The RhoA Activator GEF-H1/Lfc Is a Transforming Growth Factor-β Target Gene and Effector That Regulates α-Smooth Muscle Actin Expression and Cell Migration

Anna Tsapara,* Phillip Luthert,† John Greenwood,* Caroline S. Hill,‡ Karl Matter,* and Maria S. Balda*

Departments of *Cell Biology and †Ocular Biology and Therapeutics, Institute of Ophthalmology, University College London, London EC1V 9EL, United Kingdom; and ‡Laboratory of Developmental Signalling, Cancer Research UK London Research Institute, London WC2A 3PX, United Kingdom

Submitted July 13, 2009; Revised January 11, 2010; Accepted January 13, 2010

Monitoring Editor: Keith E. Mostov

Maintenance of the epithelial phenotype is crucial for tissue homeostasis. In the retina, dedifferentiation and loss of integrity of the retinal pigment epithelium (RPE) leads to retinal dysfunction and fibrosis. Transforming growth factor (TGF)-β critically contributes to RPE dedifferentiation and induces various responses, including increased Rho signaling, up-regulation of α-smooth muscle actin (SMA), and cell migration and dedifferentiation. Cellular TGF-β responses are stimulated by different signal transduction pathways: some are Smad dependent and others Smad independent. Alterations in Rho signaling are crucial to both types of TGF-β signaling, but how TGF-β-stimulates Rho signaling is poorly understood. Here, we show that primary RPE cells up-regulated GEF-H1 in response to TGF-β. GEF-H1 was the only detectable Rho exchange factor increased by TGF-β1 in a genome-wide expression analysis. GEF-H1 induction was Smad4-dependent and led to Rho activation. GEF-H1 inhibition counteracted α-SMA up-regulation and cell migration. In patients with retinal detachments and fibrosis, migratory RPE cells exhibited increased GEF-H1 expression, indicating that induction occurs in diseased RPE in vivo. Our data indicate that GEF-H1 is a target and functional effector of TGF-β by orchestrating Rho signaling to regulate gene expression and cell migration, suggesting that it represents a new marker and possible therapeutic target for degenerative and fibrotic diseases.

INTRODUCTION

The retinal pigment epithelium (RPE) underlies the neural retina and is crucial for photoreceptor physiology and survival; hence, various retinopathies originate from changes in RPE function. Retinal detachments due to injury or surgery lead to RPE dysfunction and the development of ocular fibrotic diseases including proliferative vitreoretinopathy (Roberts et al., 2006; Saika et al., 2008). Major drivers of ocular degenerative and fibrotic diseases are transforming growth factor (TGF)-β and its downstream signaling mechanisms (Connor et al., 1989; Hiscott et al., 1999; Kon et al., 1999; Saika et al., 2004). Rho signaling is one of those mechanisms and is activated by TGF-β in fibrotic diseases of different types of epithelia including the RPE (Zheng et al., 2004; Nishikimi and Matsuoka, 2006). Therefore, identification of regulators of Rho signaling downstream of TGF-β is crucial to understand these pathological changes and to identify novel therapeutic targets.

TGF-β signaling activates different signal transduction mechanisms: they can be Smad dependent or Smad independent and activate different types of cellular responses (Zavadil and Bottinger, 2005; Schmierer and Hill, 2007; Heldin et al., 2009; Zhang, 2009). In brief, upon TGF-β binding, the type II receptor kinase activates the type I receptor kinase, leading to phosphorylation of Smad2 and Smad3, which subsequently oligomerize with Smad4 and translocate to the nucleus to regulate gene expression. Smad-dependent signaling is important for cellular responses such as migration (Levy and Hill, 2005). TGF-β-stimulated Smad-independent signaling pathways include various branches of mitogen-activated protein kinase pathways (e.g., p38, extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase) and phosphatidylinositol-3-kinase/AKT pathways depending on the cellular context. Importantly, however, the Smad-dependent and -independent responses cannot always be separated so clearly, because certain signaling mechanisms, such as RhoGTPases, are regulated by both types of responses. Hence, it is important to understand how such Rho signaling mechanisms contribute to specific TGF-β responses.

Modulation of Rho GTPase signaling plays a central role in various TGF-β-induced responses but is only partially understood. TGF-β has opposing temporal effects on RhoA activation, initially inhibition and later activation of Rho signaling. TGF-β induces dissolution of cell–cell adhesion and reorganization of the actin cytoskeleton. During the first phase, RhoA is inactivated by degradation at cell junctions, leading to reduced intercellular adhesion (Ozdamar et al., 2005). This initial phase is important for epithelial mesenchymal transition (EMT). In contrast, subsequent cellular responses leading to cytoskeletal reorganization, α-smooth muscle actin (SMA) expression and cell migration require RhoA activation; however, the molecular mechanisms and
Rho regulators by which TGF-β induces activation of RhoA signaling are poorly understood (Masszi et al., 2003; Fu et al., 2006; Kita et al., 2008). Activation of Rho GTPases is catalyzed by guanine nucleotide exchange factors (GEFs) and inactivation by GTPase activating proteins (GAPs). Understanding the functional roles of different GEFs and GAPs as well as their regulation of expression and activity in particular signaling pathways is a major challenge, and recent evidence suggests that these proteins may be potential therapeutic targets for developing drugs to treat various diseases (Bos et al., 2007).

We now identify GEF-H1 as crucial TGF-β target gene and show that GEF-H1 regulates TGF-β-induced Rho activation, responses in gene expression, and migration in primary RPE cells. GEF-H1 protein expression is also up-regulated in migratory RPE cells of patients with retinal detachments and fibrosis, indicating that the observations in the experimental model reflect processes that occur in human disease. Our data thus indicate that GEF-H1 is a crucial target and mediator of TGF-β signaling and participates in epithelial dysfunction in disease.

MATERIALS AND METHODS

Reagents, Cell Culture, and Treatments

Human RPE cells were derived from explants of neonatal fetal bovine retina (Eli Lilly, Indianapolis, Indiana) and were immortalized by CNTF. For transfection studies, cells were grown in DMEM/F12, supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. RPE cells were transfected using Lipofectamine 2000 (Invitrogen, Paisley, United Kingdom), and a total final siRNA concentration was 100 nM (Steed et al., 2003), and lysed in SDS-PAGE sample buffer for protein analysis, or levels of active RhoA were measured with the G-LISA assay kit (Cytoskeleton, Denver, CO). and used at passage 1. For TGF-β1 (20 ng/ml) was added, and the cells were analyzed after another 24 h. 

We now identify GEF-H1 as crucial TGF-β target gene and show that GEF-H1 regulates TGF-β-induced Rho activation, responses in gene expression, and migration in primary RPE cells. GEF-H1 protein expression is also up-regulated in migratory RPE cells of patients with retinal detachments and fibrosis, indicating that the observations in the experimental model reflect processes that occur in human disease. Our data thus indicate that GEF-H1 is a crucial target and mediator of TGF-β signaling and participates in epithelial dysfunction in disease.

Microarray Analysis

RPE cells were incubated for 3 d in the absence or presence of TGF-β1 (10 ng/ml), and RNA was isolated. Three samples for each condition were obtained and analyzed using a Biochip 2100 (Agilent Technologies, Palo Alto, CA). CDNA and subsequent cRNA were prepared as described previously (Chambers et al., 2003) and then hybridized to porcine GeneChip arrays according to Affymetrix (Santa Clara, CA) standard protocols (http://www.affymetrix.com). For the University College London, Institute of Child Health Gene Microarray Centre (London, United Kingdom), Labeled GeneChips were scanned, using a confocal argon ion laser (Agilent Technologies). The data were analyzed using Gene Spring 7.2 software (Agilent Technologies). Genes were excluded if the signal strength did not significantly exceed background values and if expression did not reach a threshold value for reliable detection (based on the relaxed Affymetrix MAS 5.0 probability of detection (p ≤ 0.1) in each of the samples (See et al., 2004).

Reporter Gene Assays

RPE cells were transfected using Lipofectamine 2000 (Invitrogen, Paisley, United Kingdom) with the indicated reporter promoter constructs driving firefly luciferase expression and an expression construct for GEF-H1 (pCB6-GEF-H1) or empty vector (pCB6), a reference promoter driving Renilla luciferase. The data were normalized to totalized (a total of 600 cells were counted for each condition, shown are means ± standard deviation (SD).

Transfection of siRNAs and Determination of Active RhoA

HaCaT cells were plated into 12-well plates, to determine Rho activation, or 24-well plates, to analyze protein expression. Cells were then transfected with non-targeting control siRNA pools or siRNAs specific for GEF-H1, Snail, and Slug (Thermo Fisher Scientific, Waltham, MA; and Dharmacon RNA Technology, Lafayette, CO), or with siRNA targeting RhoA (Santa Cruz Biotechnology). The siRNA was oligo(dT)20-modified (GFP-3′) and transfected with the siPort Neo transfection reagent (Ambion, Austin, TX). At 48 h post-transfection, cells were lysed for Western blotting or luciferase activity.

Inhibition of α-SMA Expression by DN-GEF-H1

RPE cells were transiently transfected with pcDNA4/TO-CTD-VSV construct driven by a CMV promoter (DN-GEF-H1) and firefly luciferase. Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 

The assay was performed as described above for transfected control cells and for DN-GEF-H1 expressing cells. The data were normalized to totalized (a total of 600 cells were counted for each condition, shown are means ± standard deviation (SD).
RESULTS

TGF-β–induced Disorganization of Cell–Cell Adhesion Correlates with Up-Regulation of GEF-H1

We used primary porcine RPE cells as a model to analyze TGF-β signaling because they form well-differentiated monolayers in culture and respond to TGF-β (Lee et al., 2001; Ablonczy and Crosson, 2007). As expected, addition of TGF-β stimulated dissolution of cell–cell adhesion structures, such as adherens and tight junctions, correlating with altered cell morphology and reduced expression of junctional proteins, such as ZO-1 and occludin (Figure 1, A–F).

RhoA is a key player in the control of the actin cytoskeleton, cell–cell adhesion and gene expression (Fujita and Braga, 2005; Hall, 2005; Posern and Treisman, 2006; Heasman and Ridley, 2008; Nelson, 2008). To identify the Rho activators that transmit the TGF-β stimulus, we performed a genome-wide expression analysis using microarrays. Total RNA was isolated from triplicate samples of control and TGF-β–treated RPE cells and used to probe Affymetrix porcine arrays. GEF-H1 was the only detectable Rho exchange factor that was up-regulated in response to TGF-β (Table 1), suggesting that induction of GEF-H1 expression is likely to be of functional relevance for TGF-β–induced responses in RPE cells.

GEF-H1/Lfc is a guanine nucleotide exchange factor for RhoA (Benais-Pont et al., 2003; Aijaz et al., 2005; Birkenfeld et al., 2008). In contrast to permanent cell lines, GEF-H1 is expressed at very low levels in primary cultures of differentiated RPE cells (Figure 1G), similar to the levels previously reported for adult epithelial tissues (Ryan et al., 2005). Stimulation with TGF-β, however, up-regulated GEF-H1 expression (Figure 1G). Similar results were obtained with GEF-H1 antibodies recognizing different epitopes (data not shown).

| Name               | Control | TGF-β1 | UniGene   |
|--------------------|---------|--------|-----------|
| GEF-H1/Lfc         | 110.2 ± 0.7 | 191.0 ± 29.4 | Ssc.8984  |
| GEPf432/HERC1 (for ARF) | 139.6 ± 17.1 | 154.9 ± 21.8 | Ssc.6629  |
| VAV1               | 2.83 ± 1.7  | 2.5 ± 1.2   | Ssc.5910  |
| RAPGEF5/MR-GEF     | 5.6 ± 0.4   | 7.3 ± 0.6   | Ssc.4792  |
| GRINCHGEGF/ARHGEF10L | 9.7 ± 2.0   | 9.0 ± 2.0   | Ssc.4246  |
| Lsc/ARHGEF1       | 20.1 ± 1.4  | 24.5 ± 2.5  | Ssc.27637 |
| RAPGEPF3/EPAC      | 12.5 ± 2.8  | 14.7 ± 1.1  | Ssc.19027 |
| LARG/ARHGEF12     | 4.0 ± 0.8   | 28.1 ± 1.7  | Ssc.18932 |
| DeGELF/SEGRGEF    | 50.2 ± 5.2  | 56.5 ± 10.4 | Ssc.17909 |
| CoolI/βPIX         | 79.1 ± 1.1  | 80.8 ± 3.7  | Ssc.1606  |
| eEF1α (for eEF1α) | 509.8 ± 29.8 | 536.8 ± 27.6 | Ssc.1439  |
| RAPGGEF2/PDZGEF1  | 31.5 ± 2.9  | 37.2 ± 2.27 | Ssc.10907 |

Porcine RPE primary cultures were treated for 3 days with TGF-β, and total RNA was isolated. After RNA quality control, the samples were processed for microarray analysis. The data were analyzed using Gene Spring 7.2 software (Agilent Technologies). Media of normalized intensity with SEs of three determinations for each condition are shown. GEFs for different types of GTpases that could be detected and identified based on the available porcine genome information are shown.
Figure 2. Inhibition of TGF-β–induced Rho activity by GEF-H1 depletion. (A) HaCaT cells were stimulated for 3 d with TGF-β and were then analyzed for expression of GEF-H1 and α-tubulin. Expression of GEF-H1 was monitored with two different antibodies, an mAb antibody and a polyclonal (pAb) antibody that recognize different epitopes. (B and C) HaCaT cells were transfected with control or GEF-H1 targeting siRNAs, and, after 24 h, were incubated with or without TGF-β for the next 3 d. The cells were then lysed to monitor expression of GEF-H1 (B) or analyzed for active RhoA levels (C; shown are means ± 1 SD, n = 3; indicated are p values obtained from t tests comparing TGF-β-treated cells with nontreated control siRNA-transfected cells and, respectively, GEF-H1 depleted TGF-β–treated cells with control siRNA transfectants TGF-β–treated cells).

Figure 3. TGF-β1-induced GEF-H1 up-regulation is Smad4 dependent. (A) RT-PCR analysis for GEF-H1 mRNA levels in control and TGF-β1–treated (3-d) samples; GAPDH was used as a loading control. Note that the increase observed by RT-PCR (2+ fold) is similar to the increase obtained from the microarray analysis. (B) RPE cells were preincubated with actinomycin D (ActD) or cycloheximide (CHX) and then stimulated or not with TGF-β1 for 2 d. Immunoblot of total RPE cell extracts probed for GEF-H1 and α-tubulin. Note that the TGF-β1–induced increase was blocked by >85% by both actinomycin D and cycloheximide. (C) RPE cells were preincubated with SB431542, TGF-β receptor type I kinase inhibitor, and then stimulated with TGF-β1 for 3 d and tested for GEF-H1 and α-tubulin expression. (D) HaCaT-TR-S4, a stable cell line permitting inducible depletion of Smad4, and the parental cell line HaCaT-TR were treated with tetracycline for 2 d to reduce Smad4 expression and were then stimulated with TGF-β1 for 4 d. Total cell extracts were probed for GEF-H1 and Smad4. Note: A threefold up-regulation of GEF-H1 was observed in HaCaT cells that was blocked by Smad4 depletion. (E) HaCaT cells were transfected with control or Slug-targeting siRNAs. After 24 h, the cells were incubated with fresh medium without or with TGF-β3 for 3 d before analysis of Slug, GEF-H1 and tubulin expression. Note that no change in GEF-H1 expression was observed upon depletion of Slug. (F and G) RPE cells plated at very low density were grown in the absence or presence of the ALK5 inhibitor SB431542 for 14 d. (E) Phase contrast images of control and treated cells. (G) Immunoblot for GEF-H1, α-tubulin, and α-SMA.

Figure 3A shows that increased GEF-H1 mRNA levels in response to TGF-β were also observed if analyzed by semi-quantitative RT-PCR instead of microarrays: in both types of assays, an approximately twofold up-regulation of GEF-H1 mRNA was observed. To determine whether transcription was required for up-regulation of GEF-H1 protein, we treated the cells with actinomycin D or cycloheximide. Both drugs inhibited induction of GEF-H1, indicating that transcription is required (Figure 3B).

We next tested whether up-regulation of GEF-H1 involves the canonical Smad pathway (Derynck and Zhang, 2003). Treatment of RPE cells with the ALK5 kinase inhibitor

**TGF-β Transcriptionally Up-Regulates GEF-H1 Expression through a Smad4-dependent Pathway**

In response to TGF-β, activated Smad2 and Smad3 form complexes with Smad4 and accumulate in the nucleus, where they regulate expression of TGF-β target genes (Ross and Hill, 2008). Therefore, we next analyzed whether the TGF-β–induced up-regulation of GEF-H1 at the protein level and the increased mRNA levels observed by microarray analysis were due to changes at the transcriptional level and whether up-regulation depended on Smad4.
SB431542 abrogated GEF-H1 expression (Figure 3C), indicating that TGF-β type I receptor kinase activity is necessary for GEF-H1 expression. To test involvement of the Smad pathway directly, we used again HaCaT cells that stably express a tetracycline-inducible shRNA targeting Smad4 (HaCaT-TR-S4 cells) (Levy and Hill, 2005). TGF-β1 induced the expression of GEF-H1 in control HaCaT cells, and Smad4 depletion inhibited TGF-β1 induced GEF-H1 up-regulation (Figure 3D), revealing that GEF-H1 induction by TGF-β1 requires Smad4. These observations thus indicate that up-regulation of GEF-H1 involves activation of the TGF-β type I receptor kinase and the Smad pathway.

Microarray analysis previously identified two populations of TGF-target genes: Smad-dependent and -independent genes (Levy and Hill, 2005). Because the Smad-dependence groups TGF-β-responsive genes are into different functional groups, the observed Smad-dependence for GEF-H1 suggests that it may function in Smad-dependent processes such as cell migration. Certain other TGF-β-stimulated processes, such as EMT, are Smad-independent and require the up-regulation of other transcription factors, such as Snail and Slug (Levy and Hill, 2005). Hence, we tested whether Snail and Slug are involved in GEF-H1 up-regulation by transfecting cells with siRNAs targeting the two transcription factors before TGF-β stimulation. Figure 3E shows that up-regulation of GEF-H1 was not prevented by down-regulation of Slug. We were not able to detect Snail in HaCaT cells using two different antibodies, suggesting that Snail does not become up-regulated in these cells. This is in agreement with previous observations (Levy and Hill, 2005).

Primary RPE cells in culture transdifferentiate into myofibroblast-like cells not only when treated with TGF-β but also spontaneously when plated at low density (Grisanti and Guidry, 1995; Lee et al., 2001; Wiencke et al., 2003). Transdifferentiated RPE cells up-regulate both α-SMA and GEF-H1, supporting a myofibroblast-like phenotype (Figure 1G). Strikingly, treatment of low-density cultures with the ALK5 inhibitor prevented morphological changes (Figure 3F) as well as α-SMA and GEF-H1 up-regulation (Figure 3G), further supporting the correlation between TGF-β signaling and expression of α-SMA and GEF-H1.

TGF-β modulates cellular phenotypes not only by regulating α-SMA expression but also of nonmuscle myosin isoforms (Sinha et al., 2004; Obara et al., 2005). Therefore, we examined whether TGF-β stimulation affects myosin-IIA expression. Indeed, TGF-β increased myosin-IIA expression with similar kinetics as expression of GEF-H1 (Figure 4A). Although the ALK5 kinase inhibitor abrogated myosin-IIA expression, primary RPE cells in culture transdifferentiate into myofibroblast-like cells not only when treated with TGF-β but also spontaneously when plated at low density (Grisanti and Guidry, 1995; Lee et al., 2001; Wiencke et al., 2003). Transdifferentiated RPE cells up-regulate both α-SMA and GEF-H1, supporting a myofibroblast-like phenotype (Figure 1G). Strikingly, treatment of low-density cultures with the ALK5 inhibitor prevented morphological changes (Figure 3F) as well as α-SMA and GEF-H1 up-regulation (Figure 3G), further supporting the correlation between TGF-β signaling and expression of α-SMA and GEF-H1.

TGF-β modulates cellular phenotypes not only by regulating α-SMA expression but also of nonmuscle myosin isoforms (Sinha et al., 2004; Obara et al., 2005). Therefore, we examined whether TGF-β stimulation affects myosin-IIA expression. Indeed, TGF-β increased myosin-IIA expression with similar kinetics as expression of GEF-H1 (Figure 4A). Although the ALK5 kinase inhibitor abrogated myosin-IIA expression, primary RPE cells in culture transdifferentiate into myofibroblast-like cells not only when treated with TGF-β but also spontaneously when plated at low density (Grisanti and Guidry, 1995; Lee et al., 2001; Wiencke et al., 2003). Transdifferentiated RPE cells up-regulate both α-SMA and GEF-H1, supporting a myofibroblast-like phenotype (Figure 1G). Strikingly, treatment of low-density cultures with the ALK5 inhibitor prevented morphological changes (Figure 3F) as well as α-SMA and GEF-H1 up-regulation (Figure 3G), further supporting the correlation between TGF-β signaling and expression of α-SMA and GEF-H1.

TGF-β modulates cellular phenotypes not only by regulating α-SMA expression but also of nonmuscle myosin isoforms (Sinha et al., 2004; Obara et al., 2005). Therefore, we examined whether TGF-β stimulation affects myosin-IIA expression. Indeed, TGF-β increased myosin-IIA expression with similar kinetics as expression of GEF-H1 (Figure 4A). Although the ALK5 kinase inhibitor abrogated myosin-IIA expression, primary RPE cells in culture transdifferentiate into myofibroblast-like cells not only when treated with TGF-β but also spontaneously when plated at low density (Grisanti and Guidry, 1995; Lee et al., 2001; Wiencke et al., 2003). Transdifferentiated RPE cells up-regulate both α-SMA and GEF-H1, supporting a myofibroblast-like phenotype (Figure 1G). Strikingly, treatment of low-density cultures with the ALK5 inhibitor prevented morphological changes (Figure 3F) as well as α-SMA and GEF-H1 up-regulation (Figure 3G), further supporting the correlation between TGF-β signaling and expression of α-SMA and GEF-H1.
Figure 6. Rho signaling and GEF-H1 regulate α-SMA expression induced by TGF-β. (A) RPE cells were incubated for 3 d with or without TGF-β. During the last 2 d, membrane-permeable C3 transferase was added as indicated. Expression of GEF-H1 and α-tubulin was then analyzed by immunoblotting. (B and C) RPE cells were transiently transfected with DN-GEF-H1 and treated with TGF-β for 3 d. The cells were then fixed and processed for immunofluorescence using antibodies against α-SMA and VSV, to detect DN-GEF-H1. Shown is an example of obtained images (B), and quantifications of percentages of α-SMA-positive cells in the control (VSV-negative) and DN-GEF-H1 expressing (VSV-positive) cell populations. (D) RPE cells were infected with control (LNT-control) or DN-GEF-H1 (LNT-DN-GEF-H1) lentivirus and stimulated with TGF-β for 3 d, and then α-SMA and fibronectin expression was analyzed in total cell extracts. The graphs show densitometric analysis of scanned immunoblot data. Note: Dominant-negative GEF-H1 inhibits TGF-β-induced α-SMA expression.

We next used RNA interference to down-regulate GEF-H1 expression in RPE cells. However, various control siRNAs already repressed α-SMA levels, indicating an unspecific effect of siRNAs in RPE cells. Therefore, we made use of a dominant-negative (DN) construct containing the C-terminal domain (CTD) of GEF-H1 and a C-terminal VSV epitope as a tag (DN-GEF-H1). SRE reporter assays confirmed that DN-GEF-H1 is able to suppress SRE-driven transcription (Supplemental Figure 2).

We next used a transient transfection assay to determine whether DN-GEF-H1 is able to counteract α-SMA-induction by TGF-β. Double immunofluorescence revealed that most DN-GEF-H1–expressing cells failed to up-regulate α-SMA (Figure 6B). Quantification demonstrated that only 25% of the DN-GEF-H1–expressing cells were positive for α-SMA, whereas 60% of the control cells expressed the EMT marker (Figure 6C). For biochemical quantification, we repeated the experiment with lentiviral vectors to transduce RPE cells with DN-GEF-H1 (LNT-DN-GEF-H1) or a control lentivirus (LNT-control) and then stimulated with TGF-β. Immunoblot analysis showed that LNT-DN-GEF-H1 transduction resulted in a 2.7-fold decrease in the α-SMA expression compared with LNT-control (Figure 6D). If the samples were probed for expression of fibronectin, a TGF-β target gene that is up-regulated in a Smad-independent manner (Tsuda et al., 2003), no inhibition of up-regulation was observed. These results show that expression of DN-GEF-H1 counteracts the TGF-β–induced increase in α-SMA expression.

Treatment of cultures plated at low-density (0.5 × 10⁴ cells/cm²) with the ALK5 inhibitor prevented morphological changes (Figure 3F) as well as α-SMA and GEF-H1 up-regulation (Figure 3G). ALK5 inhibitor also prevented the generation of gaps and monolayer detachment and contraction of older primary cultures (>3 wk) that were plated at high density (6 × 10⁴ cells/cm²) on fibronectin after they had reached confluence, indicating that it was also caused by endogenous TGF-β production (Figure 7A). Because inhibition of GEF-H1 counteracts up-regulation of α-SMA ex-
pression, we next tested whether it also inhibits monolayer contraction and cell detachment.

Figure 7B shows that monolayers formed by RPE cells infected with a control lentivirus (LNT-control) started to detach and contract, whereas those infected with a virus encoding DN-GEF-H1 (LNT-DN-GEF-H1) did not. Quantification of such images confirmed that expression of dominant-negative GEF-H1 counteracted the appearance of cell-free areas even after 4 d of culture (Figure 7C). Thus, these results indicate that GEF-H1 drives morphological changes such as cell contraction and detachment induced by TGF-β1. Because contraction and detachment were measured by quantification of cell-free areas (Figure 7), further analysis will be necessary to identify whether cell-free areas are due to contraction only or also to reduced adhesion and/or increased cell death.

**GEF-H1 Is Up-Regulated in RPE from Patients with Disorganized Retina and Pigment Epithelium**

TGF-β signaling and expression of α-SMA have been related to the ability of RPE cells to form periretinal membranes and are thought to contribute to retinal detachments in proliferative vitreoretinopathy (PVR) and in response to trauma (Fuchs et al., 1991; Saika et al., 2004; Zheng et al., 2004). Therefore, we next studied the expression of GEF-H1 in eye sections from patients with retinal detachments due to different types of insults.

In control RPE cells, there was little or no immunoreactivity for GEF-H1 (Figure 8A1), confirming the observations we made in nonstimulated primary porcine cultures and further supporting the conclusion that expression of high levels of GEF-H1 requires a stimulus in most adult tissues. In contrast, in pathological specimens, there was consistent GEF-H1 immunoreactivity in subsets of RPE cells that had migrated away from their normal location between photoreceptor outer segments and Bruch’s membrane in nine of the ten investigated samples (Figure 8). There were four pigmented RPE cell phenotypes associated with this staining: migratory cells that remained configured as a monolayer, RPE cells around blood vessels, individual migratory cells, or apex to apex islands of RPE cells (Fig. 8, A2–A5). RPE cells were identified on the basis of intense pigmentation and a side-to-side arrangement typical of epithelia except for when arranged as individual cells. Furthermore, their cytoarchitecture was generally cuboidal or polygonal rather than rounded, as would be expected for macrophages that had engulfed uveal pigment. Clusters of CD68 expressing macrophages were, however, identified and they were also strongly immunoreactive (data not shown). These observations indicate that up-regulation of GEF-H1 occurs in response to ocular insults and can be observed in migratory RPE cells in vivo.

**GEF-H1 Regulates Cell Migration**

The observed up-regulation of GEF-H1 in migratory pigmented RPE cells in vivo, suggests that the exchange factor plays a role in TGF-β-stimulated migration, a process that involves RhoA activation and that is thought to be one of the underlying reasons for failure of retinal detachment surgery due to PVR (Kon et al., 1999; Kim et al., 2006). As TGF-β-induced migration is abolished after silencing of Smad4 in HaCaT cells (Levy and Hill, 2005) as is up-regulation of GEF-H1 (Figure 3D), we next tested whether GEF-H1 contributes to TGF-β-induced RPE migration using manual and electrical wound-healing assays.

RPE cells were infected with LNT-DN-GEF-H1 or LNT-control, pre-stimulated with TGF-β and then wounded either manually or with a strong electrical field. Wound closure was then followed microscopically or by measuring impedance of the monolayer. Figure 8, B and C, shows that expression of DN-GEF-H1 impaired wound closure in both assays. This indicates that the exchange factor indeed regulates TGF-β-induced RPE cell migration.

To test the importance of GEF-H1 for cell migration with a different cell type, we used spontaneously immortalized MDCK cells that constitutively express high levels of the exchange factor (Benais-Pont et al., 2003; Aijaz et al., 2005). We took advantage of previously generated cell lines that permit the tetracycline-induced depletion of GEF-H1 by RNA interference (Benais-Pont et al., 2003; Aijaz et al., 2005). Figure 8D shows that depletion of GEF-H1 resulted in a
strong retardation of wound closure in the electrical wound healing assay. Visual inspection of the slides confirmed that the failure in wound healing was due to reduced migration of cells into the induced wound as compared with control RNA interference cells. Thus, GEF-H1 regulates migration of different epithelial cell types and may be of general importance for epithelial migration.

**DISCUSSION**

TGF-β-induced expression of alpha-smooth muscle actin and cell migration occurs during the development of different tissues and in several diseases including cancer and fibrosis, a common complication after tissue damage and surgery (Liu, 2006; Roberts et al., 2006). Our results demonstrate that the Rho guanine nucleotide exchange factor GEF-H1 is a novel target gene and functional effector of two crucial TGF-β-driven processes: α-SMA up-regulation, a marker for transdifferentiation, and cell migration.

TGF-β activates Smad-dependent and independent signaling pathways that regulate various cellular responses including cell migration, adhesion, proliferation and EMT (Derynck and Zhang, 2003; Ikenouchi et al., 2003; Peinado et al., 2007; Ross and Hill, 2008; Thual et al., 2008; Heldin et al., 2009). Via the Smad-independent pathway, TGF-β receptor II triggers PAR6 mediated down-regulation of RhoA signaling at cell-cell junctions, which initiates dissociation of cell-cell adhesion (Ozdamar et al., 2005). However, Smad-dependent and independent processes then require RhoA activation in a spatially and temporally controlled manner. Interestingly, certain processes only require one branch of TGF-β signaling, as, for example, Smad4 is required for TGF-β-induced migration, but not EMT, which is Slug dependent but smad4 independent in HaCaT cells (Levy and Hill, 2005). Here, we found that the Smad4-dependent pathway up-regulates GEF-H1 expression induced by TGF-β. Hence, one way by which Smad4-dependent signaling drives the migratory phenotype is by controlling the expression of GEF-H1 and, thereby, Rho activation.

In epithelial cells, GEF-H1, a guanine nucleotide exchange factor for RhoA, associates with tight junctions; and functions in the regulation of paracellular permeability, cell proliferation and junction disassembly (Benais-Pont et al., 2003; Aijaz et al., 2005; Birukova et al., 2006; Samarlin et al., 2007). We now found that GEF-H1 also supports α-SMA expression and cell migration. The activity of Rho GTPases has to be carefully timed and controlled to guide epithelial proliferation and differentiation (Fujita and Braga, 2005; Heasman and Ridley, 2008; Nelson, 2008; Wheelock et al., 2008; Yu et al., 2008). In epithelial cells in culture, the endogenous levels of expression of GEF-H1 are generally high; hence, it was previously poorly understood how expression of GEF-H1 is stimulated. In adult epithelial tissues, however, GEF-H1 levels are low. The same is true for the RPE as both primary culture and in vivo experiments indicate that expression of GEF-H1 is low in differentiated cells (Figures 1 and 8). Our data now show that TGF-β induces a striking up-regulation of GEF-H1 in Smad4-dependent pathway and in two different epithelial models.

In primary RPE cells in culture, transdifferentiation can be induced when cells are plated at low density, resulting in were cultured with the antibiotic and then subjected to a high electric field to induce a wound in the center of each monolayer and impedance was measured to monitor wound closure. Shown is a representative experiment performed in duplicates.
increased expression of α-SMA (Grisanti and Guidry, 1995; Lee et al., 2001; Wiencke et al., 2003) as well as GEF-H1 (Figure 3). As it has been suggested that at low confluence RPE cell secrete TGF-β, we inhibited the TGF-β receptor I with the ALK5 inhibitor and indeed found that it prevented morphological degeneration as well as α-SMA and GEF-H1 up-regulation. Thus, exogenous as well as autocrine TGF-β induces GEF-H1, indicating that TGF-β is a major driver of GEF-H1 expression in epithelial cells. Although Smad4, but not Slug, is required for GEF-H1 up-regulation, how transcription is induced is not clear yet. The late and sustained expression of GEF-H1 indicates that it may be an indirect target of Smad4-dependent signaling. Recent evidence also shows that GEF-H1 activation is regulated by phosphorylation and TNF-alpha (Zenke et al., 2004; Callow et al., 2005; Chang and Lee, 2006; Fujishiro et al., 2008; Kakiashvili et al., 2009; Nie et al., 2009), suggesting that GEF-H1 regulation occurs at different levels and is target by different signaling pathways. Nevertheless, as most adult tissues express little GEF-H1, up-regulation represents an important step in activation of GEF-H1 signaling.

Regulation of Rho activity has previously been linked to TGF-β stimulation in different cell types (Bhowmick et al., 2001; Bakin et al., 2002; Edlund et al., 2002). TGF-β also enhances the expression of RhoB (Engel et al., 1998) as well as NET1, a RhoA-specific guanine exchange factor (Shen et al., 2001; Levy and Hill, 2005). However, we have not been able to detect NETI in RPE cells treated with TGF-β1 (not shown). We also failed to detect up-regulation of other Rho exchange factors such as ARHGEF18 by immunoblotting (Supplemental Figure 3) as well as by means of cDNA arrays (Table 1). Hence, RPE cells seem to up-regulate GEF-H1 specifically, indicating that the exchange factor is a major TGF-β target gene in respect to Rho signaling.

As activation of the α-SMA promoter seems to involve Rho signaling in TGF-β-induced transdifferentiation of renal epithelial cells (Masszi et al., 2003), we assessed α-SMA promoter activity and protein expression in RPE cells stimulated with TGF-β in the presence or absence of GEF-H1 inhibition. Our results indicate that GEF-H1 mediates Rho stimulation to induce α-SMA expression by activation of its promoter. Therefore, GEF-H1 is not only a target gene of TGF-β, but functionally contributes to the expression of marker genes associated with transdifferentiation and fibrosis. Hence, GEF-H1 represents a possible target to inhibit α-SMA expression for the treatment of fibrosis.

Although Rho signaling is thought to be important for fibrosis, the mechanisms that drive Rho activation in fibrosis had previously not been identified. We have observed strong increases in GEF-H1 expression in RPE cells of patients with retinal detachments due to different types of insults that triggered retinopathies and disorganization of the pigment epithelium (i.e., dislocation from Bruch’s membrane). RPE cells have been suggested to contribute to retinal detachments in PVR and in response to trauma (Fuchs et al., 1991; Saika et al., 2004; Zheng et al., 2004), and inhibition of the Rho-kinase pathway suppresses the expression of α-SMA in rabbit RPE cells in culture and attenuates retinal detachment in a rabbit PVR model (Zheng et al., 2004; Kita et al., 2008). Furthermore, the analysis of expression of GEF-H1 in eye sections from patients with retinal detachments demonstrated that GEF-H1 is up-regulated in migratory RPE cells (Figure 8), suggesting that increased expression of GEF-H1 is an early event in the translocation of RPE from their normal location at the back of the retina and is likely to contribute to transdifferentiation in vivo. Thus, GEF-H1 represents a possible therapeutic target to attenuate RPE migration and retinal detachments after injury or surgery.

TGF-β is involved in cell migration in different cell types using Smad-dependent or -independent pathways. Rho also plays a role in cell migration. Our results show that GEF-H1 regulates Rho activation and migration induced TGF-β in primary RPE cells, HaCaT as well as MDCK cells, a spontaneously immortalized cell line that constitutively expresses high levels of GEF-H1. When this article was under revision, a study was published that suggested that GEF-H1 also regulates migration in a tumor cell line (Nalbant et al., 2009). Thus, activation of Rho signaling by GEF-H1 seems to be connected to cell migration in different cellular contexts, indicating that GEF-H1 represents a link by which TGF-β stimulates molecular mechanisms of general importance for cell migration and gene expression.

In summary, we have identified a new target and functional effector of TGF-β signaling, the Rho guanine nucleotide exchange factor GEF-H1 that regulates expression genes related to transdifferentiation, such as α-SMA, and epithelial cell migration. Up-regulation of GEF-H1 occurs in migratory RPE in patients with retinal detachments, suggesting that GEF-H1 is a marker and novel therapeutic target for retinal detachments, and may be a crucial signaling protein to be targeted during the manipulation of RPE cells for transplantation and in fibrotic diseases.

ACKNOWLEDGMENTS

We are thankful to Dr. G. K. Ovens (University of Virginia, Charlottesville, VA) for the α-SMA promoter constructs and to Christine Gaughan for the immunoblotting (Supplemental Figure 3) as well as by means of cDNA arrays (Table 1). Therefore, GEF-H1 is a microtubule-regulated Rho-GEF: is altered GEF-H1 increased expression of α-SMA/β-SMA in rabbit RPE cells in culture and attenuates retinal barrier dysfunction. Am. J. Physiol. Lung Cell Mol. Physiol. 290, L540–L548.

Aijaz, S., D’Atri, F., Citi, S., Balda, M. S., and Matter, K. (2005). Binding of GEF-H1 to the tight junction-associated adaptor cingulin results in inhibition of Rho signaling and G1/S phase transition. Dev. Cell 8, 777–786.

Bainbridge, J. W., Stephens, C., Parsley, K., Demaison, C., Halfyard, A., Thrasher, A. J., and Ali, R. R. (2001). In vivo gene transfer to the mouse eye using an HIV-based lentiviral vector: efficient long-term transduction of corneal endothelium and retinal pigment epithelium. Gene Ther. 8, 1665–1668.

Bakin, A. V., Rinehart, C., Tomlinson, A. K., and Arteaga, C. L. (2002). p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. J. Cell Sci. 115, 3193–3206.

Benais-Pont, G., Punn, A., Flores-Maldonado, C., Eckert, J., Raposo, G., Fleming, T. P., Ceretijido, M., Balda, M. S., and Matter, K. (2003). Identification of a tight junction-associated guanine nucleotide exchange factor that activates Rho and regulates paracellular permeability. J. Cell Biol. 160, 729–740.

Bhowmick, N. A., Ghiasi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L., and Moses, H. L. (2001). Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol. Biol. Cell 12, 27–36.

Birkenfeld, J., Nalbant, P., Yoon, S. H., and Bokoch, G. M. (2008). Cellular functions of GEF-H1, a microtubule-regulated Rho-GEF: is altered GEF-H1 activity a crucial determinant of disease pathogenesis? Trends Cell Biol. 18, 210–219.

Birukova, A. A., Adyshev, D., Gorskikh, B., Bokoch, G. M., Birukov, K. G., and Verin, A. D. (2006). GEF-H1 is involved in agonist-induced human pulmonary endothelial barrier dysfunction. Am. J. Physiol. Lung Cell Mol. Physiol. 290, L540–L548.

Bos, J. L., Rehmann, H., and Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. Cell 129, 865–877.

Callow, M. G., Zozulya, S., Gishizky, M. L., Jallal, B., and Smeal, T. (2005). PAK4 mediates morphological changes through the regulation of GEF-H1. J. Cell Sci. 118, 1861–1872.
Chambers, R. C., Leoni, P., Kaminski, N., Laurent, G. J., and Heller, R. A. (2003). Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. Am. J. Pathol. 162, 533–546.

Chang, Z. F., and Lee, H. H. (2006). RhoA signaling in phorbol ester-induced apoptosis. J. Biomed. Sci. 13, 173–180.

Coleman, M. L., Sahai, E. A., Yee, M., Bosch, M., Dewar, A., and Olson, M. F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat. Cell Biol. 3, 339–345.

Connor, T. B., Jr., et al. (1989). Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. J. Clin. Invest. 83, 1661–1666.

Derynck, R., and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425, 577–584.

Edlund, S., Landstrom, M., Heldin, C. H., and Aspstenstrom, P. (2002). Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. Mol. Biol. Cell 13, 902–914.

Engel, M. E., Datta, P. K., and Moses, H. L. (1998). RhoB is stabilized by transforming growth factor beta and antagonizes transcriptional activation. J. Biol. Chem. 273, 9921–9926.

Fu, P., Liu, F., Su, S., Wang, W., Huang, X. R., Entman, M. L., Schwartz, R. J., Wei, L., and Lan, H. Y. (2006). Signaling mechanism of renal fibrosis in unilateral ureteral obstructive kidney disease in ROCK I knockout mice. J. Am. Soc. Nephrol. 17, 3105–3114.

Fuchs, U., Kivel, T., and Tarikaanen, A. (1991). Cytoskeleton in normal and reactive human retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 32, 3178–3186.

Fujisoh, S. H., Tanimura, S., Mure, S., Kashimoto, Y., Watanabe, K., and Kohno, M. (2008). ERK1/2 phosphorylate GEF-H1 to enhance its guanine nucleotide exchange activity toward RhoA. Biochem. Biophys. Res. Commun. 368, 162–167.

Fujita, Y., and Braga, V. (2005). Epithelial cell shape and Rho small GTPases. Novartis Found. Symp. 269, 144–155; discussion 155–148, 223–230.

Grisanti, S., and Guidry, C. (1995). Transdifferentiation of retinal pigment epithelial cells from epithelial to mesenchymal phenotype. Invest. Ophthalmol. Vis. Sci. 36, 391–405.

Hall, A. (2005). Rho GTPases and the control of cell behaviour. Biochem. Soc. Trans. 33, 891–895.

Heasman, S. J., and Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat. Rev. Mol. Cell Biol. 9, 690–701.

Heldin, C. H., Landstrom, M., and Moustakas, A. (2009). Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Curr. Opin. Cell Biol.

Hill, C. S., Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell 81, 1159–1170.

Hisscott, P., Sheridan, C., Magee, R. M., and Grierson, I. (1999). Matrix and the transforming growth factor-beta family signalling. Nature 357, 1170–1176.

Kakashvili, E., Speight, P., Waheed, F., Seth, R., Lodgya, M., Tanimura, S., Kohno, M., Rotein, O. D., Kapus, A., and Szczepanski, K. (2009). GEF-H1 mediates tumor necrosis factor-alpha-induced Rho activation and myosin phosphorylation: role in the regulation of tubular paracellular permeability. J. Biol. Chem. 284, 11454–11466.

Keese, C. R., Wegener, J., Walker, S. R., and Giaever, I. (2004). Electrical wound-healing assay for cells in vitro. Proc. Natl. Acad. Sci. USA 101, 15544–15549.

Kim, J. S., et al. (2006). Transforming growth factor-beta1 regulates macrophage migration via RhoA. Blood 108, 1821–1829.

Kita, T., et al. (2008). Role of TGF-beta in proliferative vitreoretinal diseases and ROCK as a therapeutic target. Proc. Natl. Acad. Sci. USA 105, 17504–17509.

Kon, C. H., Oclesten, N. L., Aylward, G. W., and Khaw, P. T. (1999). Expression of vitreous cytokines in proliferative vitreoretinopathy: a prospective study. Invest. Ophthalmol. Vis. Sci. 40, 705–712.
Seo, J., Bakay, M., Chen, Y. W., Hilmer, S., Shneiderman, B., and Hoffman, E. P. (2004). Interactively optimizing signal-to-noise ratios in expression profiling: project-specific algorithm selection and detection p-value weighting in Affymetrix microarrays. Bioinformatics 20, 2534–2544.

Shen, X., Li, J., Hu, P. P., Waddell, D., Zhang, J., and Wang, X. F. (2001). The activity of guanine exchange factorネット1 is essential for transforming growth factor-beta-mediated stress fiber formation. J. Biol. Chem. 276, 15362–15368.

Sinha, S., Hoofnagle, M. H., Kingston, P. A., McCanna, M. E., and Owens, G. K. (2004). Transforming growth factor-beta1 signaling contributes to development of smooth muscle cells from embryonic stem cells. Am. J. Physiol. Cell Physiol. 287, C1560–C1568.

Steed, E., Rodrigues, N. T. L., Balda, M. S., and Matter, K. (2009). Identification of MarvelD3 as a tight junction associated transmembrane protein of the occludin family. BMC Cell Biol. 10, 95.

Thuault, S., Tan, E. J., Peinado, H., Cano, A., Heldin, C. H., and Moustakas, A. (2008). HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition. J. Biol. Chem. 283, 33457–33466.

Tsuchida, K., Zhu, Y., Siva, S., Dunn, S. R., and Sharma, K. (2003). Role of Smad4 on TGF-beta-induced extracellular matrix stimulation in mesangial cells. Kidney Int. 63, 2000–2009.

Wamhoff, B. R., Bowles, D. K., and Owens, G. K. (2006). Excitation-transcription coupling in arterial smooth muscle. Circ. Res. 98, 868–878.

Wheelock, M. J., Shintani, Y., Maeda, M., Fukumoto, Y., and Johnson, K. R. (2008). Cadherin switching. J. Cell Sci. 121, 727–735.

Wiencke, A. K., Kiilgaard, J. F., Nicolini, J., Bundgaard, M., Ropke, C., and La Cour, M. (2003). Growth of cultured porcine retinal pigment epithelial cells. Acta Ophthalmol. Scand. 81, 170–176.

Yu, W., Shewan, A. M., Brakeman, P., Eastburn, D. J., Datta, A., Bryant, D. M., Fan, Q. W., Weiss, W. A., Zegers, M. M., and Mostov, K. E. (2008). Involvement of RhoA, ROCK I and myosin II in inverted orientation of epithelial polarity. EMBO Rep. 9, 923–929.

Zavadil, J., and Bottinger, E. P. (2005). TGF-beta and epithelial-to-mesenchymal transitions. Oncogene 24, 5764–5774.

Zenke, F. T., Krendel, M., DerMardirossian, C., King, C. C., Bohl, B. P., and Bokoch, G. M. (2004). p21-activated kinase 1 phosphorylates and regulates 14-3-3 binding to CEF-H1, a microtubule-localized Rho exchange factor. J. Biol. Chem. 279, 18392–18400.

Zhang, Y. E. (2009). Non-Smad pathways in TGF-beta signaling. Cell Res. 19, 128–139.

Zheng, Y., Bando, H., Ikuno, Y., Oshima, Y., Sawa, M., Ohji, M., and Tano, Y. (2004). Involvement of rho-kinase pathway in contractile activity of rabbit RPE cells in vivo and in vitro. Invest. Ophthalmol. Vis. Sci. 45, 668–674.