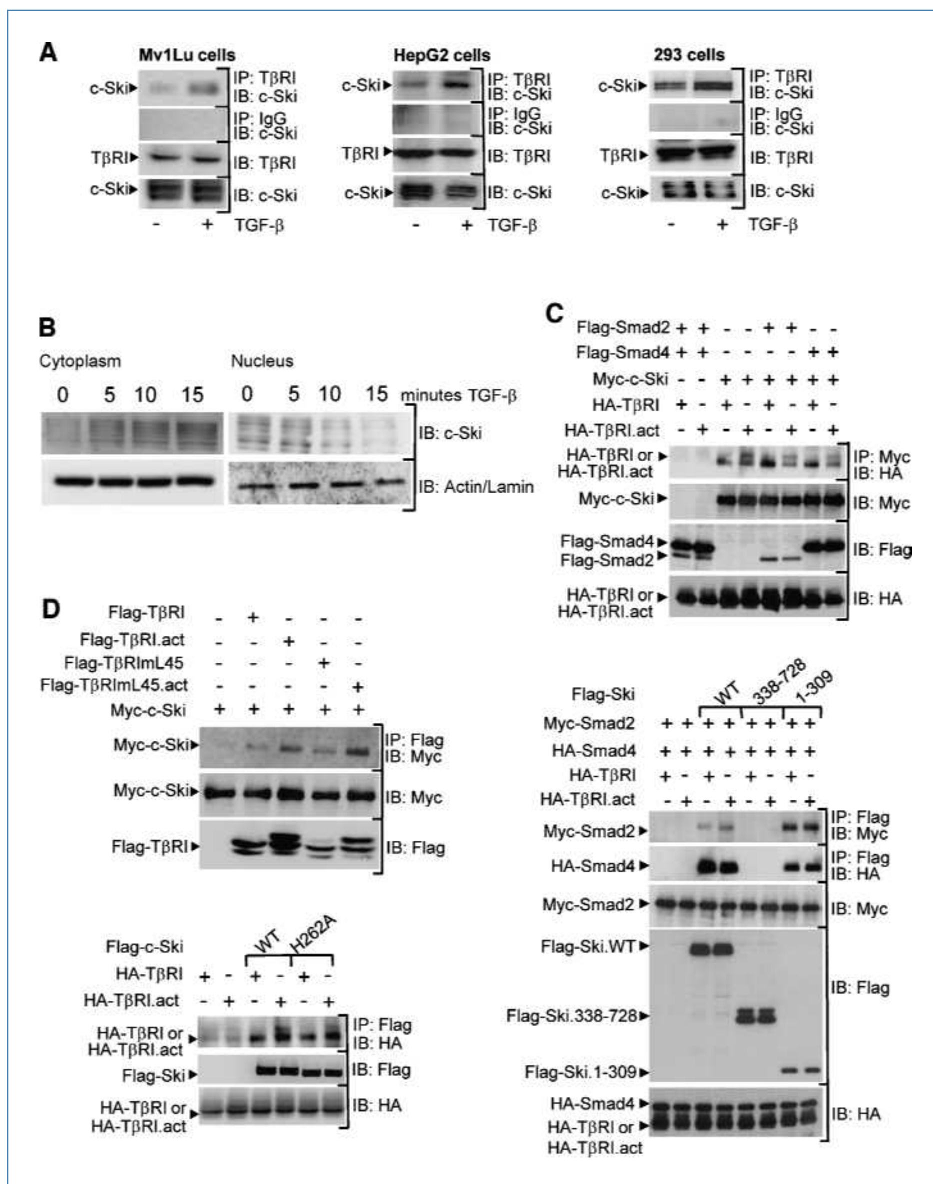

Figure 2. Endogenous association of c-Ski and TβRI. A, Mv1Lu, HepG2, and 293 cell lysates were immunoprecipitated with rabbit IgG or anti-TβRI followed by immunoblotting with anti-Ski. B, 293 cells were treated with TGF-β for the indicated time and immunoblotted with anti-c-Ski, anti-lamin (nucleus), or anti-actin (cytoplasm). C, lysates from transfected COS-7 cells were immunoprecipitated with anti-Myc (top) directed towards c-Ski or anti-Flag (bottom) directed towards various c-Ski fragments, and were immunoblotted with anti-HA (top) that recognizes HA-TβRI and HA-TβRI.act or with anti-Myc and anti-HA (bottom) that recognize Smad2 and Smad4, respectively. D, lysates from transfected COS-7 cells were immunoprecipitated with anti-Flag directed towards TβRI (top) or c-Ski mutants (bottom) and immunoblotted with anti-Myc that recognizes Myc-c-Ski (top) or with anti-HA that recognizes HA-TβRI and HA-TβRI.act (bottom).

the dissociation of R-Smad from TβRI, which could contribute to its ability to suppress TGF-β/Smad signaling.

Next, we investigated the effect of c-Ski on the association of Smad2 with TβRI under physiologic conditions. It is noteworthy that the association of Smad2 with TβRI is transient and cannot be visualized *in vivo* without disrupting the phosphorylation site in Smad2 (SSMV), because phosphorylation of Smad2 decreases the affinity of Smad2 for TβRI (25). This fact precluded the possibility of any investigation of the association between endogenous TβRI and Smad2 in cells deficient in endogenous c-Ski. To circumvent this limitation, we examined whether controlled expression of c-Ski could stabilize the association between endogenous Smad2 and TβRI. To this end, we engineered a 293 cell line ectopically expressing a doxycycline-inducible c-Ski (293-TR-c-Ski). Induction of c-Ski efficiently suppressed TGF-β–induced transcriptional activation (Supplementary Fig. S1), attesting to the functionality of c-Ski in this cell line. Crucially, we found that induction of c-Ski stabilized the association of endogenous Smad2 and TβRI (Fig. 3B). In the course of these analyses, we also employed HepG2 cells in which TGF-β–induced signaling is highly sensitive to the amount of c-Ski, as shown by a dramatic decrease in TGF-β–induced expression of endogenous PAI-1 and JunB upon overexpression of c-Ski (Supplementary Fig. S2). As in the doxycycline-inducible 293 cells, ectopic expression of c-Ski enhanced the association of endogenous Smad2 and TβRI, and this was further increased in response to TGF-β signaling (Fig. 3C).

Previous studies by our laboratory and others have shown that overexpression of c-Ski culminates in a constitutive

formation of a nonproductive R-Smad/Smad4 that no longer accumulates into the nucleus (14, 15, 26). These observations together with the findings that c-Ski facilitates the formation of a stable TβRI/R-Smad complex prompted us to consider the possibility that c-Ski may induce the formation of a nonfunctional complex comprising R-Smad, Smad4, and TβRI. In transient transfections, we observed that Smad2 or Smad3 can simultaneously interact with cotransfected Smad4 and TβRI in cells overexpressing c-Ski, suggesting that c-Ski may enforce the retention of a nonfunctional R-Smad/Smad4 complex at the level of TβRI (Fig. 3A). Similar results were obtained when the effect of c-Ski on endogenous Smad2/Smad4, Smad2/TβRI, or Smad4/TβRI complexes was examined (Fig. 3C). This phenomenon seems to be specific to members of the c-Ski family, as expression of SnoN could also promote the constitutive association of the Smad2/Smad4 complex with TβRI (Fig. 3D). On the other hand, we were unable to detect an association of the Smad2/Smad4 complex with TβRI in the presence of TGIF (Fig. 3D).

To gain further insights into the mechanism by which c-Ski prevents dissociation of Smad2 from TβRI, we investigated the requirement of the c-Ski/Smad2 interaction in this process. For this, we used v-Ski, which is defective in its ability to interact with Smad2 (27). As shown in Fig. 4A, v-Ski increased the association of Smad2 with TβRI to the same

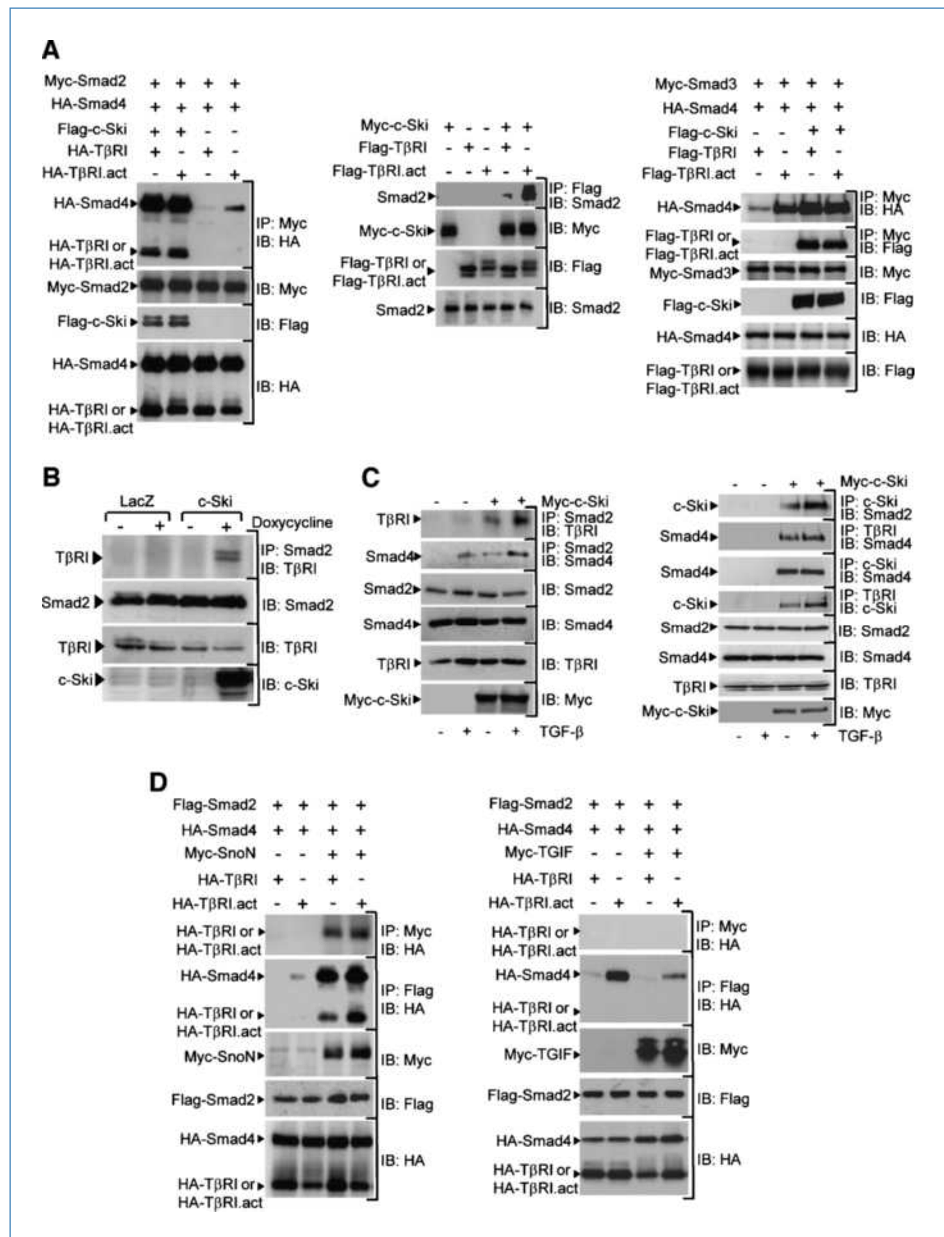

Figure 3. c-Ski induces a stable association of Smad2 with TβRI. A, lysates from transfected COS-7 cells were immunoprecipitated with anti-Myc (left and right) or anti-Flag (middle) followed by immunoblotting with anti-HA (left and right) or with anti-Smad2 (middle). B, 293-TR-c-Ski or 293-TR-LacZ cells were treated with doxycycline for 24 hours and lysates were immunoprecipitated with anti-Smad2 before immunoblotting with anti-TβRI. C, HepG2 treated with TGF-β for 1 hour were immunoprecipitated with anti-Smad2, anti-TβRI, or anti-c-Ski, and blotted with anti-TβRI, anti-Smad4, anti-Smad2, or anti-c-Ski. D, COS-7 cells were immunoprecipitated with anti-Myc directed towards SnoN (left) or TGIF (right) and with anti-Flag directed towards Smad2 and blotted with anti-HA that recognizes HA-TβRI, HA-TβRI.act, and Smad4.

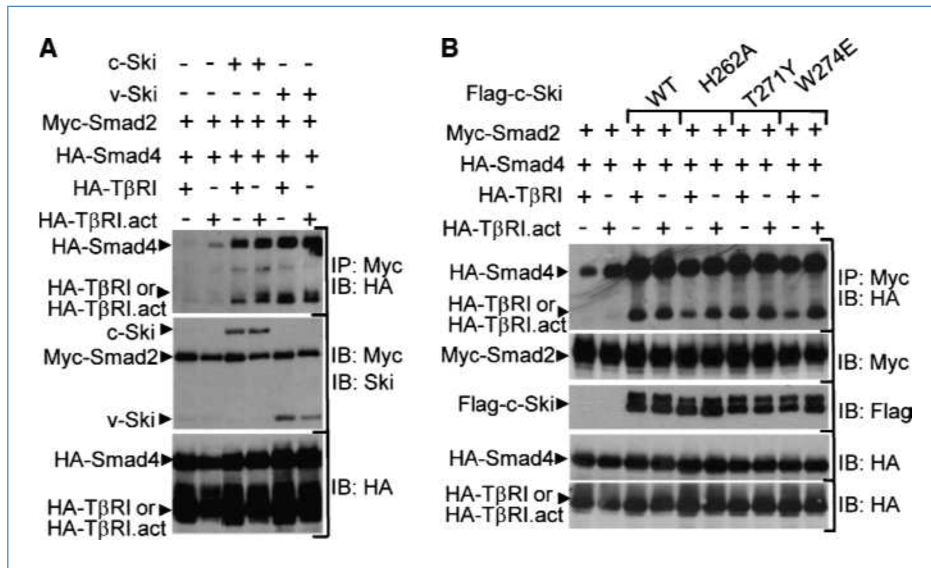

Figure 4. c-Ski induces stable association of Smad2 with TβRI in a manner independent of its association with Smad2 or Smad4. A and B, lysates from transfected COS-7 cells were subjected to immunoprecipitation with anti-Myc directed towards Myc-Smad2 before immunoblotting with anti-HA that recognizes HA-TβRI, HA-TβRI.act, and HA-Smad4.

extent as c-Ski, suggesting that the association of c-Ski with Smad2 is dispensable for the ability of c-Ski to prevent the dissociation of Smad2 from TβRI. We also investigated the requirement of the c-Ski/Smad4 interaction in c-Ski–induced constitutive interaction of Smad2 with TβRI, employing several c-Ski mutants (c-Ski.H262A, c-Ski.T271Y, c-Ski.W274E), which are defective in their ability to interact with Smad4 (19). Similar to the Smad2/c-Ski interaction, we found that the Smad4/c-Ski interaction was dispensable for c-Ski–induced association of Smad2 with TβRI, because the mutants c-Ski.H262A, c-Ski.T271Y, and c-Ski.W274E retained their capacity to interact with TβRI (Fig. 4B).

c-Ski interferes with nuclear translocation of the R-Smad/Smad4 complexes

The finding that c-Ski induces stabilization of the complex containing TβRI, R-Smad, and Smad4 prompted us to investigate whether c-Ski could prevent translocation of the R-Smad/Smad4 complex to the nucleus. As evidenced by immunofluorescence (Fig. 5A), c-Ski expression suppressed TGF-β–induced nuclear accumulation of endogenous Smad2. Cell fractionation provided further evidence that c-Ski can effectively suppress the ability of TGF-β to induce nuclear translocation of Smad2 (Fig. 5B). Similar results were obtained when the translocation of Smad3 and Smad4 was examined. Thus, it is likely that c-Ski may interfere with the dissociation of R-Smad from TβRI, thereby blocking translocation of the R-Smad/Smad4 complex into the nucleus. To confirm these findings, we generated a 293 cell line stably expressing a specific shRNA against c-Ski (293-shRNA-c-Ski; Fig. 5C). We found that depletion of c-Ski enhanced TGF-β–induced nuclear translocation of endogenous Smad2, Smad3, and Smad4. Consistent with this finding, we found that depletion of c-Ski in 293 cells also enhanced TGF-β–induced phosphorylation of endogenous Smad2 (Fig. 5C). Conversely, enforced expression of c-Ski blocked phosphorylation of Smad2 (Fig. 5B). Combined, these findings suggest that c-Ski might prevent the phosphorylation of R-Smad, leading to retention of the R-Smad/Smad4 complex in the cytoplasm.

c-Ski overexpression in tumors correlates with defective nuclear translocation of Smad2

To provide further evidence that c-Ski prevents nuclear accumulation of the Smad complex, we took advantage of previous observations that some types of human cancers, such as melanoma, express high levels of c-Ski. Using human melanoma tumors samples, we detected low c-Ski immunoreactivity in nonmalignant skin (24 samples), when compared with malignant melanoma (56 samples) and metastatic malignant melanoma (20 samples; Fig. 6A). Of note, metastatic melanoma displays more pronounced cytoplasmic localization of c-Ski than nonmetastatic melanoma (Fig. 6A), consistent with published data (14). To evaluate the consequence of c-Ski overexpression in melanoma, we analyzed the staining of Smad2 in these tumors. Interestingly, we observed that Smad2 is mainly localized to the cytoplasm when c-Ski is overexpressed (Fig. 6A).

To confirm the correlation between the level of c-Ski and nuclear translocation of Smad2, we first analyzed c-Ski expression in a panel of cancer cell lines (Fig. 6B). We found that the pulmonary adenocarcinoma Calu-6 and A427 cells express high levels of c-Ski, compared with the pulmonary adenocarcinoma A549 cells. We also found that the mammary adenocarcinoma MDA-MB231 and BT-20 cells express high levels of c-Ski compared with the untransformed mammary HMT3522-S1 cells. Here again, we observed that the TGF-β–induced nuclear translocation of Smad2 is suppressed in the tumor cell lines that overexpress c-Ski (Fig. 6C). Thus, we suggest that overexpression of c-Ski in human tumors might culminate in cytoplasmic retention of Smad2, in turn setting an attenuated TGF-β signaling.

Discussion

In the present study, we report that c-Ski can restrict TGF-β signaling by a mechanism dependent on its ability to engage R-Smad and Smad4 in a nonproductive complex. We propose a model in which c-Ski directly associates with TβRI and enforces sequestration of the R-Smad/Smad4 complex by this receptor, thereby preventing nuclear accumulation of a functional R-Smad/Smad4 complex that is required for transcriptional activation of TGF-β target genes. Thus, our findings underscore the existence of a previously unknown mechanism by which c-Ski achieves effective suppression of TGF-β signaling.

c-Ski–mediated inhibition of TGF-β superfamily signaling was initially thought to occur exclusively in the nucleus via recruitment of a general nuclear corepressor complex containing HDAC to TGF-β–activated Smad proteins. There is overwhelming evidence, however, that c-Ski also resides in the cytoplasm, leaving open to speculation whether the inhibitory function of c-Ski may also rely on extranuclear mechanisms unrelated to transcriptional regulation. Nonetheless, the function of the cytoplasmic c-Ski remains to be fully elucidated. The findings outlined in the present study provide molecular evidence that c-Ski also acts at the level of the TGF-β receptor to interfere with the initiation of TGF-β signaling. Notably, we found that c-Ski promotes the assembly of a nonproductive Smad2/Smad4 complex that fails to translocate to the nucleus, presumably due to a failure of Smad2 to dissociate from the TGF-β receptor when the latter is engaged in a complex with c-Ski. Thus, through

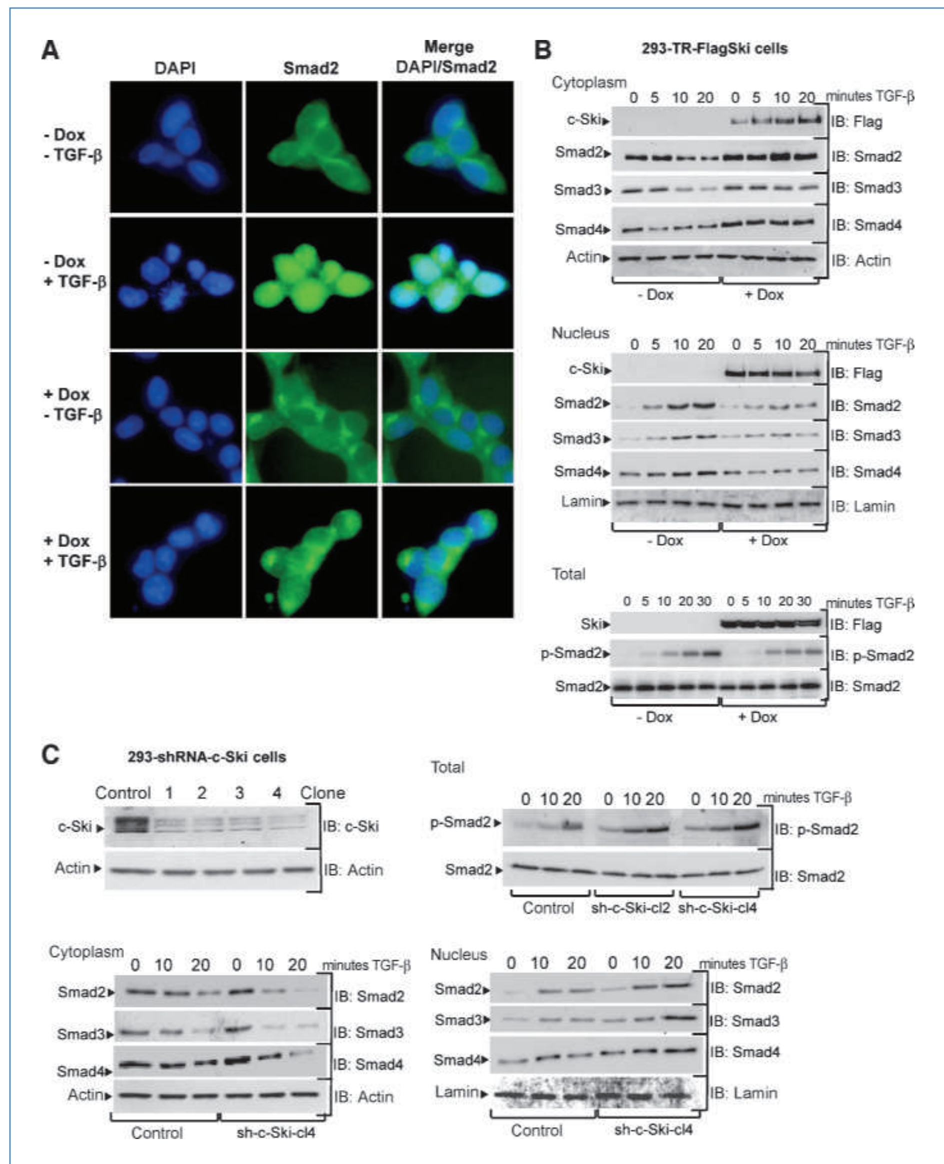

Figure 5. c-Ski prevents nuclear accumulation of R-Smad. 293-TR-c-Ski cells were pretreated with doxycycline (Dox) for 24 hours and then with TGF-β for 30 minutes (A) or various times (B). The localization of endogenous Smad2 (green) and nuclei (blue) was visualized by a fluorescence microscope (A). DAPI, 4′, 6-diamidino-2-phenylindole. Cytoplasmic and nuclear extracts were analyzed by immunoblotting with the indicated antibodies (B, top and middle). To analyze the phosphorylation of Smad2, cells were immunoblotted with anti-phosphoSmad2 (B, bottom). C, 293 cells were stably transfected with pBlockIT-Scrambled (control) or pBlockIT-shRNA-c-Ski. By blotting cell lysates with anti-c-Ski, we selected four clones based on their reduced expression of c-Ski (top, left). Cells were treated with TGF-β for various times and extracts were analyzed by immunoblotting with anti-phospho-Smad2 (top, left). Nuclear and cytoplasmic extracts from three clones were analyzed by immunoblotting with the indicated antibodies. Data obtained from clone 4, which exhibits the lowest expression of c-Ski, are shown (bottom, right and left); similar results were obtained with the other clones.

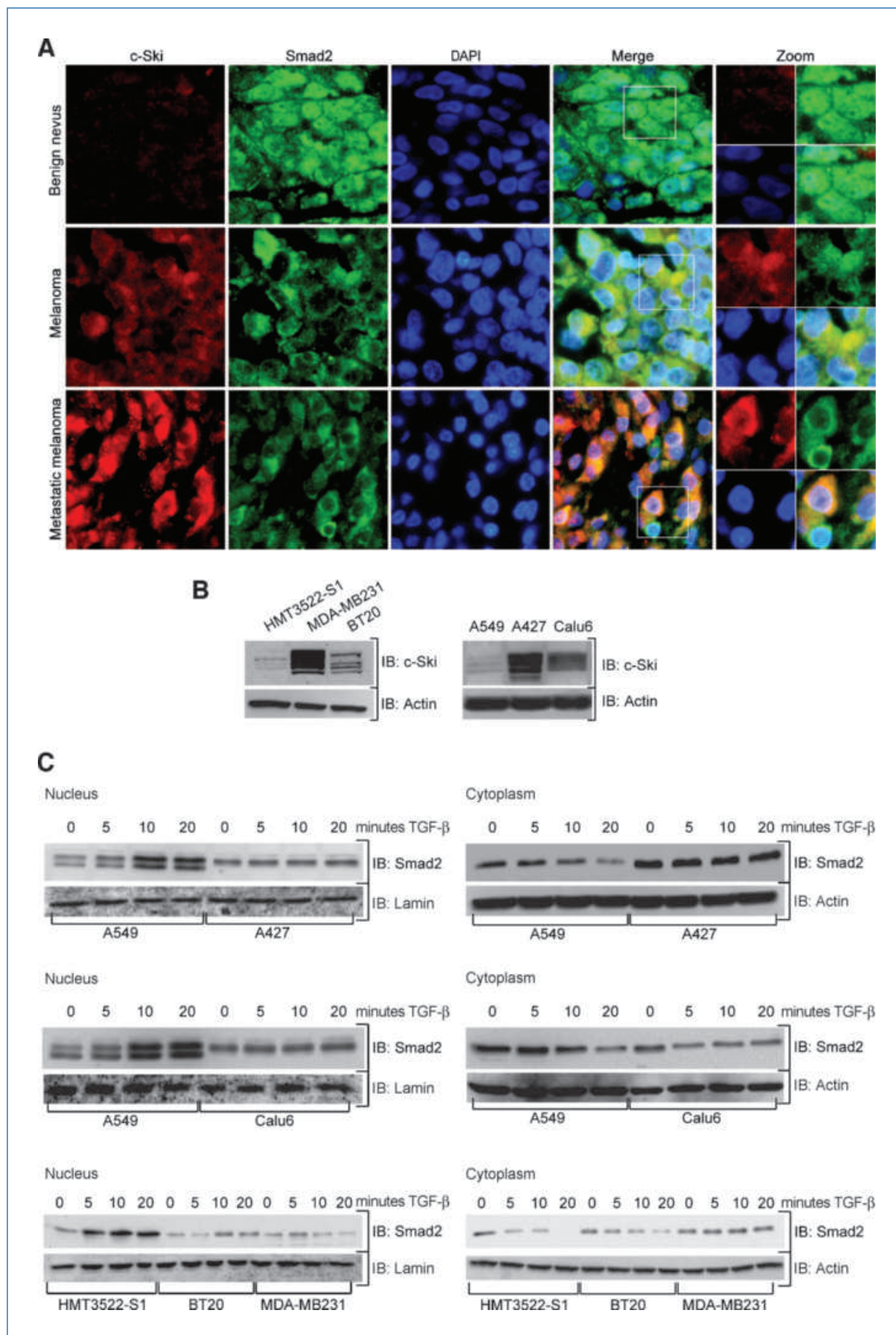

Figure 6. Overexpression of c-Ski in tumors correlates with defective nuclear translocation of Smad2. A, normal skin, melanoma, and metastatic melanoma were immunostained with anti-c-Ski (red), anti-Smad2 (green), and DAPI (blue), and the localization of c-Ski, Smad2, and nuclei was visualized by a fluorescence microscope. B, the expression level of c-Ski in lung adenocarcinoma lines (A549, Calu-6, A427) and in untransformed mammary cells HMT3522-S1 or breast cancer cell lines (MDA-MB231, BT-20) was analyzed by immunoblotting with anti-c-Ski. C, nuclear and cytoplasmic extracts from cancer cell lines cells (see B) were analyzed by immunoblotting with anti-Smad2, anti-actin (cytoplasm), or anti-lamin (nucleus).

its ability to function in the early steps of TGF-β signaling, c-Ski fulfills its inhibitory activity in an important regulatory position in this signaling pathway. Such a mechanism may impose additional constraints to allow effective inhibition of TGF-β–induced signaling by c-Ski in the presence of high levels of the ligand. Alternatively, this inhibitory function could act to set either a threshold level or a ceiling for TGF-β–mediated transcription depending on cell types, the level of c-Ski expressed, or the physiopathologic conditions.

Based on the analysis of the crystal structure of a c-Ski fragment bound to Smad4, Wu and colleagues proposed a model in which c-Ski prevents the accumulation of a functional Smad2/Smad4 complex in the nucleus (19). On the other hand, other studies have shown that c-Ski was able to induce assembly of nonproductive R-Smad/Smad4

complexes (15, 18). Although these findings clearly show that expression of c-Ski can impinge on the R-Smad/Smad4 complexes, they did not provide any indication of how this process is achieved or how it may be regulated. Our having shown that c-Ski induces the assembly of a complex between R-Smad and Smad4 that fails to dissociate from TβRI provides important insights into the molecular mechanism by which c-Ski achieves this new inhibitory function in TGF-β signaling. For instance, the molecular details of how c-Ski enforces sequestration of the R-Smad complex by TβRI are still to be explored. Because c-Ski can form a physical complex with TβRI, it is tempting to speculate that c-Ski may impose a conformational change in the receptor that prevents the dissociation of the Smad2/Smad4 complex. Activation of the TGF-β receptor can also culminate in activation of several noncanonical pathways; thus, we are currently exploring the possibility that the association of c-Ski with TβRI may provoke a general block of all aspects of TGF-β signaling.

c-Ski is considered an oncoprotein owing to its ability to cause transformation of chicken and quail embryo fibroblasts. Although the endogenous levels of c-Ski are low in normal cells, its expression is elevated in many human cancers cell lines. The data outlined in the present study showed that c-Ski is mainly localized to the cytoplasm of tumor cells, and this distribution pattern is associated with impaired nuclear translocation of Smad2. Interestingly, our data showed that the cytoplasmic localization of c-Ski became more pronounced in metastatic melanomas, which is in agreement with published investigations using metastatic melanomas (14). Based on these findings, it is becoming increasingly clear that c-Ski preferentially accumulates in the cytoplasm at the late stages of carcinogenesis in which most cancer cells already have acquired resistance to the growth inhibitory response of TGF-β. Thus, one of the principal challenges for the future is to determine whether the mechanism involving the association between c-Ski and TβRI may precisely take place at late stages of carcinogenesis to influence cell invasion and metastasis.

In summary, our present findings provide evidence for the existence of a direct and specific interaction between TβRI and c-Ski. Crucially, this interaction was detected in several mammalian cell systems, either under overexpressed or endogenous conditions, highlighting the physiologic relevance of this interaction. Thus, c-Ski now emerges as an important negative regulator that acts to suppress early steps of the canonical TGF-β/Smad signaling by associating with the TGF-β type I receptor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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