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ORIGINAL ARTICLE

The *gep* oncogenes, $G\alpha_{12}$ and $G\alpha_{13}$, upregulate the transforming growth factor-β1 gene

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Transforming growth factor-β1 (TGFβ1) plays a role in neoplastic transformation and transdifferentiation. $G\alpha_{12}$ and $G\alpha_{13}$, referred to as the *gep* oncogenes, stimulate mitogenic pathways. Nonetheless, no information is available regarding their roles in the regulation of the *TGFβ1* gene and the molecules linking them to gene transcription. Knockdown or knockout experiments using murine embryonic fibroblasts and hepatic stellate cells indicated that a $G\alpha_{12}$ and $G\alpha_{13}$ deficiency reduced constitutive, auto-stimulatory or thrombin-inducible *TGFβ1* gene expression. In contrast, transfection of activated mutants of $G\alpha_{12}$ and $G\alpha_{13}$ enabled the knockout cells to promote TGFβ1 induction. A promoter deletion analysis suggested that activating protein 1 (AP-1) plays a role in *TGFβ1* gene transactivation, which was corroborated by the observation that a deficiency of the G-proteins decreased the AP-1 activity, whereas their activation enhanced it. Moreover, mutation of the AP-1-binding site abrogated the ability of $G\alpha_{12}$ and $G\alpha_{13}$ to induce the *TGFβ1* gene. Transfection of a dominant-negative mutant of Rho or Rac, but not Cdc42, prevented gene transactivation and decreased AP-1 activity downstream of $G\alpha_{12}$ and $G\alpha_{13}$. In summary, $G\alpha_{12}$ and $G\alpha_{13}$ regulate the expression of the *TGFβ1* gene through an increase in Rho/Rac-dependent AP-1 activity, implying that the G-protein-coupled receptor (GPCR)-$G\alpha_{12}$ pathway is involved in the TGFβ1-mediated transdifferentiation process.

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Introduction

The members of the $G\alpha_{12}$ family, $G\alpha_{12}$ and $G\alpha_{13}$, were initially isolated as the transforming *gep* oncogenes (Xu *et al*., 1993, 1994). Activated mutants of $G\alpha_{12}$ and $G\alpha_{13}$ are extremely potent in inducing neoplastic transformation (Chan *et al*., 1993; Dhanasekaran and Dermott, 1996; Goldsmith and Dhanasekaran, 2007). $G\alpha_{12}$ and $G\alpha_{13}$ regulate various cellular processes, such as cell transformation, proliferation, migration, actin-stress fiber formation, neurite retraction, platelet aggregation, gene induction and apoptosis in a context-specific manner (Kawanabe *et al*., 2002; Kang *et al*., 2003; Fujii *et al*., 2005; Kelly *et al*., 2007). They regulate small GTP-binding proteins (that is, the Ras and Rho family) through guanine nucleotide exchange factors and modulate the activity of transcription factors, including activating protein 1 (AP-1), serum response factor (SRF), signal transducer and activator of transcription 3 (STAT3), nuclear factor of activated T cells (NFAT) and nuclear factor-κB (Kuner *et al*., 2002; Kurose, 2003; Fujii *et al*., 2005; Brown *et al*., 2006; Kumar *et al*., 2006; Ki *et al*., 2007). The ligands of the G-protein-coupled receptors (GPCRs) that interact with the $G\alpha_{12}$ family members, such as thrombin, angiotensin-II, endothelin-1/2 and thromboxane A_2, contribute to transdifferentiation and proliferation, as well as hemodynamic alterations (Pinzani *et al*., 1996; Bataller *et al*., 2000; Graupera *et al*., 2003; Fiorucci *et al*., 2004).

Transforming growth factor-β1 (TGFβ1) is a key mediator that regulates many cellular processes, including epithelial–mesenchymal transition, growth and extracellular matrix (ECM) formation (Friedman, 1993; Qi *et al*., 1999; Bataller and Brenner, 2005; Zhao *et al*., 2008). Although the role of TGFβ1 is very intriguing because it acts as both a tumor suppressor and a promoter of invasion and metastasis, it has also been reported that the level of TGFβ1 increased in both the blood and urine of hepatocellular carcinoma (HCC) patients, correlating with worsened prognosis and survival, and thus representing an important cancer marker (Giannelli *et al*., 2005; Fransvea *et al*., 2008). The *TGFβ1* gene is known to be regulated at two levels as follows: transcriptional regulation is orchestrated by transcription factors, whereas post-translational regulation depends on the maturation of precursors bound to

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TGFβ1-binding proteins (Kim *et al*., 1989; Oklu and Hesketh, 2000; Giannelli *et al*., 2005; Fransvea *et al*., 2008). Regulation of the *TGFβ1* gene involves AP-1, Zf9, NF1 and SP1 (Kim *et al*., 1989).

In the liver, the hepatic stellate cell (HSC) activation coincides with HCC development (Olaso *et al*., 1997; Lee *et al*., 2007). Activated HSCs, which migrate to and accumulate around transformed cells, might be responsible for remodeling and deposition of tumor-associated ECM, and thus be involved in the growth and invasion of HCC cells (Lee *et al*., 2007). Moreover, activated HSCs synthesize not only TGFβ1 but also angiogenic factors (Ankoma-Sey *et al*., 2000). Results from our laboratory and others show that $G\alpha_{12}$ and/or $G\alpha_{13}$ are responsible for production of inflammatory cytokines and inducible nitric oxide synthase (Kang *et al*., 2003), and that certain Gα proteins, such as $G\alpha_s$, regulate the activation of HSCs (Solis-Herruzo *et al*., 1998; Kang *et al*., 2003). Nevertheless, there have been few studies on the signaling molecules that control the *TGFβ1* gene in association with G proteins. In particular, no information is available regarding the roles of $G\alpha_{12}$/$G\alpha_{13}$ in the regulation of the *TGFβ1* gene or the molecules linking them to gene transcription.

This study examined whether or not the *gep* oncogenes, $G\alpha_{12}$ and $G\alpha_{13}$, have regulatory roles in *TGFβ1* gene expression. Here, we used murine embryonic fibroblast (MEF) cells, whose $G\alpha_{12}$ and/or $G\alpha_{13}$ were disrupted, to examine the cell signaling pathways that control *TGFβ1* gene expression. To investigate the roles of $G\alpha_{12}$ and $G\alpha_{13}$, $G\alpha_{12}$ and $G\alpha_{13}$ knockout (KO) cells were transfected with plasmids encoding activated mutants of $G\alpha_{12}$ ($G\alpha_{12}$QL) and $G\alpha_{13}$ ($G\alpha_{13}$QL), and the levels of TGFβ1 and AP-1 transactivation were determined. Moreover, we examined the effects of $G\alpha_{12}$ and $G\alpha_{13}$ deficiency on *TGFβ1* gene expression in HSCs. In this study, we found that $G\alpha_{12}$ and $G\alpha_{13}$ have regulatory functions in *TGFβ1* gene induction through Rho/Rac-dependent AP-1 activation.

Results

$G\alpha_{12}$/$G\alpha_{13}$ regulation of the basal TGFβ1 gene expression

The effects of a deficiency in $G\alpha_{12}$/$G\alpha_{13}$ on *TGFβ1* gene transcription were examined in MEF cells. Rhodopsin kinase-null (RK−/−) MEF cells were used as the KO control in association with the G-protein because the physiological function of RK is restricted to phototransduction (Xu *et al*., 1997; Ki *et al*., 2007). The TGFβ1 transcript levels in the RK−/− cells were identical to those in wild-type (WT) cells. Semi-quantitative reverse transcription PCR (RT–PCR) assays showed that the TGFβ1 mRNA level was weakly altered by a deficiency in either $G\alpha_{12}$ or $G\alpha_{13}$ as compared with that in controls, whereas it was completely abolished by a deficiency in both $G\alpha_{12}$ and $G\alpha_{13}$ (Figure 1a, left panel). A real-time PCR analysis confirmed that *TGFβ1* gene repression was caused by a $G\alpha_{12}$/$G\alpha_{13}$ deficiency (Figure 1a, right panel). Knockdowns of $G\alpha_{12}$ and $G\alpha_{13}$ by siRNA transfection (12 h) also inhibited luciferase expression from pGL3-1132 (TGFβ1 luciferase reporter) in MEF cells (Figure 1b, left panel), which is consistent with the result showing significant inhibition of gene transactivation by simultaneous transfection of minigenes that disturb the GPCR-$G\alpha_{12}$/$G\alpha_{13}$ coupling by the C-terminal peptide expression of $G\alpha_{12}$/$G\alpha_{13}$ (Figure 1b, right panel) (Qi *et al*., 1999; Ki *et al*., 2007). *TGFβ1* gene expression was significantly increased in $G\alpha_{12}$/$G\alpha_{13}$−/− cells after transfection of constitutively active mutants of $G\alpha_{12}$ ($G\alpha_{12}$QL) and $G\alpha_{13}$ ($G\alpha_{13}$QL) (Figure 1c). These results provide evidence that $G\alpha_{12}$ and $G\alpha_{13}$ are required for basal *TGFβ1* gene expression.

$G\alpha_{12}$/$G\alpha_{13}$ regulation of TGFβ1 gene induction by TGFβ1 or thrombin

Next, we examined the role of $G\alpha_{12}$ and $G\alpha_{13}$ in the auto-induction of the *TGFβ1* gene. The level of TGFβ1 mRNA in control cells gradually increased after TGFβ1 treatment, reaching its maximal level at 12 h, and reducing from the maximum at 24 h. In contrast, a deficiency in both $G\alpha_{12}$ and $G\alpha_{13}$ abolished the basal expression level and auto-induction of the gene (Figure 2a, left panel). Similarly, treating control cells with TGFβ1 resulted in a marked increase in the level of luciferase expression from a TGFβ1 promoter construct, but this was not observed in cells deficient in $G\alpha_{12}$ and $G\alpha_{13}$ (Figure 2a, right panel). We also confirmed a decrease in TGFβ1-inducible *TGFβ1* gene expression in primary cultured MEFs prepared from $G\alpha_{12}$/$G\alpha_{13}$ +/− mice (Figure 2b). Previously, we reported the coupling of $G\alpha_{12}$/$G\alpha_{13}$ with PAR (thrombin receptor) in macrophages (Kang *et al*., 2003). TGFβ1 mRNA levels in KO MEF cells were measured after thrombin treatment to associate the effect of GPCR ligand with *TGFβ1* gene induction. As expected, thrombin increased the level of TGFβ1 mRNA in control MEF cells. However, this was attenuated by a deficiency in $G\alpha_{12}$/$G\alpha_{13}$ (Figure 2c). These data indicate that $G\alpha_{12}$ and $G\alpha_{13}$ play a role in *TGFβ1* gene induction not only by TGFβ1 but also by thrombin.

Given the decreases in basal and inducible TGFβ1 expression by a $G\alpha_{12}$/$G\alpha_{13}$ deficiency, we next examined whether TGFβ1 treatment activates $G\alpha_{12}$ and $G\alpha_{13}$. As the activated forms of $G\alpha_{12}$ and $G\alpha_{13}$ bind to the tetratricopeptide repeat (TPR) domain of Ser/Thr phosphatase type 5, the interaction between the TPR domain of Ser/Thr phosphatase type 5 and $G\alpha_{12}$ or $G\alpha_{13}$ can be utilized to assess the activation status of the G-proteins (Yamaguchi *et al*., 2003; Radhika *et al*., 2005). GST-TPR pulldown assays showed that TGFβ1 treatment did not allow $G\alpha_{12}$ or $G\alpha_{13}$ to bind to the TPR domain (Figure 2d). Conversely, either $G\alpha_{12}$QL or $G\alpha_{13}$QL interacted with GST-TPR. These results provide evidence that TGFβ1 may not activate $G\alpha_{12}$ or $G\alpha_{13}$.

$G\alpha_{12}$/$G\alpha_{13}$ regulation of TGFβ1 gene induction in HSCs and in the liver

To verify our results from the MEF cell model, we next assessed the role of $G\alpha_{12}$ and $G\alpha_{13}$ in the regulation of the *TGFβ1* gene in HSCs and in the mouse liver. In

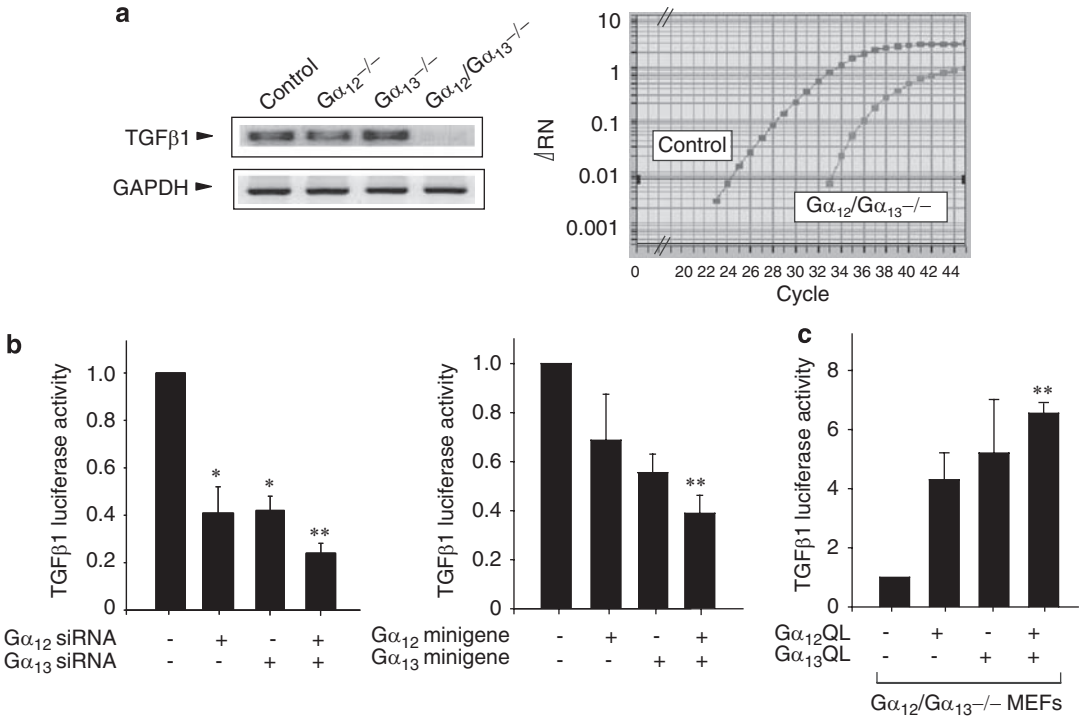


Figure 1 The constitutive *TGFβ1* gene expression. (**a**) The relative TGFβ1 mRNA levels in MEF cells were assessed using reverse transcription PCR (RT–PCR) (left panel) and real-time PCR (right panel) analyses. The relative mRNA levels were normalized by those of GAPDH. The amplification curve of TGFβ1 mRNA was right-shifted by a deficiency in $G\alpha_{12}$ and $G\alpha_{13}$ with the increase in C_T value. Results were confirmed by repeated experiments (control, RK−/− MEFs). (**b**) Repression of TGFβ1 reporter activity. Control WT MEF cells were transfected with scrambled siRNA (scRNA) or siRNAs directed against $G\alpha_{12}/G\alpha_{13}$. Data represented the mean ± s.e. of three separate experiments (significantly different as compared with scRNA, $^*P<0.05$, $^{**}P<0.01$; scRNA transfection, 1) (left panel). Repression of the basal *TGFβ1* gene expression by $G\alpha_{12}$ and/or $G\alpha_{13}$ minigene(s). MEFs were transfected with a TGFβ1 reporter construct (1 μg) and $G\alpha_{12}/G\alpha_{13}$ minigene(s) (500 ng each). pCMV was used for mock transfection. Data represented the mean ± s.e. of three separate experiments (significantly different as compared with vehicle, $^{**}P<0.01$; pCMV transfection, 1) (right panel). (**c**) The increase in the TGFβ1 reporter activity by $G\alpha_{12}$QL and $G\alpha_{13}$QL. $G\alpha_{12}/G\alpha_{13}$−/− cells were transfected with pCMV (1 μg) or a plasmid encoding $G\alpha_{12}$QL and/or $G\alpha_{13}$QL (500 ng each) (significantly different as compared with pCMV, $^{**}P<0.01$).

HSCs, knockdowns of $G\alpha_{12}$ and $G\alpha_{13}$ by siRNA transfection (12 h) decreased the level of TGFβ1 mRNA and inhibited luciferase expression from a TGFβ1 reporter gene (Figure 3a). Moreover, induction of TGFβ1 mRNA by thrombin was also inhibited by a deficiency in $G\alpha_{12}/G\alpha_{13}$ in HSCs (Figure 3b).

Studies in animal models have shown that exposure to dimethylnitrosamine (DMN), a hepatocarcinogen, causes HSC activation within 3–6 weeks (Jenkins *et al*., 1985; George and Chandrakasan, 1996; George *et al*., 2001; Kang *et al*., 2002a). In this study, after 4 weeks of DMN treatments, the levels of TGFβ1 transcript were measured in the livers of WT or double heterozygous deletion mutants of $G\alpha_{12}/G\alpha_{13}$ ($G\alpha_{12}/G\alpha_{13}+/-$) mice. As the $G\alpha_{12}$ members exhibited overlapping roles in transducing the signals for *TGFβ1* gene expression *in vitro*, here we focused on double heterozygous deletion mutants of $G\alpha_{12}/G\alpha_{13}$. A semi-quantitative RT–PCR assay showed that hepatic TGFβ1 mRNA levels were significantly higher in WT mice after 4 weeks of the DMN treatment compared with vehicle-treated control (Figure 3c, left panel). It should be noted that the ability of DMN to induce TGFβ1 in the liver was almost completely prevented by a $G\alpha_{12}$ and $G\alpha_{13}$ double heterozygous deficiency ($G\alpha_{12}/G\alpha_{13}+/-$). The result of RT–PCR analyses confirmed the regulatory role of $G\alpha_{12}/G\alpha_{13}$ in *TGFβ1* gene expression in the liver (Figure 3c, right panel).

Role of AP-1 in TGFβ1 gene expression downstream from $G\alpha_{12}/G\alpha_{13}$

In an effort to study the mechanistic basis of this process, we next explored the regulatory site affected by $G\alpha_{12}/G\alpha_{13}$ in the *TGFβ1* gene. A series of TGFβ1 promoter deletion constructs prepared from the −1.36 kb region were used to examine the role of the DNA response elements, which interact with various transcription factors. The luciferase activities expressed from pGL3-1362, pGL3-1132, pGL3-731 or pGL3-453 were all lower in cells deficient in $G\alpha_{12}$ and $G\alpha_{13}$ compared with their respective controls (Figure 4a). However, the expression of TGFβ1 from pGL-323 in the absence of $G\alpha_{12}$ and $G\alpha_{13}$ was not statistically different compared with that of controls, suggesting that DNA element(s) located in the region between −453 and −323 bp interact with transcription factor(s) regulated by $G\alpha_{12}$ and $G\alpha_{13}$.

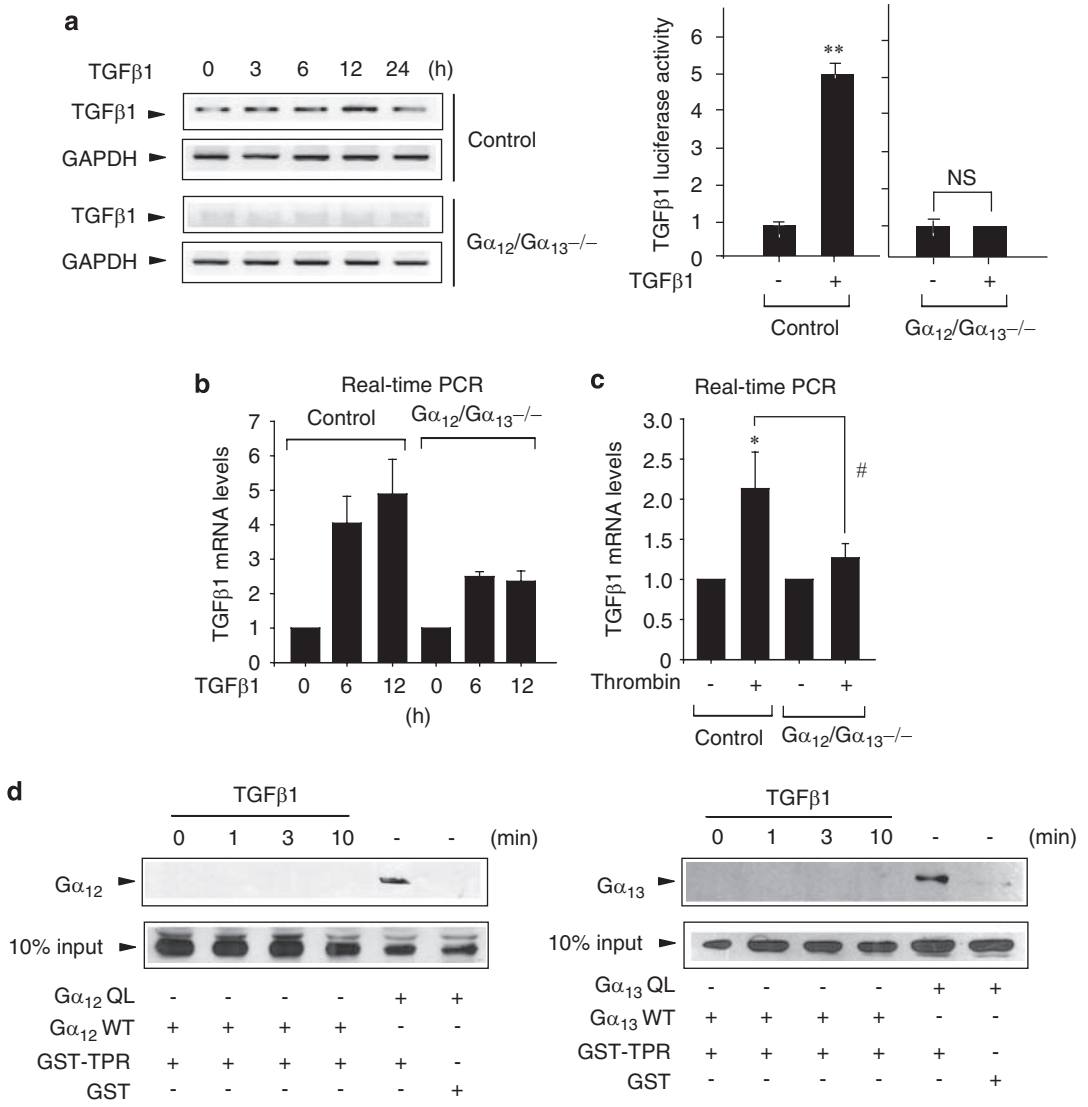


Figure 2 The effect of transforming growth factor-β1 (TGFβ1) or thrombin on the *TGFβ1* gene expression. (**a**) Reverse transcription PCR (RT–PCR) and reporter gene assays. TGFβ1 mRNA levels were measured in control or $G\alpha_{12}/G\alpha_{13}-/-$ MEF cells treated with 5 ng/ml of TGFβ1 for 3–24 h (left panel). Luciferase activity was measured in the lysates of MEF cells treated with TGFβ1 for 12 h (right panels). Results were confirmed by at least three separate experiments (significantly different as compared with the respective vehicle treatment, $^{**}P<0.01$; NS, not significant; control, WT MEFs). (**b**) TGFβ1-inducible TGFβ1 mRNA expression in primary MEF cells. MEF cells obtained from WT or $G\alpha_{12}/G\alpha_{13}+/-$ mice were treated with TGFβ1, and were subjected to real-time RT–PCR assays (control, WT MEFs). (**c**) Thrombin-inducible TGFβ1 mRNA levels. MEF cell lines were treated with 10 U/ml of thrombin for 12 h, and were subjected to real-time RT–PCR assays. Results were confirmed by at least three separate experiments (significantly different as compared with the respective vehicle treatment, $^{*}P<0.05$; significantly different as compared with primary MEF treated with thrombin, $^{\#}P<0.05$). (**d**) GST-TPR pulldown assays. Cells were transfected with a construct encoding WT $G\alpha_{12}$ (left panel) or WT $G\alpha_{13}$ (right panel), and were treated with 5 ng/ml of TGFβ1. Pulldown assays were performed as described in the experimental procedure section to assess whether TGFβ1 treatment activates $G\alpha_{12}/G\alpha_{13}$. Overall 10% of total cell lysate was used as an input control.

The role of AP-1 in *TGFβ1* gene expression was examined in association with $G\alpha_{12}/G\alpha_{13}$ because the region from −323 to −453 bp contains two AP-1 sites, the proximal serving as a critical binding site for gene transcription (Kim *et al*., 1989; Weigert *et al*., 2000). As anticipated, the siRNA knockdowns of $G\alpha_{12}$ and/or $G\alpha_{13}$ caused a decrease in the AP-1 reporter activity (Figure 4b, left panel). Similarly, transfection of control cells with the $G\alpha_{12}$ and $G\alpha_{13}$ minigenes also resulted in inhibition (Figure 4b, right panel). The AP-1 activity increased in KO cells simultaneously transfected with $G\alpha_{12}$QL and $G\alpha_{13}$QL (Figure 4c), and a $G\alpha_{12}/G\alpha_{13}$ deficiency abrogated a TGFβ1-dependent AP-1 reporter activity (Figure 4d, left panel), confirming that $G\alpha_{12}$ and $G\alpha_{13}$ regulate AP-1.

Next, we assessed the causal relationship between AP-1 activation and *TGFβ1* gene induction using a

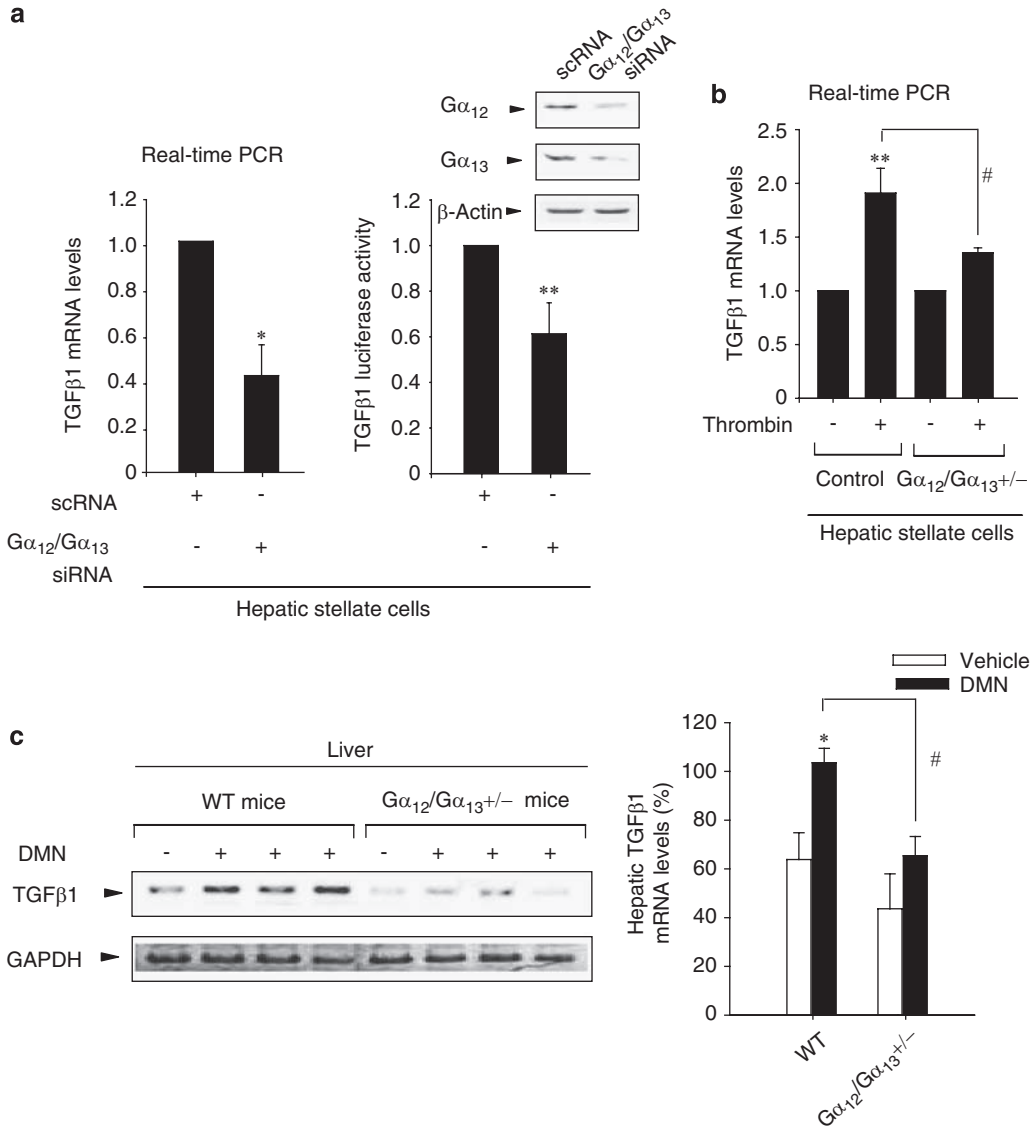
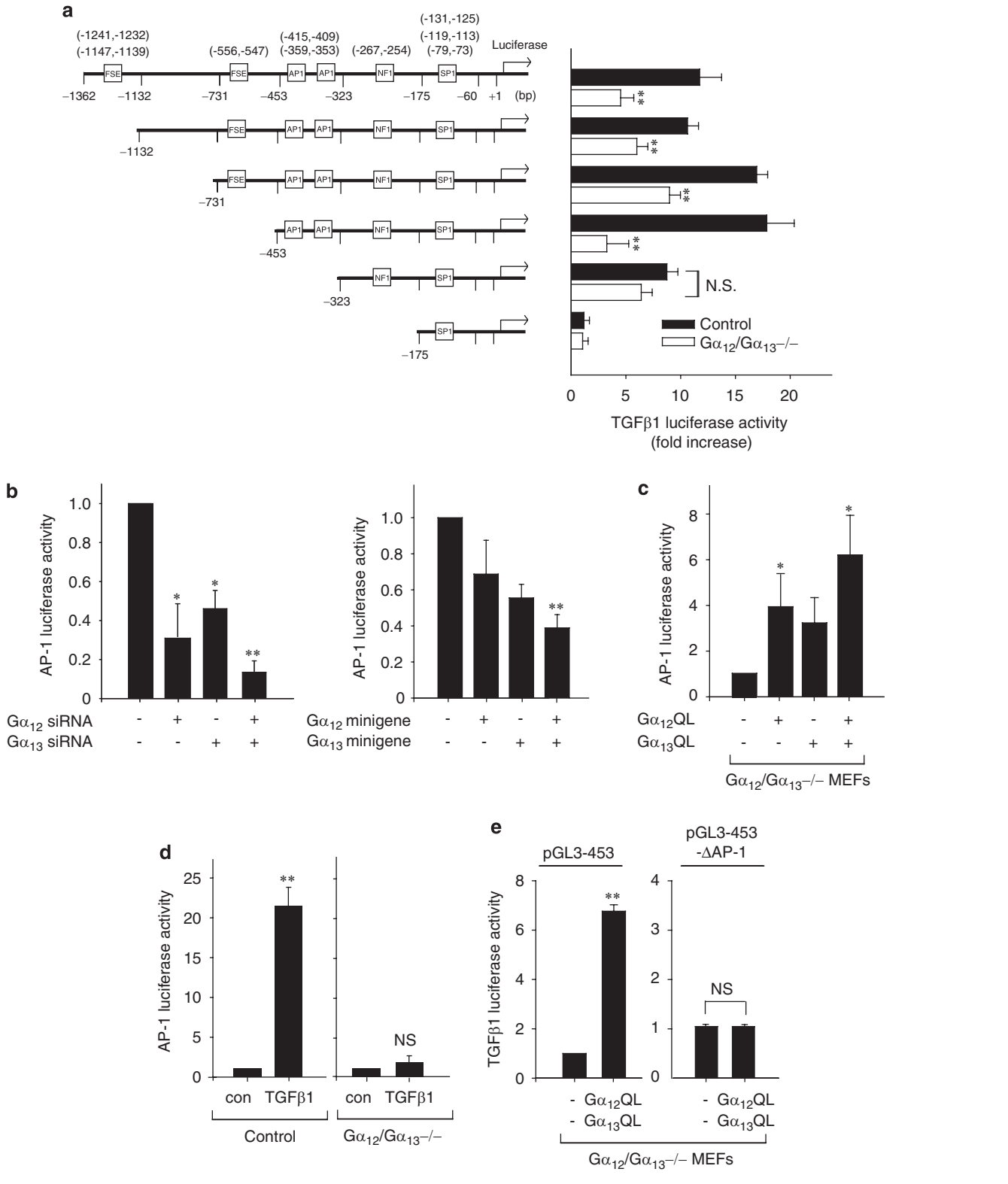


Figure 3 The regulatory role of $G\alpha_{12}/G\alpha_{13}$ in the *TGFβ1* gene induction in HSCs or in the liver. (**a**) The effects of siRNA knockdown of $G\alpha_{12}/G\alpha_{13}$ on the TGFβ1 mRNA level (left panel) and TGFβ1 luciferase activity (right panel) in HSCs. *TGFβ1* gene expression was determined in HSCs transfected with siRNA(s). Knockdowns of $G\alpha_{12}$ and $G\alpha_{13}$ were confirmed by immunoblottings. (**b**) The effect of thrombin on TGFβ1 mRNA level in HSCs. Primary HSCs isolated from WT or $G\alpha_{12}/G\alpha_{13}+/-$ mice were treated with thrombin (10 U/ml of thrombin, 12 h), and were subjected to real-time RT–PCR assays, as described in Figure 2c. Results were confirmed by at least three separate experiments (significantly different as compared to the respective vehicle treatment, $^{**}P<0.01$; significantly different as compared with WT HSCs treated with thrombin, $^{\#}P<0.05$). (**c**) Hepatic *TGFβ1* gene repression in mice by a double heterozygous deficiency of $G\alpha_{12}/G\alpha_{13}$. Hepatic TGFβ1 mRNA from DMN-treated WT or $G\alpha_{12}/G\alpha_{13}+/-$ mice was assessed using RT–PCR analyses, as described in the Materials and methods section. Four to six animals in each treatment group were used to confirm the data (significantly different as compared with the respective vehicle treatment, $^{*}P<0.05$; significantly different as compared with WT mice treated with DMN, $^{\#}P<0.05$; WT mice treated with DMN, 100%).

Figure 4 AP-1-dependent *TGFβ1* gene expression downstream of $G\alpha_{12}/G\alpha_{13}$. (**a**) A promoter deletion analysis of the *TGFβ1* gene. The sizes of the flanking insert in TGFβ1 chimeric gene constructs were indicated as bp numbers (left panel). Luciferase expression from each construct was determined in control or $G\alpha_{12}/G\alpha_{13}+/-$ MEF cells (right panel). Data represent the mean ± s.e. of three separate experiments (significantly different as compared with respective constructs in control cells, $^{*}P<0.05$, $^{**}P<0.01$; control, RK−/− cells). (**b**) Inhibition of AP-1 reporter activity by $G\alpha_{12}/G\alpha_{13}$ siRNA or minigenes (significantly different as compared with scRNA, $^{*}P<0.05$, $^{**}P<0.01$; left panel) (significantly different as compared with pCMV, $^{**}P<0.01$; right panel). (**c**) AP-1 reporter activity. $G\alpha_{12}/G\alpha_{13}-/-$ MEFs were transfected with pCMV or plasmids encoding $G\alpha_{12}$QL and $G\alpha_{13}$QL (significantly different as compared with pCMV, $^{*}P<0.05$). (**d**) TGFβ1-inducible AP-1 reporter activity. MEF cells transfected with an AP-1 reporter construct were treated with TGFβ1 for 12 h. Results were confirmed by at least three separate experiments (significantly different as compared with the respective vehicle treatment, $^{**}P<0.01$; NS, not significant; control, WT MEFs). (**e**) The effect of AP-1-binding site mutation on *TGFβ1* gene induction. A TGFβ1 reporter activity was determined in $G\alpha_{12}/G\alpha_{13}-/-$ cells that had been transfected with pGL3-453 or pGL3-453-AP-1 in combination with pCMV or $G\alpha_{12}$QL/$G\alpha_{13}$QL. Data represent the mean ± s.e. of three separate experiments (significantly different as compared with pCMV transfection, $^{*}P<0.05$, $^{**}P<0.01$; NS, not significant).

mutagenesis assay. A specific point mutation of the proximal AP-1 box caused the complete loss of pGL3-453 responsiveness to $G\alpha_{12}$QL and $G\alpha_{13}$QL, strengthening the notion that AP-1 plays a role in *TGFβ1* gene induction by $G\alpha_{12}/G\alpha_{13}$ (Figure 4e). Our results support the concept that $G\alpha_{12}$ and $G\alpha_{13}$ regulate the signals required for AP-1 activation and thereby control AP-1-dependent *TGFβ1* gene expression.



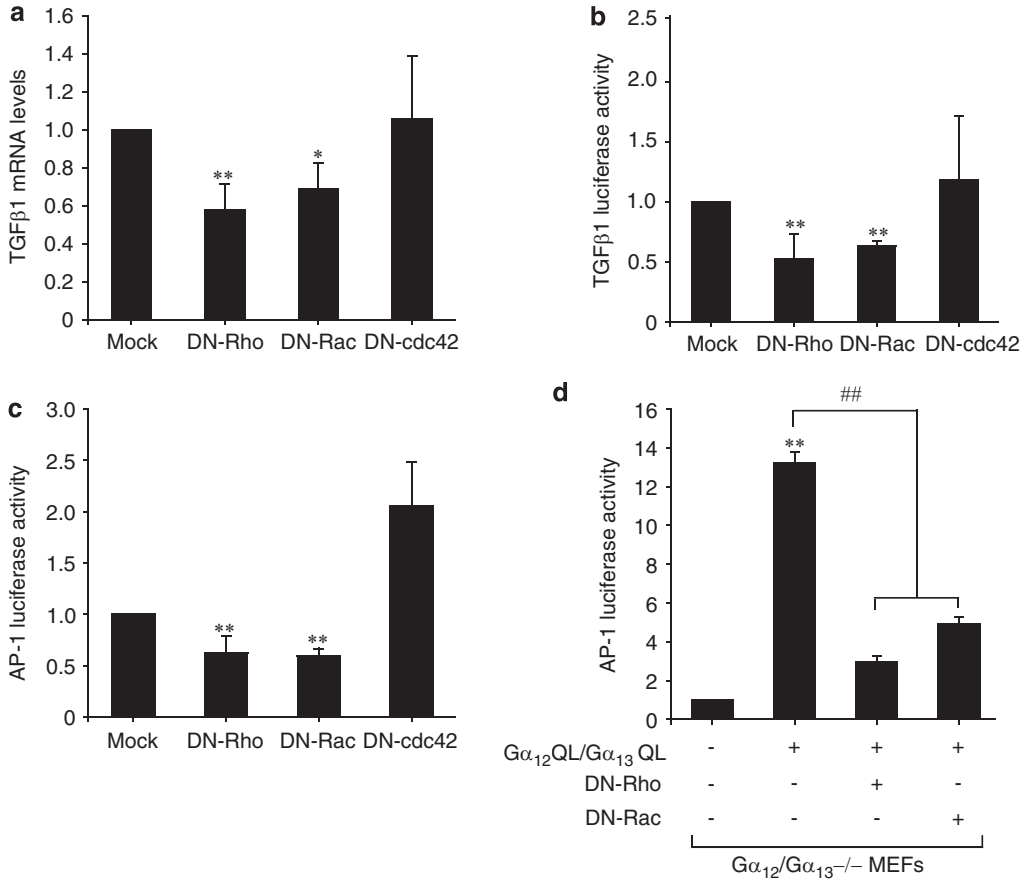


Figure 5 The role of Rho/Rac in the *TGFβ1* gene expression or the AP-1 reporter activity downstream of $G\alpha_{12}/G\alpha_{13}$. (**a**) Real-time PCR assays. TGFβ1 mRNA levels were measured in MEF cells transfected with a plasmid encoding a dominant-negative mutant (DN) of Rho, Rac or Cdc42 (1 μg each). (**b**) TGFβ1 luciferase reporter activity. (**c**) AP-1 luciferase activity. Data represent the mean ± s.e. of at least three separate experiments (significantly different as compared with pCMV transfection, $^{**}P<0.01$). (**d**) Inhibition of $G\alpha_{12}$QL/$G\alpha_{13}$QL-dependent AP-1 activity by DN-Rho or DN-Rac. An AP-1 reporter activity was measured in $G\alpha_{12}/G\alpha_{13}-/-$ cells that had been transfected with $G\alpha_{12}$QL/$G\alpha_{13}$QL (500 ng each) in combination with DN-Rho or DN-Rac (1 μg). pCMV was used for mock transfection. Data represent the mean ± s.e. of three separate experiments (significantly different as compared with mock transfection, $^{**}P<0.01$; significantly different as compared with $G\alpha_{12}$QL and $G\alpha_{13}$QL transfection, $^{\#\#}P<0.01$).

Rho/Rac-mediated AP-1-dependent TGFβ1 gene expression

To address the downstream effectors between $G\alpha_{12}/G\alpha_{13}$ and AP-1 activation, we finally examined the role of small GTP-binding proteins. Among small GTPase family members, Rho members serve as the central molecules regulated by many receptors including GPCRs, and are involved in a variety of cellular functions. We found that a dominant-negative mutant (DN) of Rho or Rac, but not Cdc42, attenuated not only the level of TGFβ1 mRNA but also the TGFβ1 reporter activity in MEF cells (Figures 5a and b). Our data identify the role of Rho and Rac in *TGFβ1* gene transactivation. Consistently, DN-Rho or DN-Rac transfection inhibited the AP-1 activity (Figure 5c). Moreover, they substantially decreased AP-1 activation induced by $G\alpha_{12}$QL and $G\alpha_{13}$QL in cells deficient in $G\alpha_{12}$ and $G\alpha_{13}$ (Figure 5d). These results showed that Rho and Rac are the downstream effectors responsible for AP-1 activation by $G\alpha_{12}$ and $G\alpha_{13}$, contributing to *TGFβ1* gene expression.

Discussion

TGFβ1 signaling is considered as an important hallmark of cancer transdifferentiation and metastasis (Coulouarn *et al*., 2008). In our study, the results of real-time PCR and reporter gene assays in conjunction with siRNA knockdowns and minigene or activated mutant transfection experiments indicated the role of $G\alpha_{12}$ and $G\alpha_{13}$ in constitutive or inducible *TGFβ1* gene transcription. To expand our results from the MEF cell studies, we examined the functional role of $G\alpha_{12}/G\alpha_{13}$ in primary HSCs. Knockdowns of $G\alpha_{12}/G\alpha_{13}$ in cultured HSCs caused inhibition of TGFβ1 transactivation. The concept that interruption of the G-protein signal abrogated *TGFβ1* gene induction is strengthened by the finding that TGFβ1- or thrombin-inducible *TGFβ1* gene expression is also decreased by a $G\alpha_{12}$ and $G\alpha_{13}$ deficiency, implying that the G-protein signal may provide a central upstream mechanism that amplifies the induction of TGFβ1. Moreover, we found that a deficiency in $G\alpha_{12}/G\alpha_{13}$ inhibits HSC activation,

which might be because of the lack of $G\alpha_{12}/G\alpha_{13}$-dependent autocrine loops, which activate *TGFβ1* gene expression.

In this study, promoter deletion analyses revealed the DNA region responsible for *TGFβ1* gene repression in the absence of $G\alpha_{12}$ and $G\alpha_{13}$ to be the segment containing the AP-1 boxes. Subsequent experiments on AP-1 DNA-binding and reporter activities using siRNA, minigenes or activated mutants demonstrated that $G\alpha_{12}$ and $G\alpha_{13}$ may regulate the signal for basal or TGFβ1-inducible AP-1 activity. Moreover, the role of AP-1 in TGFβ1 induction downstream of $G\alpha_{12}$ and $G\alpha_{13}$ was supported by our specific AP-1 mutation result for gene expression. Our additional gel shift studies indicated that Jun-D might be involved in the AP-1 complex formation for *TGFβ1* gene expression (unpublished data), which is consistent with an earlier observation (Smart *et al*., 2006). We also found that the mRNA levels of Jun-D and of its target genes (matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1) were reduced by a deficiency in $G\alpha_{12}/G\alpha_{13}$. Hence, $G\alpha_{12}$ and $G\alpha_{13}$ may regulate the remodeling and deposition of ECM, which is essential for the invasion and metastasis of tumor cells. The present results showing the enhanced AP-1 activation by TGFβ1 and the repression of inducible AP-1 activity by the absence of $G\alpha_{12}/G\alpha_{13}$ recapitulate the importance of AP-1 in the auto-stimulatory mechanism of *TGFβ1* gene transcription. This supports the concept that $G\alpha_{12}$ and $G\alpha_{13}$ regulate the basal and TGFβ1-inducible activation of AP-1. Our results agree with earlier reports that AP-1 serves as the transcription complex for induction of the *TGFβ1* gene and inflammatory genes, such as tumor necrosis factor-α, interleukin-1β and -6 (Kim *et al*., 1989; Rhoades *et al*., 1992; Dokter *et al*., 1995; Weigert *et al*., 2000).

Our observation that the small GTPases, Rho and Rac, play crucial roles in relaying the signaling by $G\alpha_{12}/G\alpha_{13}$ that results in the AP-1-dependent TGFβ1 induction (Figure 5d) is consistent with the observation that $G\alpha_{12}$ and $G\alpha_{13}$ have been known to regulate specific signaling pathways through Rho family of GTPases (Kurose, 2003; Fujii *et al*., 2005; Brown *et al*., 2006). However, the competitively inhibitory mutants of Rho (DN-Rho) and Rac (DN-Rac) were not able to completely attenuate the basal TGFβ1 mRNA levels (Figure 5a), TGFβ1-luciferase activity (Figure 5b) or AP-1 luciferase activity (Figure 5c). This is fully in agreement with the findings that the expression of TGFβ can be regulated by many different pathways that are independent of $G\alpha_{12/13}$ or Rho/Rac (Yue and Mulder, 2000). Thus, although the basal regulation of TGFβ1 levels is not fully dependent on Rho or Rac (Figures 5a–c), the $G\alpha_{12}/G\alpha_{13}$-mediated upregulation of AP-1 promoter activity involved in TGFβ1 expression is critically dependent on Rho and Rac, as indicated by the observation (Figure 5d) that DN-Rho and DN-Rac inhibit $G\alpha_{12}/G\alpha_{13}$-mediated AP-1 luciferase activity by 80 and 60%, respectively.

Quiescent HSCs, which lack AP-1 activity, transiently express c-Fos, Fra-1, c-Jun and Jun-B during culture, whereas myofibroblast-like (that is, activated) HSCs show persistent AP-1 activity consisting of Jun-D, Fos-B and Fra-2 (Bahr *et al*., 1999). Hence, the $G\alpha_{12}/G\alpha_{13}$ regulation of the *TGFβ1* gene in HSCs may also be mediated with the AP-1-dependent signaling cascade. As a large amount of TGFβ1 is produced from activated HSCs in the liver (Bataller and Brenner, 2005), these cells may be involved in the growth and migration of HCCs. In fact, HCCs have been shown to accompany HSC activation (Olaso *et al*., 1997; Lee *et al*., 2007). Moreover, co-transplantation of malignant hepatocytes with activated HSCs increases the potency of tumor formation, which might be mediated with activation of the TGFβ1 signaling pathway (Mikula *et al*., 2006). Thus, TGFβ1 produced from activated HSCs during liver disease is likely to be a potent inducer of cellular transformation.

In the hepatic carcinogenesis, HSCs serve as a liver-specific pericyte and contribute to the remodeling and deposition of tumor-associated ECM (Olaso *et al*., 1997). In an additional effort to verify the regulatory role of $G\alpha_{12}$ and $G\alpha_{13}$ in *TGFβ1* gene expression, we examined the TGFβ1 mRNA level in the liver of mice challenged with DMN (a hepatocarcinogen). A $G\alpha_{12}$ and $G\alpha_{13}$ double heterozygous deficiency prevented the ability of DMN to increase the TGFβ1 mRNA level, which corroborates the findings obtained from cellular models. Moreover, we found the hepatic accumulation of ECM to be inhibited during a deficiency in $G\alpha_{12}/G\alpha_{13}$ (manuscript in preparation), which is consistent with our finding showing decrease in the level of TGFβ1 mRNA in HSCs. Our studies also indicated that the level of CYP2E1 after DMN treatments was not significantly different among the KO animals, suggesting that a deficiency in $G\alpha_{12}/G\alpha_{13}$ might not change DMN activation. During the inflammatory period, the destruction of hepatocytes stimulates pro-inflammatory cells to produce toxic cytokines, which then trigger cellular changes, leading to liver disease through HSC activation (Schook *et al*., 1992; Friedman, 1993; Bataller and Brenner, 2005; Lee *et al*., 2007). Our result showing no significant difference in tumor necrosis factor-α levels between WT and $G\alpha_{12}/G\alpha_{13}+/-$ mice in response to DMN excludes the possibility that a deficiency in $G\alpha_{12}/G\alpha_{13}$ inhibits the tumor necrosis factor-α-dependent inflammatory process.

The vasoconstrictive mediators (for example, thrombin, angiotensin II and endothelin-1/2) induce cell transdifferentiation and proliferation (Pinzani *et al*., 1996; Bataller *et al*., 2000; Gaca *et al*., 2002; Fiorucci *et al*., 2004). Blockage of the GPCRs interacting with these ligands attenuates HCC growth and hepatocarcinogenesis through the inhibition of neovascularization (Molteni *et al*., 2006; Kaufmann *et al*., 2007; Yoshiji *et al*., 2007). In this study, we verified that thrombin treatment increased the level of TGFβ1 mRNA in MEFs or HSCs through AP-1, which was attenuated by a deficiency in $G\alpha_{12}/G\alpha_{13}$. Hence, it is plausible that the $G\alpha_{12}$ and $G\alpha_{13}$ pathway for AP-1 activation becomes more sensitive to elevated levels of the appropriate GPCR ligands, which contribute to TGFβ1 production.

Thus, $G\alpha_{12}$ and $G\alpha_{13}$ regulate expression of the *TGFβ1* gene, and a deficiency in $G\alpha_{12}/G\alpha_{13}$ prevents *TGFβ1* gene expression through a process mediated by a decrease in AP-1 activity. These observations lend support to the hypothesis that the activation of the GPCR-$G\alpha_{12}$ pathway is associated with TGFβ1-mediated transdifferentiation process.

Materials and methods

Cell culture and treatment

Immortalized MEFs were generated from genetically engineered mice that contained gene KOs for rhodopsin kinase (RK) and $G\alpha_{12}$ and/or $G\alpha_{13}$ (Xu *et al*., 1997; Vogt *et al*., 2003). RK, whose physiological function is restricted to photoreduction (Xu *et al*., 1997; Ki *et al*., 2007), was used as the KO control in association with G-proteins. As gene expression and induction responses in RK−/− MEFs were similar to those in WT MEFs (Ki *et al*., 2007), both RK−/− and WT MEFs were used as control cells. Cells were grown to 80–90% confluency and were subjected to no more than 20 cell passages. HSCs were isolated from WT or $G\alpha_{12}/G\alpha_{13}+/-$ mice, according to the published method (Kang *et al*., 2002b). After incubation for 7 days, the cells were sub-cultured to a six-well plate for further experiments. The degree of liver perfusion was important to ensure the purity of the HSCs (>95%), which show fully activated myofibroblast-like phenotype in culture. Primary MEFs were isolated from the embryos of WT or $G\alpha_{12}/G\alpha_{13}+/-$ mice at embryonic days 10 and 12. Experiments were carried out using primary MEF cells between passages 1 and 2. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% of fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. After serum starvation for 12 h, the cells were treated with 5 ng/ml of TGFβ1 (R&D Systems, Minneapolis, MN, USA) or 10 U/ml of thrombin (Calbiochem, San Diego, CA, USA) for the indicated time period.

Animals

Animal experiments were conducted under the guidelines of the Institutional Animal Use and Care Committee at Seoul National University. WT and $G\alpha_{12}/G\alpha_{13}+/-$ mice at the age of 8 weeks (~30 g body weight) were used for animal experiments ($N=4-6$ for each group) (Dr M Simon, Caltech) (Gu *et al*., 2002). PCR-based genotyping and immunoblotting confirmed the genotypes (Gu *et al*., 2002). To induce the activation of HSCs, mice in each group were treated with vehicle ($N=4$) or multiple doses of DMN (10 μl per kg body weight, i.p., $N=6$; Sigma Chemical, St Louis, MO, USA) twice per week for 4 weeks, as described earlier (Kang *et al*., 2002a, b). The mice were killed on day 28, and total RNA was extracted from the liver.

Semi-quantitative RT and real-time PCR

Total RNA was isolated from cells or liver using the improved single-step method of thiocyanate–phenol–chloroform RNA extraction according to the procedures described earlier (Kang *et al*., 2002b). RT–PCR was performed using primers specific for *TGFβ1* (sense primer: 5′-CTTCAGCTCCACAGAGAA GAACTGC-3′, antisense primer: 5′-CACGATCATGTTGGA CAACTGCTCC-3′) (298 bp) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense: 5′-TCGTGGAGTCTACT GGCGT-3′, antisense: 5′-GCCTGCTTCACCACCTTCT-3′) (510 bp). Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator. The SYBR Green real-time PCR amplifications were performed in a 96-well GeneAmp PCR System 9700 coupled with an ABI Prism 7000 Sequence Detection System (AB Applied Biosystems, Alameda, CA, USA). The C_T values of TGFβ1 and GAPDH amplification were obtained by using serially diluted cDNA samples, and samples with the same C_T values were used to facilitate equal loading of cDNA.

Transient transfection and luciferase reporter assay

MEFs or HSCs were plated at a density of 5×10^5 cells/well in a six-well dish and were transfected with expression constructs, scrambled siRNA (scRNA) or siRNA directed against $G\alpha_{12}/G\alpha_{13}$ (100 pmol each) (Dharmacon, Boulder, CO, USA) using a Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The culture medium was then changed to the medium containing 1% of fetal bovine serum, and the cells were further incubated for 12 h. The specificity of siRNA was verified by $G\alpha_{12}/G\alpha_{13}$ immunoblotting. The TGFβ1 reporter constructs were kindly provided by Dr SJ Kim (National Cancer Institute, Bethesda, MD, USA). The AP-1 reporter plasmid was purchased from Stratagene (La Jolla, CA, USA). Transfected cells were treated with TGFβ1 or thrombin for 12 h. Luciferase activities were measured using a dual-luciferase assay system (Promega, Madison, WI, USA) (24). In certain experiments, the minigenes (Solis-Herruzo *et al*., 1998; Byun *et al*., 2006) or activated mutant constructs of $G\alpha_{12}/G\alpha_{13}$ (0.5 μg each) were co-transfected with the reporter gene. For certain knockdown experiments, the cells were co-transfected with scRNA, $G\alpha_{12}$ siRNA and/or $G\alpha_{13}$ siRNA with reporter constructs.

GST-TPR pulldown assays

A construct of GST-fused TPR domain was kindly provided by Dr N Dhanasekaran (Temple University, PA, USA). GST-TPR was purified from *Escherichia coli*, as described earlier (Yamaguchi *et al*., 2003). The GST-TPR pulldown assay was carried out according to the previously published method (Radhika *et al*., 2005). Briefly, MEF cells were transfected with the construct encoding WT $G\alpha_{12}$ or $G\alpha_{13}$ and were stimulated with TGFβ1 for 1–10 min. Cell lysates were incubated with GST-TPR bound to glutathione-Sepharose beads for 3 h at 4 °C. The bound proteins were resolved by SDS–polyacrylamide gel electrophoresis and immunoblotted for $G\alpha_{12}$ or $G\alpha_{13}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA). $G\alpha_{12}$QL or $G\alpha_{13}$QL was used as a positive control.

Mutagenesis assay

The proximal AP-1 box in the human *TGFβ1* gene located between −430 and −396 bp was specifically point-mutated by substituting bases using mutagenic primers (5′-TGTTTCCCAGCCTGGTTCTCCTTCCGTTCTGG-3′ and 5′-AAGGGTCGGACCAAAGAGGAAGGCAAGACCCAG-3′, underlines indicate the mutated nucleotides), according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA) (Weigert *et al*., 2000). The DNA sequence of the mutant construct, pGL3-453-ΔAP-1, was verified by using an automatic DNA sequence analyzer.

Statistical analysis

One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means.

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