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The TGF-β co-receptor endoglin modulates the expression and transforming potential of H-Ras

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Endoglin is a coreceptor for transforming growth factor-β (TGF-β) that acts as a suppressor of malignancy during mouse skin carcinogenesis. Because in this model system H-Ras activation drives tumor initiation and progression, we have assessed the effects of endoglin on the expression of *H-Ras* in transformed keratinocytes. We found that TGF-β1 increases the expression of H-Ras at both messenger RNA and protein levels. The TGF-β1-induced *H-Ras* promoter transactivation was Smad4 independent but mediated by the activation of the TGF-β type I receptor ALK5 and the Ras-mitogen-activated protein kinase (MAPK) pathway. Endoglin attenuated stimulation by TGF-β1 of both MAPK signaling activity and *H-Ras* gene expression. Moreover, endoglin inhibited the Ras/MAPK pathway in transformed epidermal cells containing an *H-Ras* oncogene, as evidenced by the levels of Ras-guanosine triphosphate, phospho-MAPK kinase (MEK) and phospho-extracellular signal-regulated kinase (ERK) as well as the expression of c-fos, a MAPK downstream target gene. Interestingly, in spindle carcinoma cells, that have a hyperactivated Ras/MAPK pathway, endoglin inhibited ERK phosphorylation without affecting MEK or Ras activity. The mechanism for this effect is unknown but strongly depends on the endoglin extracellular domain. Because the MAPK pathway is a downstream mediator of the transforming potential of Ras, the effect of endoglin on the oncogenic function of H-Ras was assessed. Endoglin inhibited the transforming capacity of H-Ras(Q61K) and H-Ras(G12V) oncogenes in a NIH3T3 focus formation assay. The ability to interfere with the expression and oncogenic potential of H-Ras provides a new face of the suppressor role exhibited by endoglin in H-Ras-driven carcinogenesis.

Introduction

Endoglin (CD105) is an auxiliary receptor for transforming growth factor-β (TGF-β) that is highly expressed in endothelial cells of the tumor vasculature and at much lower levels in tumor cells (1). So far, most of the studies on endoglin have focused on endothelial cells as it plays an important role in vascular development, remodeling and homeostasis (2). In addition to the proangiogenic role of endoglin, there is evidence supporting its involvement in cancer progression by its direct function on the tumor cells themselves (3). Thus, in cultured human prostate cancer cells, loss of endoglin expression enhances cell migration and invasiveness (4,5). Also, we have shown that endoglin plays an invasion suppressor role during mouse skin chemical carcinogenesis (6). In this model, successive applications of an initiator and a promoter of carcinogenesis result in the outgrowth of benign papillomas that progress to malignant squamous cell carcinomas (SCCs) and subsequently to highly undifferentiated spindle cell carcinomas (SpCCs). Tumors are initiated by an activating mutation in the *H-Ras* gene (7), and increases in the copy number and/or expression of mutated *H-Ras* are associated with progression to carcinomas (7–9). Although TGF-β1 acts as a tumor suppressor at early stages of carcinogenesis, it also promotes malignancy by inducing an epithelial–mesenchymal transition (EMT) associated with progression to SpCCs (10,11). Although the current evidence suggests that endoglin acts in endothelial cells as a molecular switch by balancing the signaling of TGF-β through the type I receptors ALK1 and ALK5 (12), endoglin acts in epidermal cells attenuating TGF-β/ALK5 signaling (6). Interestingly, membrane-bound endoglin is inactivated by shedding during progression from SCC to SpCC allowing an increased TGF-β signaling and malignant progression (6). Thus, both transgenic mice with targeted expression of TGF-β1 to the epidermis (11) and endoglin heterozygous ($Eng^{+/-}$) mice (13,14) show a vast acceleration of malignant progression and enhanced development of SpCCs after chemical carcinogenesis.

Since in epidermal carcinogenesis TGF-β1 promotes malignant progression and this process is associated with increased H-Ras levels, we asked whether TGF-β1 regulates *H-Ras* gene expression and whether endoglin modulates this TGF-β1 effect in transformed keratinocytes.

Materials and methods

Cell culture conditions and treatments

The epidermal mouse cell lines PDV and MCA3D (15) were cultured in Ham's F-12 medium supplemented with amino acids and vitamins (Gibco, Rockville MD). The cell lines B9 and CarC were derived from mouse skin chemically induced carcinomas (8,16). The mouse NIH3T3 cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium. Clones of PDV cells stably transfected with an expression vector encoding short hairpin RNA (shRNA) that silences mouse endoglin (shEng2 and shEng4) as well as pooled PDV cells stably transfected with an endoglin-unrelated sequence (shCont) were used (6). Stable transfectant clones of the CarC cell line expressing human L-endoglin (L-Eng1 and L-Eng2) or transfected with an empty vector (pcDNA3) have been described (6). CarC cells retrovirally infected with the viral plasmids pWZL (empty vector), pWZL-L-Eng FL (full-length endoglin), pWZL-L-Eng ΔCt (lacking the cytoplasmic domain) and pWZL-L-Eng ΔPDZ (lacking the PDZ domain) were generated as described (17). Hemagglutinin (HA)-tagged pWZL-L-Eng ΔEC (lacking the extracellular domain) was generated using HA-TMCT-Endo construct described previously (18). Retrovirally transduced CarC cells were cultured in Dulbecco's modified Eagle's medium containing 400 μg/ml hygromycin B (Invitrogen, Paisley, UK). All culture media were supplemented with 10% fetal bovine serum (Invitrogen) and 80 μg/ml gentamycin. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. For TGF-β1 treatment, cells were incubated with human recombinant TGF-β1 (R and D Systems, Abingdon, UK) at a final concentration of 10 ng/ml for the indicated times. Treatments with the chemical inhibitor of MAPK kinase (MEK) UO125 (Calbiochem-Merck, Darmstadt, Germany) were carried out at a concentration of 5 μM in 0.1% dimethyl sulfoxide.

Abbreviations: EMT, epithelial–mesenchymal transition; ERK, extracellular signal-regulated kinase; GTP, guanosine triphosphate; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; SCC, squamous cell carcinoma; shRNA, short hairpin RNA; SpCC, spindle cell carcinoma; TGF-β, transforming growth factor-β.

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Plasmids, expression vectors, transfections and reporter assays

The expression vector encoding HA-tagged full-length endoglin in pDisplay vector (Invitrogen) has been reported (18). Expression vectors encoding HA--tagged constitutively active ALK5 (T204D) and kinase-deficient ALK5 (K232R) were provided by Dr Liliana Attisano (University of Toronto, Canada). Plasmid pMEXneo encoding a dominant-negative H-Ras mutant (RasN17) was described by Feig and Cooper (19). Plasmids encoding dominant-negative versions of Raf1 and HA-tagged MEK1 were provided by Dr S.Lavandero (University of Chile, Chile). Expression vectors encoding human Smad2 and Smad3 have been described (20). A dominant-negative Smad4 in pCMV was provided by Dr Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, USA). For shRNA-mediated knockdown of endoglin, double-stranded oligonucleotides encoding shRNA, that silence the mouse *endoglin* gene, and inserted into the pSUPER-GFP vector (6) was used. The pSUPER-GFP vector containing an endoglin-unrelated sequence was used as a negative control. The retroviral vector pWZL and the endoglin-derived constructs L-Eng FL, L-Eng ΔCt, L-Eng ΔPDZ and HA-L-Eng ΔEC described above are based on the pBABE retrovirus, which uses an internal ribosomal entry site to drive hygromycin resistance (21). Amplification of the viruses was carried out in the packaging cell line 293-T.

The reporter vector pGL2-Ha-Ras containing the rat H-Ras proximal promoter region −1500/−80 (supplementary Figure 1 is available at *Carcinogenesis* Online) was kindly provided by Dr Mikheev (Fred Hutchinson Cancer Research Center, University of Washington, Seattle, USA) (22). The reporters (SRE)-luc, containing a serum-responsive element, and cyclin A-luc driven by the promoter of cyclin A were provided by Dr A.Corbí (Centro de Investigaciones Biológicas, Madrid, Spain). The reporter pCMV-Gal4-Elk1 (1-147) containing an Ets-like transcription factor (ELK) 1 activation domain that confers mitogen-activated protein kinase (MAPK) specificity was purchased from Stratagene (La Jolla, CA). The pcFos-luc reporter, whose luciferase expression is driven by c-Fos promoter (23), was constructed in pGL3 vector (Promega, Madison, WI). The Smad3-responsive promoter construct (CA-GA)$_{12}$-Luc was provided by Dr Peter ten Dijke (24).

Cells were transfected with Superfect (Qiagen, Hilden, Germany) according to manufacturer's instructions. Reporter assays were performed as described (25) in the presence or absence of TGF-β1, as indicated. Basically, cells were pretreated with TGF-β1, transfected with the reporter vector and then incubated with TGF-β1. One day after transfection, luciferase activity was determined. All transfections were performed in combination with pSV40-LacZ encoding beta-galactosidase, as a control for transfection efficiency and normalization. Luciferase activity was determined by using a commercial kit (Promega), whereas beta-galactosidase activity was determined using the Galacto-Light Kit (Applied Biosystem, Carlsbad, CA). Light emission was measured in a TD20/20 luminometer (Promega). The experiments were performed in triplicates at least three times and representative experiments are shown in the figures.

Antibodies, pull-down and immunoblotting assays

The broad specific anti-Ras monoclonal antibody was purchased from R and D Systems (clone #342404). For immunodetection of endoglin proteins, the mAbs MJ7/18 and P4A4, which recognize epitopes within the extracellular domains of murine and human endoglin, respectively, were used (14). Recombinant proteins with the HA epitope (endoglin, ALK5 and MEK1,2-DN) were detected with 12CA5 monoclonal antibody (Roche Diagnostics, Mannheim, Germany). Recombinant Smad proteins with the Flag epitope were detected with anti-Flag M2 monoclonal antibody (Sigma, Madrid, Spain). The antibodies against phospho-extracellular signal-regulated kinase (ERK)1,2, ERK1,2, phospho-MEK1,2 and MEK1,2 were purchased from Cell Signaling (Danvers, MA). Anti-c-Fos (sc-52) and anti-Raf1 (C12; sc-133) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin antibody was purchased from Sigma. The secondary antibody coupled to horseradish peroxidase was purchased from DAKO (Barcelona, Spain). To analyze the level of active Ras in cell lysates, the ability of Ras-guanosine triphosphate (GTP) to bind the Ras-binding domain of Raf-1 (RBD) was used in pull-down assays as described previously (26) and using a commercial kit (Cytoskeleton, Denver, CO). For immunoblotting experiments, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes as described (25). Membranes were blocked with 4% milk (diluted in Tris-buffered saline and 0.5% Tween 20) and incubated with the appropriate antibody at 4°C overnight. The targeted proteins were detected by enhanced chemiluminescence as indicated by the manufacturer (Pierce, Rockford, IL).

Semiquantitative reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from keratinocytes with Trizol (Gibco BRL, Grand Island, NY). To evaluate the transcript levels of H-Ras and endoglin, total RNA was subjected to reverse transcription–polymerase chain reaction using H-Ras (9) and endoglin-(27) specific primers. The cycling parameters for the polymerase chain reactions were as follows: 10 min at 94°C, 30 cycles of 94°C for 15 s, 55°C for 15 s and 72°C for 90 s, followed by 10 min extension at 72°C. The polymerase chain reaction of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was also performed to ensure equivalency in all samples.

Foci formation assay

Foci formation assays were performed in mouse NIH3T3 fibroblasts. For transformation with H-RasQ61K, cells were transfected using Superfect reagent (Qiagen) with plasmids bearing human L-endoglin in pDisplay (18), si-SuperGFP-endoglin (6) and H-RasQ61K pAL8-Ras (28). Next day, each plate was splitted in three and incubated with Dulbecco's modified Eagle's medium containing 5% donor calf serum (Invitrogen). For transformation with H-Ras-G12V, cells were infected with retrovirus bearing H-Ras-G12V and endoglin. Retroviruses were obtained using the Linx packaging cell line. NIH3T3-infected plates were centrifuged at 1500 r.p.m. for 1 h at room temperature and then incubated at 32°C for 6–8 h. Then, plates were transferred to 37°C. Both H-RasQ61K and H-Ras-G12V transduced cells were incubated for around 2 weeks changing media every 2 days. Then, cells were fixed and stained with crystal violet, and foci were counted.

Statistics

Data are given as means ± SD from at least three independent experiments. When necessary, statistical significance was evaluated using the Student's *t*-test. Differences were considered to be significant at a value of $P < 0.05$.

Results

TGF-β1 stimulates H-Ras promoter activity in premalignant and malignant keratinocytes

PDV transformed keratinocytes express both normal and oncogenic versions of the *H-Ras* gene and produce well-differentiated SCC upon injection in mice (15). Chronic treatment of PDV cells with TGF-β1 promotes an EMT and the conversion to a spindle cell tumor phenotype (10). Since progression from SCC to SpCC is associated with increased levels of activated H-Ras (8,29), we analyzed in PDV cells the effect on *H-Ras* gene expression of a chronic treatment with TGF-β1. As shown in Figure 1A, a series of sequential increases in H-Ras protein levels were observed in PDV cells incubated with TGF-β1, reaching a plateau after ∼3 days of treatment. In agreement with these data, a similar kinetics of *H-Ras* stimulation was obtained by analyzing the messenger RNA levels by semiquantitative reverse transcription–polymerase chain reaction and the activity of an exogenous *H-Ras* promoter (22) (Figure 1B). This stimulatory effect on *H-Ras* was also observed in other epidermal cell lines, such as MCA3D premalignant keratinocytes and B9 squamous carcinoma cells (Figure 1C).

TGF-β1 stimulation of the H-Ras promoter is mediated by the ALK5/Ras/MAPK signaling pathway

TGF-β signals through Smad-dependent and Smad-independent pathways (30). To find out the signaling pathway that mediates TGF-β1-induced transactivation of the *H-Ras* promoter, PDV cells were cotransfected with the *H-Ras* promoter and plasmids encoding different normal or mutant components of the Smad and Ras/MAPK pathways. Constitutively active TGF-β type I receptor ALK5 (ALK5-T204D) enhanced basal *H-Ras* promoter activity to the same level as treatment with TGF-β, whereas kinase dead ALK5 (ALK5-K232R) blocked the TGF-β1-induced *H-Ras* promoter transactivation (Figure 1D), indicating that activation of ALK5 is necessary for TGF-β1 stimulation of the *H-Ras* promoter. The behavior of the *H-Ras* promoter when overexpressing Smad2/Smad3 or a dominant-negative Smad4 was similar to that of the control empty vector (Figure 1D), suggesting the involvement of an Smad4-independent pathway. In contrast, stimulation of the *H-Ras* promoter by TGF-β1 depends on a fully active Ras/MAPK pathway since expression of a dominant-negative form of Ras, Raf or MEK1,2 or treatment with UO125, a pharmacological inhibitor of MEK1,2, completely blocked the TGF-β1 effect (Figure 1E).

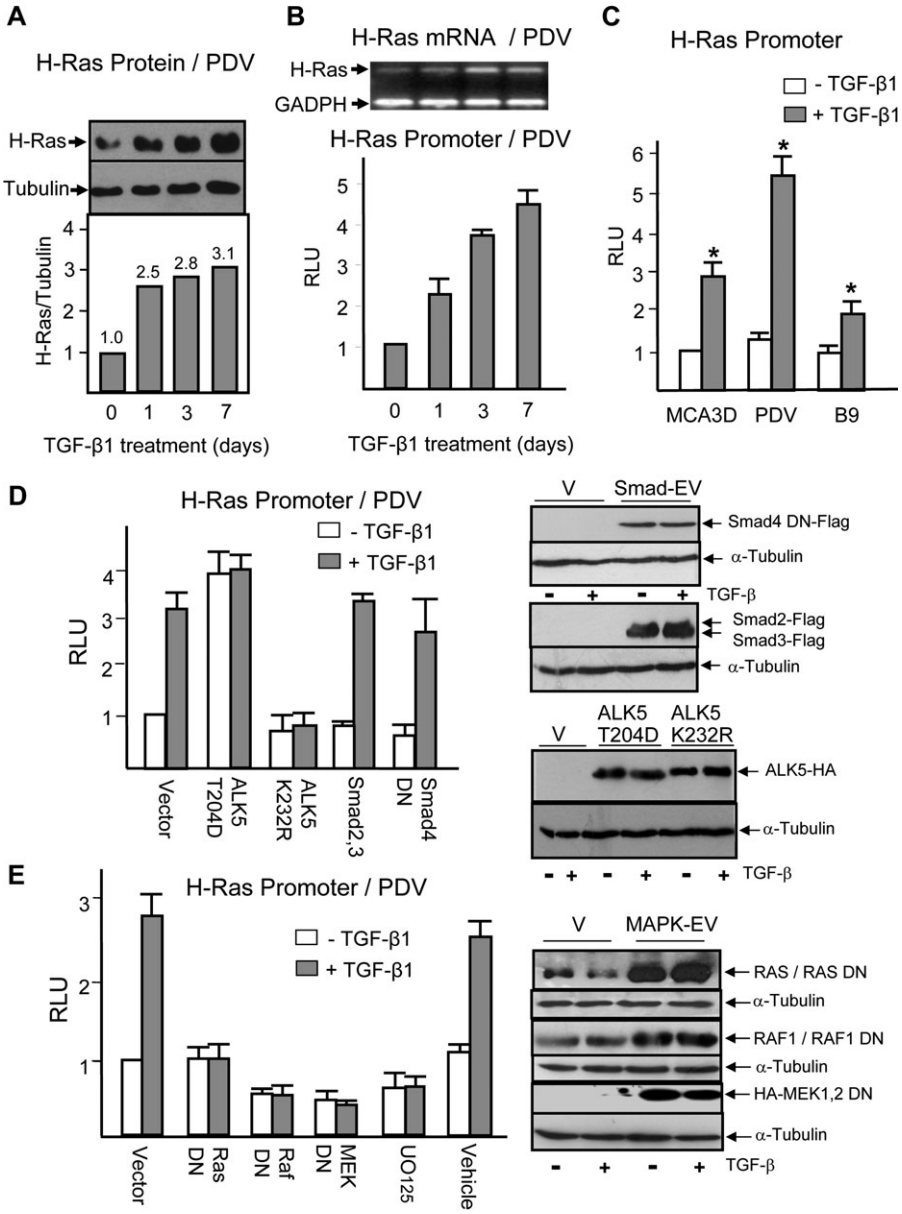

Fig. 1. TGF-β enhances *H-Ras* expression in transformed keratinocytes by activating the Ras-MAPK pathway. Keratinocytes were incubated in the presence or absence of TGF-β1 for the indicated times. (**A**) Analysis of H-Ras protein levels. PDV cells were incubated in the presence or absence of TGF-β1 and the H-Ras protein levels were visualized by western blot. Normalized H-Ras protein levels were measured relative to α-tubulin. (**B**) Kinetic study of the TGF-β1-dependent transcriptional regulation of *H-Ras*. PDV cells were incubated in the presence or absence of TGF-β1. For analysis of H-Ras messenger RNA levels, total RNA was extracted and subjected to semiquantitative reverse transcription–polymerase chain reaction and H-Ras transcript levels were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (top). For *H-Ras* promoter activity assays, 1 day before completion of TGF-β treatment, treated and untreated cells were transfected with a reporter construct driven by the *H-Ras* promoter and the luciferase activity was measured 24 h later (bottom). (**C**) Analysis of *H-Ras* promoter activity in the carcinoma cell lines MCA3D, PDV and B9. Cells were transfected with a reporter construct driven by the *H-Ras* promoter and incubated for 24 h. Then, cells were treated in the presence or absence of TGF-β1 for 2 days and the luciferase activity was measured. Asterisks indicate statistically significant differences respect to untreated cells ($P < 0.005$). (**D** and **E**) Analysis of the signaling pathway involved. The luciferase experiments were performed in triplicates at least three times and representative experiments are shown in the figures. RLU, relative luciferase units. (D) Analysis of ALK5 and Smad involvement. PDV cells were cotransfected with a reporter construct driven by the *H-Ras* promoter and expression vectors (EV) coding for the constitutively active ALK5 (ALK5-T204D), the kinase dead form of ALK5 (ALK5-K232R), Smad2/Smad3 or the dominant negative (DN) form of Smad4. Twenty-four hours after transfection, cells were incubated or not with TGF-β1 for 1 day and the luciferase activity was measured (histogram on the left). Right, the ectopic expression of Flag-tagged Smad4-DN, Smad2 and Smad3 in transfected PDV cells was revealed by western blot analysis using anti-Flag antibodies. The ectopic expression of HA-tagged ALK5 (T204D) and ALK5 (K232R) in transfected PDV cells was revealed by western blot analysis using anti-HA antibodies. As a negative control, transfection with an empty vector (V) was carried out. As a loading control, the presence of α-tubulin is included. (E) Analysis of Ras-MAPK involvement. PDV cells were cotransfected with a reporter construct driven by the *H-Ras* promoter and expression vectors coding for dominant negative (DN) forms of Ras, Raf or MEK1,2. A chemical inhibitor of ERK (UO125) was also used. Twenty-four hours after transfection, cells were treated or not with TGF-β1 for 1 day and the luciferase activity was measured (histogram on the left). A control transfection with an empty vector, as well as a control incubation with the vehicle (0.1% dimethyl sulfoxide) used in the treatment with UO125, were included. Right, the ectopic expression of Ras-DN, Raf1-DN or HA-tagged MEK1,2-DN in transfected PDV cells was revealed by western blot analysis using specific antibodies against Ras, Raf and HA. Note that polyclonal antibodies against Ras and Raf1 also recognize the endogenous proteins, but the signals are substantially higher upon expression of the DN forms of Ras and Raf1. As a loading control, the presence of α-tubulin is included. V, empty vector; MAPK-EV, expression vector encoding components of the MAPK pathway.

Endoglin interferes with TGF-β1-induced Ras/MAPK activation and TGF-β1-stimulated H-Ras gene expression

Because stimulation of *H-Ras* expression by TGF-β1 occurs via ALK5, and endoglin negatively modulates TGF-β1/ALK5 signaling in epidermal cells (6), we reasoned that endoglin should modulate this TGF-β1 effect. To test this hypothesis, increasing amounts of exogenous endoglin were transfected in PDV cells and the activity of *H-Ras* promoter determined before and after treatment with TGF-β1 (Figure 2A). As expected, PDV cells expressing low (endogenous) levels of endoglin (13) showed a 2.8-fold increase of the *H-Ras* promoter activity upon treatment with TGF-β1. This stimulatory effect decreased in a dose-dependent fashion as increasing levels of human endoglin were expressed. These results suggest that endoglin interferes with TGF-β1-dependent stimulation of the *H-Ras* promoter. To assess whether the stimulation of H-Ras messenger RNA levels by TGF-β1 are regulated by endoglin, a semiquantitative reverse transcription–polymerase chain reaction analysis was carried out in control and human endoglin-transfected PDV cells. As shown in Figure 2B, overexpression of exogenous endoglin blocked the 3-fold stimulation of H-Ras transcript levels observed after treatment with TGF-β1.

Because TGF-β1-dependent *H-Ras* transactivation is mediated by Ras-MAPK signaling activity (Figure 1E), we investigated whether endoglin was able to modulate this pathway. To this aim, the effect of TGF-β1 on the activity of reporter constructs specific for the MAPK signaling pathway was analyzed in the presence or absence of endoglin. These reporter genes included (SRE)-luc containing a serum-responsive element, pCMV-Gal4-Elk1, comprising an Ets-like transcription factor (ELK1) activation domain, and pcFos containing the *c-Fos* promoter. All three reporter genes were activated by TGF-β1 in the absence but not in the presence of exogenous endoglin (Figure 2C). Interestingly, endoglin also decreased basal activity of these reporter genes, suggesting that the TGF-β coreceptor not only inhibits stimulation of this pathway by TGF-β1 but also inhibits the basal MAPK signaling activity. Alternatively, endoglin inhibition of the basal reporter activity could be explained by an endoglin effect on autocrine TGF-β responses. As a positive control, endoglin abolished the TGF-β1 stimulation of the Smad3-responsive construct (CAGA)12-Luc (Figure 2C). As a negative control, endoglin did not affect the basal activity nor the TGF-β1-induced inhibition of the p38-dependent and ERK-independent promoter activity of cyclin A, further supporting the specificity of endoglin within the MAPK pathway.

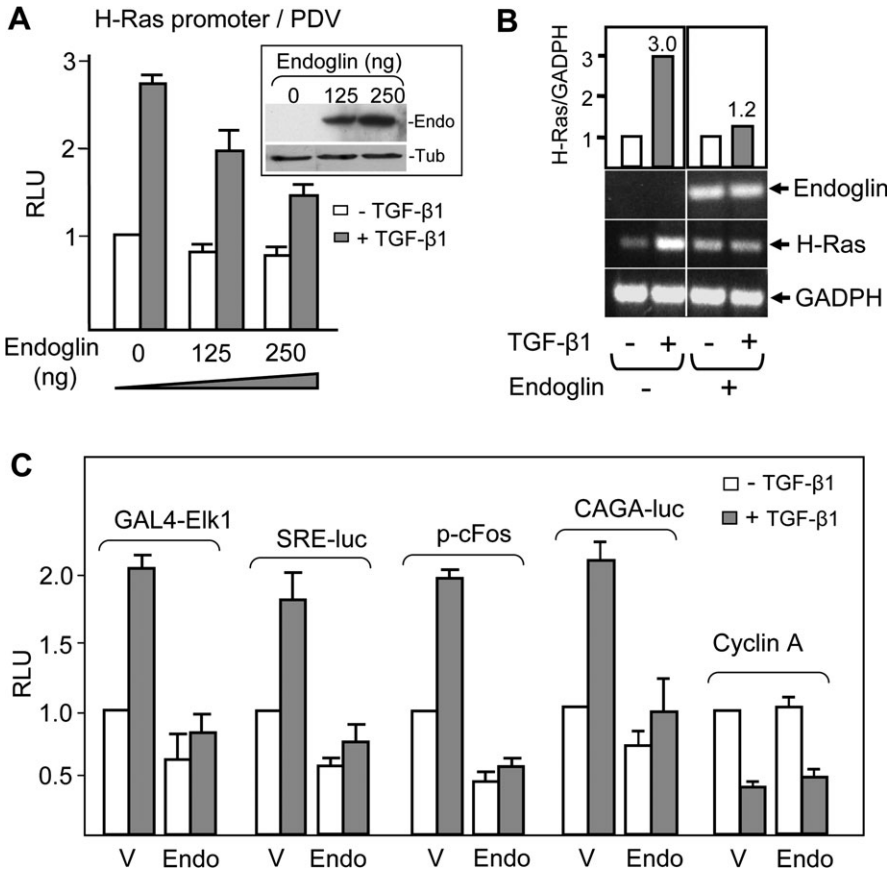

Fig. 2. Endoglin inhibits the induction of *H-Ras* by TGF-β1. (**A**) Effect of endoglin on the stimulation of *H-Ras* promoter activity by TGF-β1. PDV cells were cotransfected with a reporter construct driven by the *H-Ras* promoter and increasing amounts of an expression vector coding for human endoglin. After 24 h, cells were treated in the presence or absence of TGF-β1 for 2 days. Then, cells were lysed and the luciferase activity was measured. The inset shows the recombinant expression of endoglin in transfected PDV cells, as evidenced by western blot analysis using antiendoglin antibodies. As a loading control, the presence of α-tubulin is included. (**B**) Effect of endoglin on the TGF-β1 stimulation of H-Ras transcript levels. PDV cells were transfected or not with endoglin and incubated or not with TGF-β1 for 7 days as indicated. Total RNA was extracted and subjected to semiquantitative reverse transcription–polymerase chain reaction to determine endoglin, H-Ras and glyceraldehyde 3-phosphate dehydrogenase levels. Normalized H-Ras transcript levels relative to glyceraldehyde 3-phosphate dehydrogenase are shown in the histogram. (**C**) Effect of endoglin on the activation of MAPK target genes by TGF-β1. PDV cells were cotransfected with the reporters SRE-luc, containing a serum-responsive element, pCMV-Gal4-Elk1, that confers MAPK specificity, pcFos-luc containing the *c-Fos* promoter and a reporter construct driven by the *cyclin A* promoter, as well as an endoglin expression vector, as indicated. As controls, mock transfections in the presence of an empty vector (V) or transfections with the Smad3-responsive promoter construct $(CAGA)_{12}$-Luc were carried out. Twenty-four hours after transfection, cells were treated or not with TGF-β1 for 1 day and, then, the luciferase activity was measured.

Endoglin inhibits the MAPK pathway in transformed epidermal cells

We have shown previously in PDV cells that TGF-β1 transiently stimulates ERK1,2 phosphorylation and translocation from the cytoplasm to the nucleus, with a maximum at 30 min, whereas the upstream Ras activation was a much earlier event (26). In order to analyze whether endoglin interferes basal ERK1,2 phosphorylation and/or the kinetic profile of ERK1,2 activation by TGF-β1, we utilized PDV cells in which endoglin expression can be markedly reduced (50–70%) by shRNA interference (6). Figure 3A shows that specific suppression of endoglin expression associates with increased basal levels of phospho-ERK1,2 (∼2-fold) and enhanced c-Fos expression; also, no changes in the total protein levels of ERK1,2 were

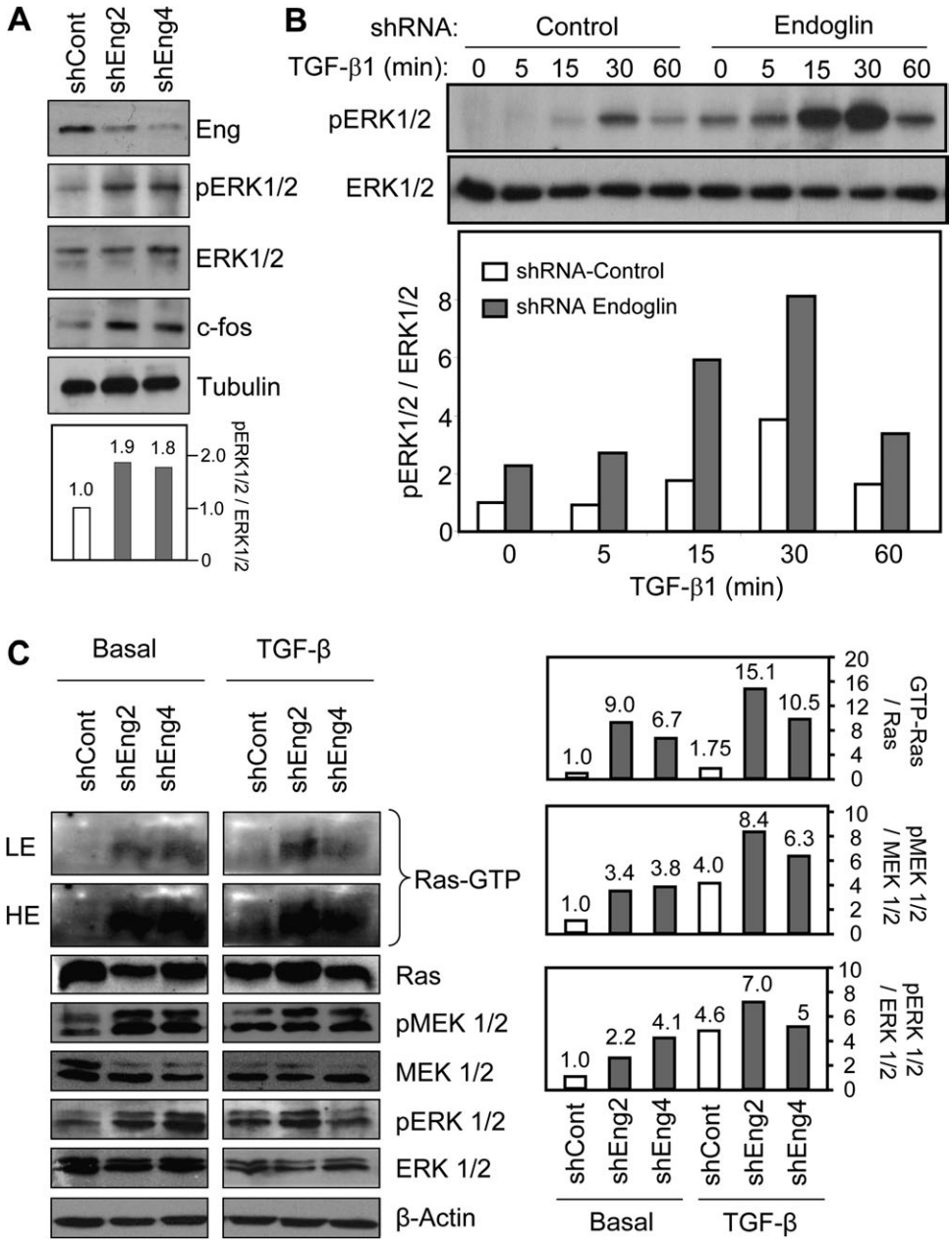

Fig. 3. Endoglin modulates basal and TGF-β-dependent activation of MAPK in transformed keratinocytes. (**A**) Effect of endoglin knockdown on basal ERK phosphorylation. Western blot analysis of endoglin (Eng), phospho-ERK1,2 (pERK1,2), total ERK1,2 and c-Fos in PDV cells stably transfected with shRNA endoglin (shEng2 and shEng4) or shRNA control (shCont). No differences between shCont and mock-transfected cells were observed in several independent experiments (data not shown). Anti-α-tubulin antibodies were used as a control for protein loading. Normalized pERKs levels relative to total ERK proteins are shown in the histogram. This is a representative experiment of three. (**B**) Effect of endoglin knockdown on TGF-β-dependent ERK phosphorylation. shEng2 and shCont cells were incubated or not with TGF-β1 for the times indicated. Total cellular lysates were subjected to western blot analysis using specific monoclonal antibodies to phospho-ERK1,2 and total ERK1,2. Normalized pERKs levels relative to total ERK proteins are shown in the histogram. This is a representative experiment of three. (**C**) Effect of endoglin knockdown on basal and TGF-β-induced Ras and MEK activities. Cells were incubated or not with 10 ng/ml TGF-β1 for 15 min. Western blot analyses of phospho-MEK1,2, total MEK1,2, phospho-ERK1,2 and total ERK1,2, in PDV cells stably transfected with shRNA endoglin (shEng2 and shEng4) or shRNA control (shCont), are shown. The levels of total Ras and active Ras-GTP proteins were determined in total cell lysates by precipitating with RBD-sepharose, followed by western blotting with a pan-Ras monoclonal antibody. Low (LE) and high (HE) exposures of the Ras-GTP signal are shown. Anti-β-actin antibodies were used as a control for protein loading. Normalized Ras-GTP levels versus total Ras, pMEKs levels relative to total MEK and pERKs levels relative to total ERK proteins are shown in the histogram. An arbitrary value of 1 was assigned to untreated shCont samples. This is a representative experiment.

observed. In addition to an enhanced basal ERK1,2 activation, the endoglin knockdown led to increased TGF-β1-induced ERK1,2 phosphorylation (Figure 3B). In contrast, overexpression of human endoglin reduced basal and TGF-β1-induced levels of phospho-ERK1,2 in PDV cells (data not shown). Of note, the relative intensity of the two ERK isoforms varied among different experiments, probably due to the particular electrophoretic and exposure conditions used in each case. Next, the upstream components of the MAPK pathway were analyzed. Suppression of endoglin expression in PDV cells increased both basal and TGF-β-estimulated Ras and MEK activities, as evidenced by the levels of active Ras-GTP, phospho-MEK1,2 and phospho-ERK1,2 (Figure 3C), suggesting that endoglin interferes the upstream components of the Ras/Raf/MEK/ERK pathway. As expected, the TGF-β-dependent stimulation was evident in all the samples (shCont and shEng), as demonstrated by comparative densitometric analysis of the bands (Figure 3C, histogram on the right). The TGF-β-dependent upregulation of the MAPK components occurred even with Ras-GTP (Figure 3C, overexposed gel), in spite of the fact that the time point selected for this analysis (15 min) was optimized for ERK activation and it is known that maximal Ras activation occurs at an earlier time. Of note, in PDV cells, due to the *H-Ras* mutant allele, an enhanced basal activation of Ras, as compared with normal keratinocytes, was observed (data not shown), in agreement with a previous report (26).

The effect of endoglin was also assessed on the highly aggressive spindle carcinoma cell line CarC that expresses undetectable levels of membrane-associated endoglin (6). CarC contains the same mutated *H-Ras* oncogene as PDV cells, but, although PDV expresses normal H-Ras protein, CarC has lost the normal *H-Ras* allele (15,31) and represents a step further in malignant progression with respect to PDV (32). As shown in Figure 4A, two different clones of CarC cell transfectants stably expressing endoglin displayed reduced levels of phospho-ERK1,2 (∼50%) and c-Fos as compared with mock transfectants, whereas no significant change was observed in the expression of total ERK1,2 proteins. At variance with PDV cells, endoglin expression did not affect the levels of active Ras (Ras-GTP) or MEK1,2 (phospho-MEK1,2) (Figure 4A), suggesting that the TGF-β coreceptor directly inhibits ERK1,2 phosphorylation. In order to map the endoglin domain involved in this inhibitory effect, different endoglin truncated constructs were expressed in CarC cells (Figure 4B). Endoglin constructs L-Eng-ΔCt and L-Eng-ΔPDZ lacking the whole cytoplasmic domain or the PDZ-binding motif located at the carboxy-terminus, respectively, diminished the levels of phospho-ERK1,2 and c-fos in a similar fashion as the full-length endoglin construct. Interestingly, expression of the construct (HA)L-Eng-ΔEC, lacking the extracellular domain of endoglin, was unable to inhibit the phosphorylation of ERK1,2 and the expression of c-Fos (Figure 4B), and this effect was

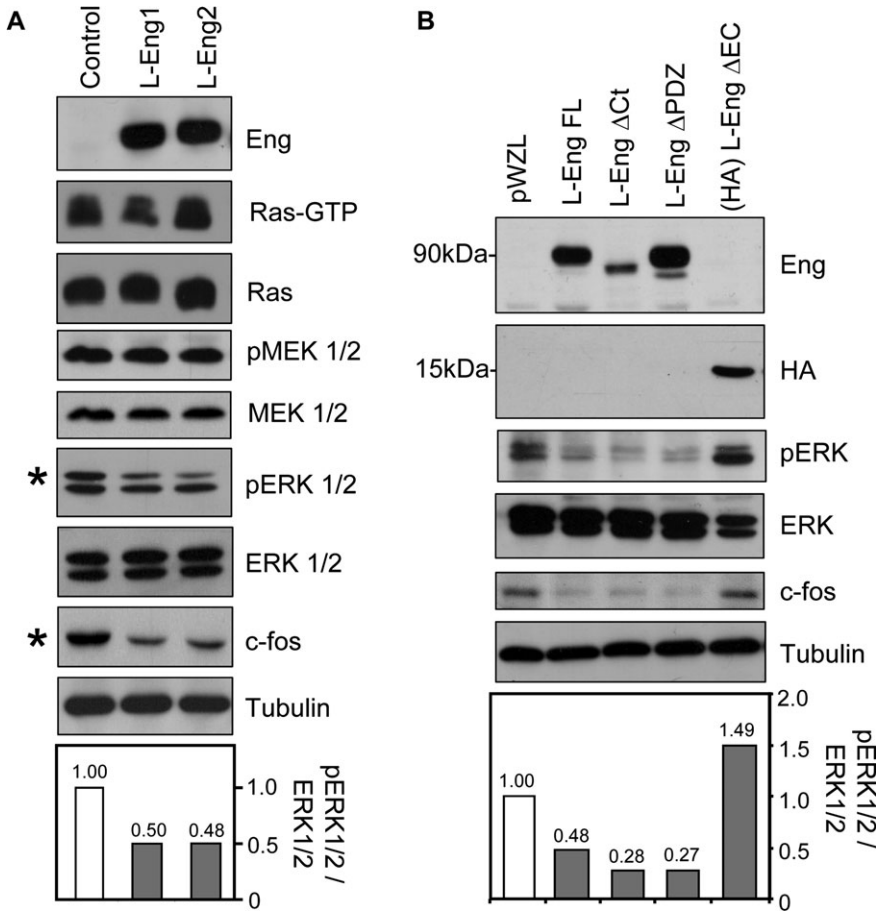

Fig. 4. Endoglin inhibits ERK phosphorylation in spindle carcinoma cells with hyperactivated MAPK activity. (**A**) Total cellular lysates from CarC cell transfectants stably expressing human endoglin (L-Eng1 and L-Eng2) and control mock transfectants were subjected to western blot analysis using specific monoclonal antibodies to endoglin (Eng), phospho-MEK1,2, total MEK1,2, phospho-ERK1,2, total ERK1,2 and c-Fos. The levels of total Ras and active Ras-GTP proteins were determined in the cell lysates by precipitating with RBD-sepharose followed by western blotting with a pan-Ras monoclonal antibody. α-tubulin was used as a control for protein loading. A representative experiment of three is shown. Inhibition of pERK 1/2 and *c-Fos* levels in the presence of endoglin was observed (asterisks). Normalized pERKs levels relative to total ERK proteins are shown in the histogram. (**B**) Inhibition of MAPK activity by endoglin in spindle carcinoma cells depends on the extracellular domain. Total cellular lysates from CarC cells retrovirally infected with the constructs pWZL (empty vector), L-Eng FL (full-length endoglin), L-Eng ΔCt (lacking the cytoplasmic domain), L-Eng ΔPDZ (lacking the PDZ domain) and the HA-tagged L-Eng ΔEC (lacking the extracellular domain) were subjected to western blot analysis using specific monoclonal antibodies to endoglin (Eng), phospho-ERK1,2, total ERK1,2, and c-Fos and α-tubulin. Normalized pERKs levels relative to total ERK proteins are shown in the histogram. A representative experiment of two is shown.

not mediated by the HA tag of the construct (data not shown). These results suggest that the extracellular but not the cytoplasmic domain of endoglin is involved in the inhibition of ERK1,2 phosphorylation.

Endoglin inhibits transformation of NIH3T3 cells by oncogenic H-Ras

Because endoglin appears to modulate ERK signaling activity and ERK is a downstream mediator of the transforming potential of Ras, we wondered whether endoglin could interfere with H-Ras oncogenic function. To address this, we assessed whether endoglin affects the transforming potential of the H-RasQ61K oncogene, which has normal glutamine 61 mutated to lysine. NIH3T3 fibroblasts express similar amounts of endogenous endoglin as PDV cells (13). Therefore, besides the parental cells, we used NIH3T3 fibroblasts in which endoglin levels were downregulated by shRNA interference as well as NIH3T3 cells transfected with the mouse endoglin complementary DNA to enhance endogenous endoglin levels. As shown in Figure 5, shRNA-mediated downregulation of endoglin increased (∼30%) the already robust capacity of H-RasQ61K for cellular transformation (33). In contrast, endoglin overexpression decreased the H-RasQ61K transforming capacity at a similar extent. In order to assess that this effect of endoglin was not restricted to a particular *H-Ras* oncogene, we also used H-RasG12V in which normal glutamine 12 was mutated to valine. As shown in the inset of Figure 5, endoglin expression also significantly reduced the transforming capacity of H-RasG12V. These results suggest that in addition to regulate TGF-β-induced *H-Ras* gene expression and MAPK signaling activity, endoglin modulates the oncogenic potential of H-Ras.

Discussion

TGF-β1 induces H-Ras gene expression in keratinocytes through activation of the Ras/MAPK pathway

In this report, we show that chronic exposure of mouse transformed keratinocytes to TGF-β1 upregulates *H-Ras* gene expression concomitantly to stimulation of cell migration/invasiveness and EMT (10,34,35). This result is in line with a previous study by Xie *et al.* showing that treatment of mouse mammary gland epithelial cells with TGF-β1 for 24 h increased ∼2.5-fold *H-Ras* expression, as determined by microarray analyses (36). The effect of the growth factor on *H-Ras* expression appears to be independent of Smad4 signaling but dependent on Ras/MAPK signaling activity. This conclusion is supported by experiments showing that in PDV cells, expression of a Smad4 dominant-negative form did not affect the TGF-β1-dependent activation of the *H-Ras* promoter, whereas dominant-negative forms of Ras, Raf or MEK1,2 as well as a MEK1,2 pharmacological inhibitor abolished the TGF-β effect (Figure 1D and E). It should be taken into account, however, that Smad4 does not need to be involved in all effects mediated by R-Smads (37,38). Thus, the effect of TGF-β1 on *H-Ras* expression may be independent of Smad4 but may require the cooperation of Smad2/3 and the Ras/MAPK pathway. The involvement of MAPK signaling activity is similar to other TGF-β cell responses associated with malignancy, such as the induction of EMT (36) or upregulation of the matrix proteinases urokinase (uPA) and MMP-9 collagenase (39,40). Altogether, these results point to activation of MAPK as a key signaling event for TGF-β1 to push malignant progression in epidermal carcinogenesis. This dependency on MAPK activity for TGF-β1 to upregulate *H-Ras* expression also suggests that it might be mediated by Ets-related and/or Sp1 transcription factors, whose putative binding motifs are widely distributed along the *H-Ras* gene promoter (supplementary Figure 1 is available at *Carcinogenesis* Online). Indeed, a sequence within the *H-Ras* promoter known as the H-Ras response element (HRE) is known to bind Ets transcription factors, and most of Ets family proteins are major nuclear effectors of the Ras/MAPK signaling pathway (41,42). In addition, the transcription factor Sp1, which is also activated through MAPK, can bind consensus GC-rich motifs in the *H-Ras* promoter and is required for *H-Ras* gene expression (43,44). Accordingly, TGF-β1 activates Sp1 and, in turn, Sp1 transactivates the *H-Ras* promoter in PDV cells (data not shown), suggesting that at least Sp1 appears to mediate

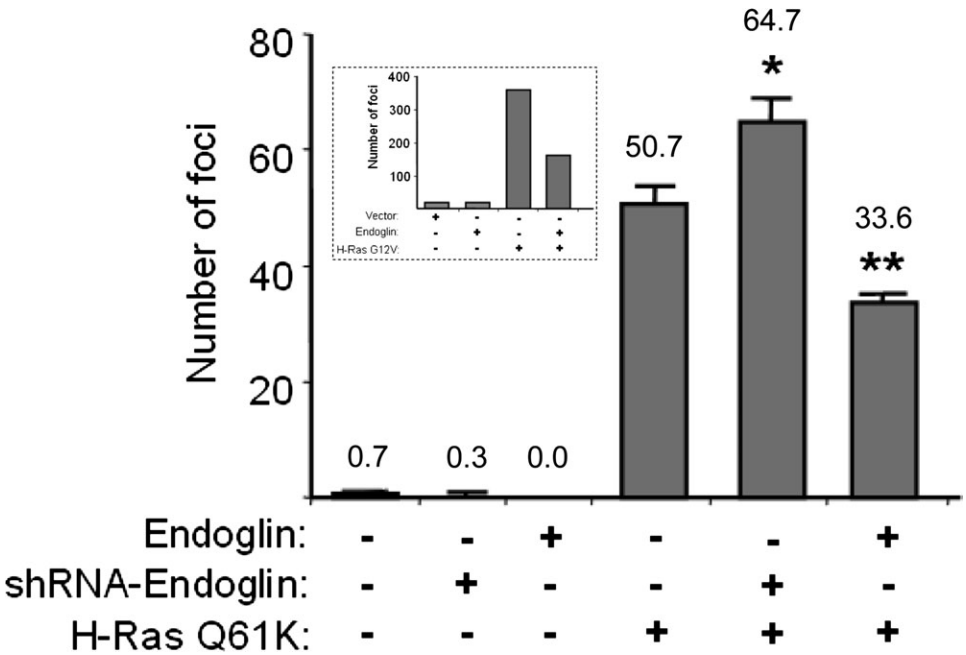

Fig. 5. Effect of endoglin on the transforming activity of the *H-Ras* oncogene. Mouse NIH3T3 fibroblasts were transfected with expression vectors encoding human endoglin (pDisplay-HA-endoglin), shRNA specific for mouse endoglin (shEng2) and H-RasQ61K (pAL8-Ras). Next day, each plate was splitted in three and incubated with Dulbecco's modified Eagle's medium containing 5% donor calf-serum for 2–3 weeks. Then, cells were fixed and stained with crystal violet, and foci were counted. This is a representative experiment of three. Figures on top of the bars stand for the mean number of foci in each condition. Statistically significant differences respect to cells transformed with H-RasQ61K alone were observed ($^{*}P < 0.005$; $^{**}P < 0.0005$). In the inset, a representative experiment is shown in which NIH3T3 fibroblasts were infected with retrovirus bearing H-Ras-G12V and human endoglin. Transduced cells were incubated at 37°C for around 2 weeks changing media every 2 days. Then, cells were fixed and stained with crystal violet, and foci were counted.

stimulation of *H-Ras* gene expression by TGF-β1 in transformed keratinocytes.

The early changes in ERK activity were analyzed as one of the primary downstream signals in response to TGF-β, which, in turn, may account for the long-term effects on *H-Ras* expression and promoter activity. However, it should be noted that although there is an early response of MAPK activity to TGF-β, in agreement with previous reports (45,46), this peak response occurs in a regular cyclic mode during long-term exposure of PDV cells to TGF-β (26). These results suggest that there may be a coupling between the changes in ERK activity and *H-Ras* expression throughout the whole period of incubation.

Differential regulation of the MAPK pathway by endoglin in PDV versus CarC transformed keratinocytes

Endoglin attenuated the TGF-β1 cell response on *H-Ras* expression (Figure 2). This is not surprising since we have shown that endoglin inhibits both basal and TGF-β1 stimulated ALK5-dependent cell responses in PDV cells (6), and, conversely, downregulation of endoglin expression in these transformed keratinocytes leads to increased basal and TGF-β1-stimulated Smad3 (6) and Ras/MAPK (Figure 3) activities. Furthermore, endoglin was shown to inhibit ERK activity in both PDV and CarC transformed epidermal cell lines (Figures 3 and 4), but the mechanism involved appears to be different in each cell type. In PDV cells, endoglin inhibited the basal activity of the entire Ras/MEK/ERK pathway, a fact probably dependent on endoglin inhibition of intrinsic ALK5 activity (6). This is in agreement with reports showing that membrane-associated endoglin physically interacts with type I TGF-β receptors impairing ALK5 activation (18,47) and inhibiting stimulation of Smad3 (48) and ERK1,2 by TGF-β1 (49,50) in different cell types. In CarC cells, on the other hand, endoglin appeared to directly reduce constitutive ERK phosphorylation without affecting the activity of upstream components of the pathway (Figure 4A).

A schematic model depicting the differences between PDV and CarC cells is shown in Figure 6. The major difference between CarC and PDV cells might be due to the strong constitutive activity of the Ras/MAPK pathway in the former cell line that somehow confers resistance to the endoglin effect at this level. Indeed, CarC cells have a hyperactivated MAPK pathway due to the presence of a mutated H-Ras oncogene and absence of a normal H-Ras allele, which increases the dosage of mutated to normal Ras protein expression within the cells (15,31). Also, CarC spindle cells have downregulated ALK5 and TβRII levels with respect to PDV cells (6). Nevertheless, CarC cells do respond to TGF-β stimulation of Smad3, although at a lower rate than PDV cells, and endoglin is able to inhibit TGF-β-mediated Smad3 activation in CarC cells (6). Taken together, these results suggest that blocking endoglin expression would restore normal TGF-β signaling through Ras/ERK/fos/H-Ras in PDV cells, whereas overexpression of endoglin would block any remaining TGF-β receptor signaling in CarC cells, which would reduce Smad3 signaling.

Involvement of additional cross talks between the MAPK and the Smad pathways

In order to understand the whole picture, we should take into account the existence of additional cross talks at different levels between the Ras/MAPK and Smad pathways. Thus, MAPK-mediated phosphorylation appears to have a dual role in Smad2/3 regulation. Mitogens and hyperactive Ras result in ERK-mediated phosphorylation of Smad3 that inhibits its activity (51). In contrast, ERK-dependent phosphorylation of Smad2 enhances its transcriptional activity (52). Therefore, through this mechanism, the hyperactivated Ras/ERK pathway may contribute to a reduced TGF-β/Smad3 signaling in CarC cells. Furthermore, Arany *et al.* have found that ERK activation by both TGF-β and oncogenic Ras is reduced in primary mouse embryo fibroblasts null for Smad3 (53). Interestingly, endoglin expression in CarC cells significantly decreased basal Smad3 activity, as

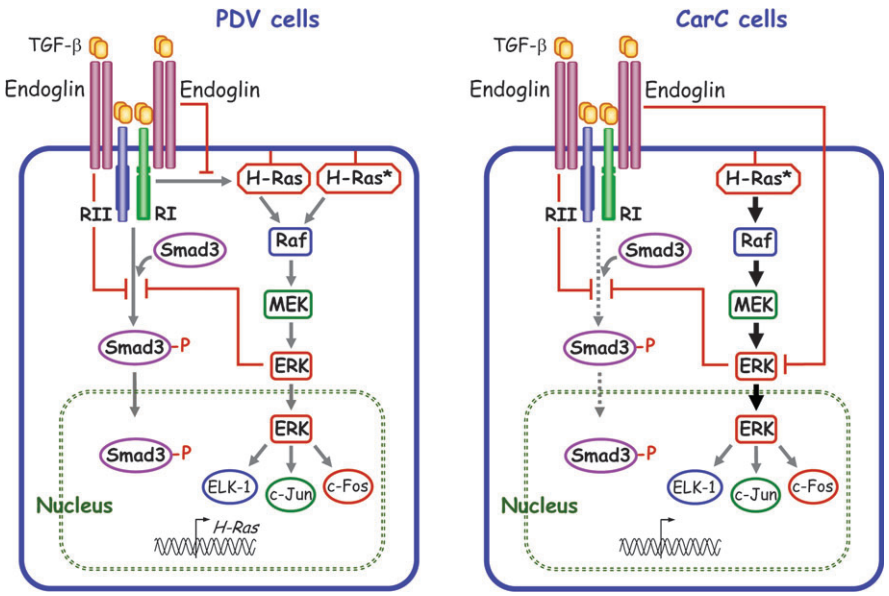

Fig. 6. Hypothetical model for the role of endoglin on the Ras/MAPK pathway in transformed keratinocytes. TGF-β binds to the signaling receptor complex formed by the serine threonine kinase receptors type I (RI) and type II (RII) and endoglin. In turn, this receptor complex can activate two different signaling pathways: (i) the Smad pathway by phosphorylating the receptor activated Smad3 protein and (ii) the Ras/MAPK pathway leading to the Raf/MEK/ERK phosphorylation cascade. In PDV cells, the TGF-β-induced activation of the Ras/MAPK pathway leads to the upregulated expression of *H-Ras*. In PDV and CarC cells, endoglin interferes with the TGF-β-dependent Smad3 activity; conversely, ERK phosphorylates and inactivates Smad3. In PDV transformed keratinocytes, containing a normal to mutated H-Ras gene dosage of ∼2:1 (15), endoglin interferes with basal and TGF-β-dependent Ras activation and downstream components. However, in CarC spindle cells, homozygous for the mutant H-Ras allele (15), endoglin inhibits ERK phosphorylation but not the upstream hyperactivated (black arrows) Ras/Raf/MEK components. H-Ras*, oncogenic H-Ras mutant. Dashed gray arrows indicate a weaker signal than straight arrows. The involvement of mitogenic growth factors and their corresponding tyrosine kinase receptors that activate the MAPK pathway, the association of phospho-Smad3 with Smad4 as well as other Ras family members different from H-Ras have been omitted for simplification. For further details, see the Discussion.

monitored by the $(CAGA)_{12}$-Luc reporter construct but has no major effect on basal Smad2 activity (6). These results suggest that the endoglin-mediated reduction of ERK phosphorylation may involve Smad3 inactivation, similarly as in Smad3 knockout cells (53). Alternatively, this effect might be mediated by the activation of a MAPK phosphatase (54). Because endoglin is known to be phosphorylated at Ser/Thr residues (18,55,56), it remains to be determined whether endoglin may be a substrate of MEK or may directly interact with MEK or ERK1/2, thus interfering with ERK1/2 phosphorylation by MEK.

Endoglin protein domains involved in ERK inhibition

Endoglin also inhibits the basal phosphorylation of ERK1,2 in myoblasts and endothelial cells that apparently have normal Ras genes (49,50). The latter authors have suggested that in endothelial cells, this effect is mediated by endoglin internalization through interaction of its cytoplasmic domain with β-arrestin. The mechanism by which endoglin inhibits ERK signaling activity in CarC cells is presently unknown, but it seems to be different from endothelial cells since it depends on the extracellular domain and not on the endodomain (Figure 4B). Nonetheless, the possible involvement of the transmembrane domain in this inhibitory effect of endoglin remains to be investigated. In this regard, the localization of TGF-β receptors (and also tyrosine kinase receptors) in lipid rafts is essential for MAPK activation [(57) and references therein]. A similar requirement may exist for the inhibitory effect exerted by the TGF-β coreceptor, involving both the extracellular and the transmembrane domains of endoglin. Thus, the observed discrepancy between endothelial and epidermal cells could be attributed to the different distribution of endoglin on the membrane microdomains and their distinct routes of internalization.

Endoglin modulates the oncogenic potential of H-Ras

Consistent with the inhibition of ERK1,2 phosphorylation, a relevant finding of this work is the ability of endoglin to protect NIH3T3 fibroblasts from H-Ras oncogenic transformation (Figure 5). Arany *et al.* (53) have shown that Smad3-deficient embryo fibroblasts are safeguarded against viral H-Ras transformation. This observation is in line with our previous results since endoglin inhibits both basal and TGF-β-stimulated Smad3 signaling in epidermal cells (6). Whether the protector effect exhibited by endoglin is mediated by reduced Ras farnesylation and suppression of the Ras/JNK MAPK pathway, as it is the case of Smad3-deficient fibroblasts (53), remains to be investigated. The inhibitory effect of endoglin on H-Ras oncogenic transformation seems to be a novel and important clue of its suppressor role in epidermal carcinogenesis (6), a system in which tumorigenesis is driven by H-Ras activation (58). Two major consequences of endoglin inactivation during tumor progression (6) would be to facilitate TGF-β-stimulated expression of *H-Ras* and to augment the oncogenic capacity of mutated *H-Ras*. Thus, TGF-β/endoglin and Ras cooperate to modulate malignant progression.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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References

1. Fonsatti,E. *et al*. (2003) Endoglin (CD105): a powerful therapeutic target on tumor-associated angiogenetic blood vessels. *Oncogene*, **22**, 6557–6563.
2. Bernabeu,C. *et al*. (2007) Novel biochemical pathways of endoglin in vascular cell physiology. *J. Cell. Biochem.*, **102**, 1375–1388.
3. Bernabeu,C. *et al*. (2009) The emerging role of TGF-β superfamily coreceptors in cancer. *Biochim. Biophys. Acta*, **1792**, 954–973.
4. Liu,Y. *et al*. (2002) Over expression of endoglin in human prostate cancer suppresses cell detachment, migration and invasion. *Oncogene*, **21**, 8272–8281.
5. Craft,C.S. *et al*. (2007) Endoglin inhibits prostate cancer motility via activation of the ALK2-Smad1 pathway. *Oncogene*, **26**, 7240–7250.
6. Pérez-Gómez,E. *et al*. (2007) A role for endoglin as a suppressor of malignancy during mouse skin carcinogenesis. *Cancer Res.*, **67**, 10268–10277.
7. Quintanilla,M. *et al*. (1986) Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. *Nature*, **322**, 78–80.
8. Buchmann,A. *et al*. (1991) Progression of squamous carcinoma cells to spindle carcinomas of mouse skin is associated with an imbalance of H-ras alleles on chromosome 7. *Cancer Res.*, **51**, 4097–4101.
9. Rodriguez-Puebla,M.L. *et al*. (1999) Increased expression of mutated Ha-ras during premalignant progression in SENCAR mouse skin. *Mol. Carcinog.*, **26**, 150–156.
10. Caulin,C. *et al*. (1995) Chronic exposure of cultured transformed mouse epidermal cells to transforming growth factor-beta 1 induces an epithelial-mesenchymal transdifferentiation and a spindle tumoral phenotype. *Cell Growth Differ.*, **6**, 1027–1035.
11. Cui,W. *et al*. (1996) TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell*, **86**, 531–542.
12. Lebrin,F. *et al*. (2004) Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction. *EMBO J.*, **23**, 4018–4028.
13. Quintanilla,M. *et al*. (2003) Expression of the TGF-beta coreceptor endoglin in epidermal keratinocytes and its dual role in multistage mouse skin carcinogenesis. *Oncogene*, **22**, 5976–5985.
14. Pérez-Gómez,E. *et al*. (2005) Characterization of murine S-endoglin isoform and its effects on tumor development. *Oncogene*, **24**, 4450–4461.
15. Quintanilla,M. *et al*. (1991) Comparison of ras activation during epidermal carcinogenesis *in vitro* and *in vivo*. *Carcinogenesis*, **12**, 1875–1881.
16. Burns,P.A. *et al*. (1991) Loss of heterozygosity and mutational alterations of the p53 gene in skin tumours of interspecific hybrid mice. *Oncogene*, **6**, 2363–2369.
17. Conley,B.A. *et al*. (2004) Endoglin controls cell migration and composition of focal adhesions: function of the cytosolic domain. *J. Biol. Chem.*, **279**, 27440–27449.
18. Guerrero-Esteo,M. *et al*. (2002) Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. *J. Biol. Chem.*, **277**, 29197–29209.
19. Feig,L.A. *et al*. (1988) Relationship among guanine nucleotide exchange, GTP hydrolysis, and transforming potential of mutated ras proteins. *Mol. Cell. Biol.*, **8**, 2472–2478.
20. Labbe,E. *et al*. (1998) Smad2 and Smad3 positively and negatively regulate TGF-beta-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol. Cell*, **2**, 109–120.
21. Conley,B.A. *et al*. (2004) Endoglin controls cell migration and composition of focal adhesions. *J. Biol. Chem.*, **279**, 27440–27449.
22. Mikheev,A.M. *et al*. (2000) CArG binding factor A (CBF-A) is involved in transcriptional regulation of the rat Ha-ras promoter. *Nucleic Acids Res.*, **28**, 3762–3770.
23. Hu,Q. *et al*. (1995) Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science*, **268**, 100–102.
24. Dennler,S. *et al*. (1998) Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.*, **17**, 3091–3100.
25. Santibanez,J.F. *et al*. (2007) Endoglin increases eNOS expression by modulating Smad2 protein levels and Smad2-dependent TGF-beta signaling. *J. Cell. Physiol.*, **210**, 456–468.

26. Iglesias,M. *et al*. (2000) Blockade of Smad4 in transformed keratinocytes containing a Ras oncogene leads to hyperactivation of the Ras-dependent Erk signalling pathway associated with progression to undifferentiated carcinomas. *Oncogene*, **19**, 4134–4145.
27. Bellon,T. *et al*. (1993) Identification and expression of two forms of the human transforming growth factor-beta-binding protein endoglin with distinct cytoplasmic regions. *Eur. J. Immunol.*, **23**, 2340–2345.
28. Sukumar,S. *et al*. (1988) Independent molecular pathways in initiation and loss of hormone responsiveness of breast carcinomas. *Science*, **240**, 524–526.
29. Oft,M. *et al*. (2002) Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat. Cell. Biol.*, **4**, 487–494.
30. Derynck,R. *et al*. (2003) Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature*, **6958**, 577–584.
31. Bremner,R. *et al*. (1990) Genetic changes in skin tumor progression: correlation between presence of a mutant ras gene and loss of heterozygosity on mouse chromosome 7. *Cell*, **61**, 407–417.
32. Zoumpourlis,V. *et al*. (2003) Alterations in signal transduction pathways implicated in tumour progression during multistage mouse skin carcinogenesis. *Carcinogenesis*, **24**, 1159–1165.
33. Der,C.J. *et al*. (1986) Biological and biochemical properties of human ras^H genes mutated at codon 61. *Cell*, **44**, 167–176.
34. Frontelo,P. *et al*. (1998) Transforming growth factor beta 1 induces squamous carcinoma cell variants with increased metastatic abilities and a disorganized cytoskeleton. *Exp. Cell Res.*, **244**, 420–432.
35. Santibanez,J.F. *et al*. (1999) Urokinase expression and binding activity associated with the transforming growth factor beta1-induced migratory and invasive phenotype of mouse epidermal keratinocytes. *J. Cell. Biochem.*, **74**, 61–73.
36. Xie,L. *et al*. (2004) Activation of the Erk pathway is required for TGF-beta1-induced EMT *in vitro*. *Neoplasia*, **6**, 603–610.
37. Yue,J. *et al*. (2000) Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a smad-dependent pathway. *J. Biol. Chem.*, **275**, 30765–30773.
38. Fink,S.P. *et al*. (2003) TGF-beta-induced nuclear localization of Smad2 and Smad3 in Smad4 null cancer cell lines. *Oncogene*, **22**, 1317–1323.
39. Santibanez,J.F. *et al*. (2000) Involvement of the Ras/MAPK signaling pathway in the modulation of urokinase production and cellular invasiveness by transforming growth factor-beta(1) in transformed keratinocytes. *Biochem. Biophys. Res. Commun.*, **273**, 521–527.
40. Santibanez,J.F. *et al*. (2002) Transforming growth factor-beta1 modulates matrix metalloproteinase-9 production through the Ras/MAPK signaling pathway in transformed keratinocytes. *Biochem. Biophys. Res. Commun.*, **296**, 267–273.
41. Wasylyk,B. *et al*. (1998) Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signalling pathway. *Trends Biochem. Sci.*, **23**, 213–216.
42. Yordy,J.S. *et al*. (2000) Signal transduction and the Ets family of transcription factors. *Oncogene*, **19**, 6503–6513.
43. Lu,J. *et al*. (1994) Start site selection by Sp1 in the TATA-less human Ha-ras promoter. *J. Biol. Chem.*, **269**, 5391–5402.
44. Kwak,H.J. *et al*. (2006) Transforming growth factor-beta1 induces tissue inhibitor of metalloproteinase-1 expression via activation of extracellular signal-regulated kinase and Sp1 in human fibrosarcoma cells. *Mol. Cancer Res.*, **4**, 209–220.
45. Hartsough,M.T. *et al*. (1995) Transforming growth factor beta activation of p44mapk in proliferating cultures of epithelial cells. *J. Biol. Chem.*, **270**, 7117–7124.
46. Hartsough,M.T. *et al*. (1996) Altered transforming growth factor signaling in epithelial cells when ras activation is blocked. *J. Biol. Chem.*, **271**, 22368–22375.
47. Blanco,F.J. *et al*. (2005) Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-beta receptor complex. *J. Cell. Physiol.*, **204**, 574–584.
48. Guo,B. *et al*. (2004) CD105 inhibits transforming growth factor-beta-Smad3 signalling. *Anticancer Res.*, **24**, 1337–1345.
49. Rodríguez-Barbero,A. *et al*. (2006) Endoglin modulation of TGF-beta1-induced collagen synthesis is dependent on ERK1/2 MAPK activation. *Cell. Physiol. Biochem.*, **18**, 135–142.
50. Lee,N.Y. *et al*. (2007) The interaction of endoglin with beta-arrestin2 regulates transforming growth factor-beta-mediated ERK activation and migration in endothelial cells. *J. Biol. Chem.*, **282**, 21507–21517.
51. Kretzschmar,M. *et al*. (1999) A mechanism of repression of TGFbeta/Smad signaling by oncogenic Ras. *Genes Dev.*, **13**, 804–816.
52. Funaba,M. *et al*. (2002) Modulation of Smad2-nediated signalling by extracellular signal-regulated kinase. *J. Biol. Chem.*, **277**, 41361–41368.
53. Arany,P.R. *et al*. (2008) Smad3 deficiency inhibits v-ras-induced transformation by suppression of JNK MAPK signaling and increased farnesyl transferase inhibition. *Oncogene*, **27**, 2507–2512.
54. Owens,D.M. *et al*. (2007) Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene*, **26**, 3203–3213.
55. Lastres,P. *et al*. (1994) Phosphorylation of the human-transforming-growth-factor-beta-binding protein endoglin. *Biochem. J.*, **301**, 765–768.
56. Koleva,R.I. *et al*. (2006) Endoglin structure and function: determinants of endoglin phosphorylation by transforming growth factor-beta receptors. *J. Biol. Chem.*, **281**, 25110–25123.
57. Zuo,W. *et al*. (2009) Specific activation of mitogen-activated protein kinase by transforming growth factor-β receptors in lipid rafts is required for epithelial cell plasticity. *Mol. Biol. Cell.*, **20**, 1020–1029.
58. Kemp,C.J. (2005) Multistep skin cancer in mice as a model to study the evolution of cancer cells. *Semin. Cancer Biol.*, **15**, 460–473.

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