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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

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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-dependant 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 NH_2-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

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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

Recombinant human TGF-β1 was from (PeproTech Rocky Hill, NJ). SB431542, actinomycin D, and cycloheximide were from Sigma Chemical (Poole, Dorset, United Kingdom). RPE cells were isolated from porcine eyes (Lee *et al*., 2001) and used at passage 1. For TGF-β1 experiments, cells were plated at 3×10^4 cells/cm^2, serum starved (0.5% fetal bovine serum) for 24 h, and then stimulated by adding 10 ng/ml TGF-β1 for the indicated times. For inhibitor studies, cells were preincubated with 10 μM SB431542, 50 ng/ml actinomycin D, or 10 μg/ml cycloheximide for 1 h and then treated with TGF-β1 in the continuous presence of the inhibitor for the specified time. For spontaneous transdifferentiation, RPE cells (passage 1) were plated at 0.5×10^4 cells/cm^2; in some experiments, cells were cultured in the presence of SB431542 (10 μM; 14 d). The human keratinocyte cell lines HaCaT-TR (stably expressing the tetrcycline [Tet] repressor) and HaCaT-TR-S4 (stably expressing the Tet-inducible Smad4 small interfering RNA (siRNA) in addition to the Tet repressor) have been characterized previously (Levy and Hill, 2005). For siRNA induction, the cells were grown for 48 h in the presence of tetracycline (2 μg/ml) and then stimulated by adding 2 ng/ml TGF-β1 for 4 d in the continuous presence of tetracycline. Madin-Darby canine kidney (MDCK) cells allowing the conditional depletion of GEF-H1 were described previously (Benais-Pont *et al*., 2003).

Immunoblotting and Immunofluorescence Microscopy

Total cell extracts were prepared in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and western blotting was performed using standard procedures. For immunofluorescence, cells were fixed in ice-cold methanol and processed for immunostaining as described previously (Benais-Pont *et al*., 2003). Photographs were obtained with an LSM510 confocal microscope (Carl Zeiss, Jena, Germany) using a 63× objective, and the manufacturer's image acquisition software. Brightness and contrast of the images were adjusted with Photoshop (Adobe Systems Mountain View, CA). Antibodies used were as follows: zona occludens-1 (Benais-Pont *et al*., 2003), occludin (mouse; Zymed Laboratories, South San Francisco, CA), cingulin (rabbit; Zymed Laboratories), GEF-H1 (Benais-Pont *et al*., 2003), α-SMA (1A4; Sigma Chemical), α-tubulin (1A2; Kreis, 1987), Smad4 (B8; Santa Cruz Biotechnology, Santa Cruz, CA), Slug (Santa Cruz Biotechnology), myosin-IIA (rabbit [Sigma Chemical] and mouse, 3/36), fibronectin (monoclonal F0791; Sigma Chemical) myosin light chain phosphates (MYPT1) and phosphorylated (T696) MYPT1 (Millipore, Billerica, MA). Secondary antibodies conjugated to horseradish peroxidase, fluorescein isothiocyanate, and cyanine 3 were from Jackson ImmunoResearch Laboratories (West Grove, PA). IRDye-680- and IRDye 80-conjugated secondary antibodies were from Li-COR Biosciences (Lincoln, NE) and were used in combination with an Odyssey fluorescence reader.

Immunocytochemistry

On approval of the ethics committee of the local health authority (REC 05/Q0504/17), eyes consented for research were obtained from Moorfields Hospital Eye Bank (London, United Kingdom). Nine evisceration specimens and one enucleation specimen were examined. In all eviscerations, there was relatively extensive disorganization of intraocular contents, generally with at least partial retinal detachment and changes of proliferative vitreoretinopathy. Trauma was the most common underlying pathology with two postinfection cases and one patient with retinopathy of prematurity. The enucleation was carried out because of a choroidal malignant melanoma. Routine, buffered Formalin-fixed, paraffin-embedded sections were cut at 4 to 5 μm in thickness. Hybridoma supernatant with anti-GEF-H1 monoclonal antibody (mAb) was diluted 1 in 3 and incubated overnight followed by washing and alkaline phosphatase-conjugated secondary antibody. Immunoreaction product was visualized using a red alkaline phosphatase-based technique and an Autostainer (Dako, Ely, Cambridgeshire, United Kingdom) after pretreatment in citrate buffer, pH 6.0, in a Pascal pressure cooker (Dako) according to manufacturer's instructions.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was reverse transcribed with specific anti-sense primers using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) for 1 h. PCR was carried out in the exponential phase (25 cycles) to allow comparison of PCR product levels. This was achieved by performing initial reactions with different amounts of template to determine optimal amounts of input. For reverse transcription, total RNA (0.5 μg in 15-μl RT reaction) was incubated for 1h at 50°C for GEF-H1 primer 5′-ACATCTGTCATCAGCAGGA-3′. For PCR 1 μl of RT reaction was used: primers 5′-TTCTCATCACCCAGTTCTCA-3′ (forward) and 5′-ACATCTGTCATCAGCAGGA-3′ (reverse) and an annealing temperature of 56°C. Other primers used were myosin-IIA (forward 5′-AAGCTGCAGGAGATGGA GGGC-3′; reverse 5′-AAAAAAGAATTCCGGCCTGGAGCT CCTCCTCTTT-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5′-ATCACTGCCACCCAGAAGAC-3′; reverse ATGAGGTCCACCACCCTGTT-3′).

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 the RNA quality analyzed using a Bioanalyzer 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) at 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 ($p \leq 0.1$) in each of the samples (Seo *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 was used to normalized the data. Reporters genes used were as follows: serum response element (SRE) (SRE containing promoter; Clontech, Mountain View, CA), α-SMA-fl (full-length α-SMA promoter), α-SMA-155 (155-base pair α-SMA promoter construct), and α-SMA-BmAm (155-base pair α-SMA promoter construct with mutated SRE elements; Liu *et al*., 2003). After 26 h, firefly and *Renilla* luciferase were measured. Where indicated, cells were incubated with 0.5 μM TAT-C3 transferase, a membrane permeable C3 transferase (Coleman *et al*., 2001) for 24 h.

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 nontargeting control siRNA pools or siRNAs specific for GEF-H1, Snail, and Slug (Thermo Fisher Scientific, Waltham, MA; and Dharmacon RNA Technologies, Lafayette, CO), using Interferin transfection reagent (Polyplus Transfection, Calne, Wilts, United Kingdom), and a total final siRNA concentration was 100 nM (Steed *et al*., 2009). Twenty-four hours after the transfection, TGF-β1 (20 ng/ml) was added, and the cells were analyzed after another 3 d of culture. The cells were then rinsed with phosphate-buffered saline (PBS) 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). The assay was performed as instructed by the manufacturer but avoiding the freezing step in all samples.

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

RPE cells were transiently transfected with pcDNA4/TO-CTD-VSV construct (DN-GEF-H1 (Aijaz *et al*., 2005) using Lipofectamine 2000 and then treated with TGF-β1 for 3 d. Samples were fixed and stained for α-SMA, vesicular stomatitis virus (VSV; transfected cells), and DNA. Random fields were photographed and the percentages of α-SMA positive cells in the control (VSV-negative) and DN-GEF-H1 expressing (VSV-positive) cells were calculated (a total of 600 cells were counted for each condition, shown are means ±

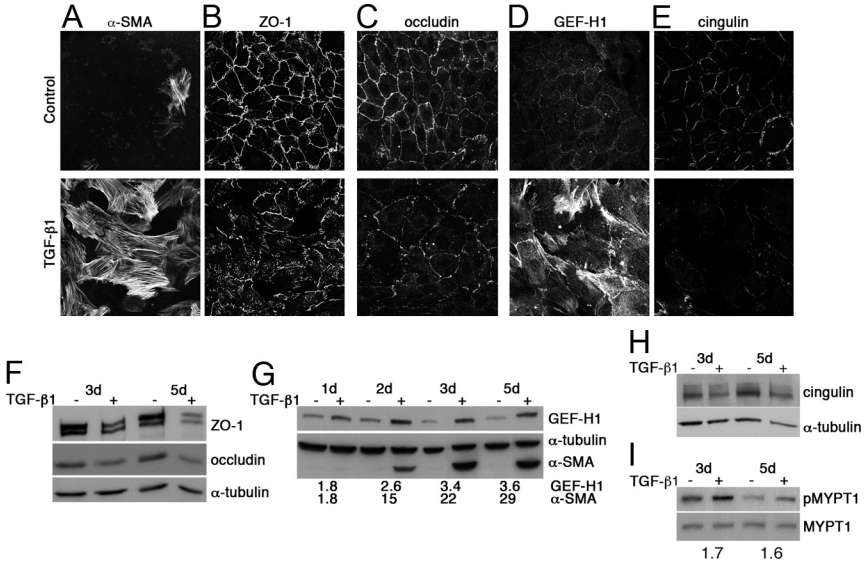

Figure 1. TGF-β1 induces junctional disruption and GEF-H1 up-regulation in RPE cells. RPE cells were stimulated with TGF-β1 (A–E for 3 d; F–I as indicated) and processed for immunofluorescence (A–E) or immunoblot (F–H) analysis. (A–C) Samples were stained for either α-SMA (A) and ZO-1 (B), occludin (C), or GEF-H1 (D) and cingulin (E). (F–H) Immunoblots of total RPE cell extracts stimulated with TGF-β1 for the indicated time were probed with antibodies against ZO-1 and occludin (by densitometry, both proteins were decreased by >50% after 3 and 5 d of TGF-β treatment; F), GEF-H1 and α-SMA (the numbers indicate the ratios of TGF-β–treated divided by control samples obtained by densitometry; all values were normalized by those obtained for tubulin in each sample; G), cingulin (H); α-tubulin was used as loading control. (I) Immunoblot of RPE cell extracts was probed for phosphorylated (p-MYPT1) and total myosin light chain phosphatase (MYPT1) (the numbers indicate the relative increase in p-MYPT1 in TGF-β–treated samples). Shown are representative results from at least two experiments.

1 SD of 3 determinations). For the lentiviral constructs, either the CTD-VSV sequence or the VSV epitope cassette were cloned into the lentiviral pHR'IN plasmid (Bainbridge *et al*., 2001), giving rise to LNT-DN-GEF-H1 or LNT-control, respectively. RPE cells were infected with control (LNT-VSV) or DN-GEF-H1 (LNT-VSV-DN-GEF-H1) lentivirus at multiplicity of infection (MOI) 100 and stimulated with TGF-β1 for 3 d (experiments were performed in triplicates). Cells lysates were analyzed for α-SMA and fibronectin expression; α-tubulin was used as loading control.

Wound-Healing and Morphological Assays

The Electric Cell-Substrate Impedance Sensing (ECIS) Model 1600R (Applied BioPhysics, Troy, NY) was used to monitor cell migration. RPE cells were plated in ECIS electrode array (8W1E) (Applied BioPhysics). The following day, they were infected with LNT-control or LN T-DN-GEF-H1 at MOI 100 and 24 h later treated with TGF-β1 (50 ng/ml) for 2 d, and electrical wounds were inflicted as described previously (Keese *et al*., 2004). In another type of wound-healing assays, manual wounds were inflicted with a pipette yellow tip, pictures were then taken after 0, 16, 24, 48 and 72 h, and the wound area was measured. The wound areas were normalized to the ones obtained at 0 h that are referred as 1 and all other areas were then expressed as fractions of the initial wound. For MDCK cells, the same wounding and impendence assay was used. GEF-H1 depletion was induced for 4 d before the wounding assay using tetracycline and was confirmed in parallel experiments as described previously (Benais-Pont *et al*., 2003).

To follow morphological changes, RPE cells confluent for 3 wk were split and plated on glass coverslips coated with fibronectin (6×10^4 cells/cm^2), cells were infected with LNT-control or LN T-DN-GEF-H1 at 200 MOI, and reached confluence after 1 d. Monolayer detachment/contraction was then followed for up to 5 d. Quantification of monolayer detachment/contraction was performed by measuring cell-free areas using ImageJ (National Institutes of Health, Bethesda, MD.

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-β1 (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-β1, however, up-regulated GEF-H1 expression (Figure 1G). Similar results were obtained with GEF-H1 antibodies recognizing different epitopes (data not

Table 1. Guanine nucleotide exchange factor mRNA expression profile of control and TGF-β 1–treated porcine RPE cells

Name	Control	TGF-β1	UniGene
GEF-H1/Lfc	110.2 ± 2.0	191.0 ± 29.4	Ssc.8984
GEFp432/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.4921
GRINCHGEF/ARHGEF10L	9.7 ± 2.0	9.0 ± 2.0	Ssc.4246
Lsc/ARHGEF1	20.1 ± 1.4	24.5 ± 2.5	Ssc.27637
RAPGEF3/EPAC	12.5 ± 2.8	14.7 ± 1.0	Ssc.19027
LARG/ARHGEF12	4.0 ± 0.8	2.8 ± 1.1	Ssc.18932
DelGEF/SERGEF	60.2 ± 5.2	65.8 ± 10.4	Ssc.17909
Cool1/βPIX	79.1 ± 1.1	80.8 ± 3.7	Ssc.1606
eEF1δ (for eEF1α)	509.8 ± 29.8	538.6 ± 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.

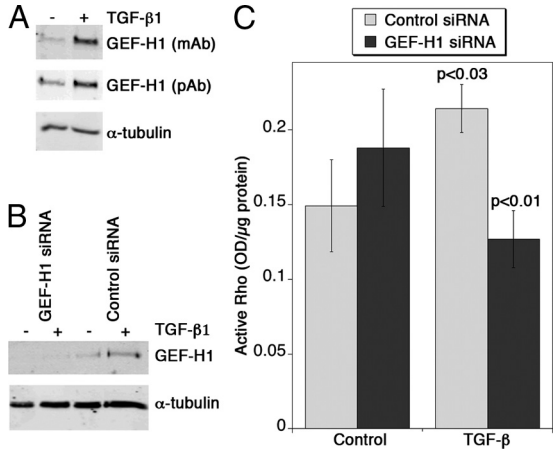

Figure 2. Inhibition of TGF-β–induced Rho activity by GEF-H1 depletion. (A) HaCaT cells were stimulated for 3 d with TGF-β and were then analyzed for expression of GEF-H1 and α-tubulin. Expression of GEF-H1 was monitored with two different antibodies, an mAb antibody and a polyclonal (pAb) antibody that 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 not-treated control siRNA-transfected cells and, respectively, GEF-H1 depleted TGF-β–treated cells with control siRNA transfected TGF-β–treated cells).

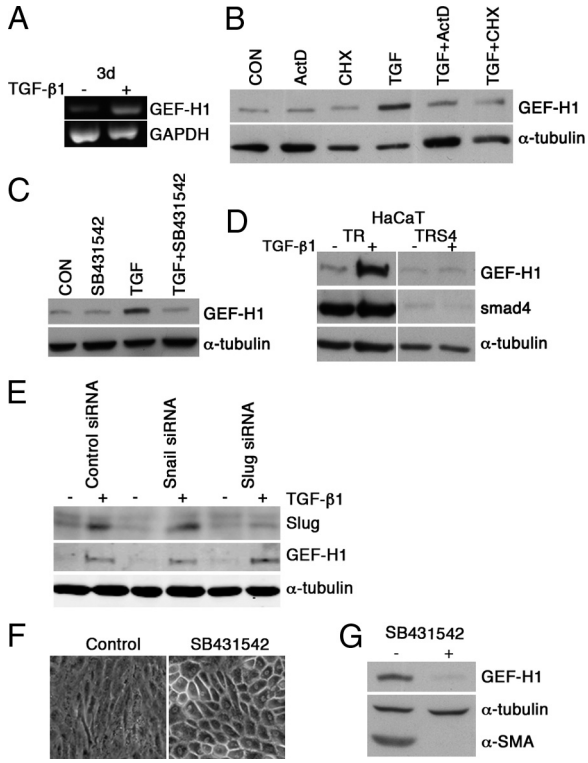

Figure 3. TGF-β1-induced GEF-H1 up-regulation is Smad4 dependent. (A) RT-PCR analysis for GEF-H1 mRNA levels in control and TGF-β1–treated (3-d) samples; GAPDH was used as a loading control. Note that the increase observed by RT-PCR (>2-fold) was similar to the increase obtained from the microarray analysis. (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-β–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-β 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.

shown). Immunofluorescence also revealed increased expression of GEF-H1 and accumulation in areas of cell protrusions (Figure 1D). Up-regulation of GEF-H1 not only correlated with increased expression of α-SMA (Figure 1G) but also enhanced phosphorylation of myosin phosphatase (Figure 1I), suggesting increased activity of the Rho–Rho kinase pathway. Thus, up-regulation of GEF-H1 by TGF-β correlates with activation of Rho signaling and α-SMA expression.

We next analyzed the importance of GEF-H1 for the activation of RhoA in response to TGF-β treatment. We used the HaCaT cells, a keratinocyte cell line, for this purpose as we could down-regulate GEF-H1 in these cells effectively with commercially available siRNAs, and control siRNAs did not cause nonspecific effects as in primary porcine RPE cells. Figure 2A shows that TGF-β treatment also resulted in increased GEF-H1 expression in HaCaT cells. Up-regulation was inhibited by transfection of GEF-H1-specific but not nontargeting control siRNAs (Figure 2B). When the levels of active RhoA was measured in identically treated cells, we found that active RhoA levels increased in response to TGF-β and that this was inhibited if up-regulation of GEF-H1 was blocked by RNA interference. These data thus indicate that the increased levels of GEF-H1 expression contribute to Rho activation in response to TGF-β.

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.

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

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). Thus, TGF-β1 induced GEF-H1 expression is smad4 dependent and does not require up-regulation of Slug.

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

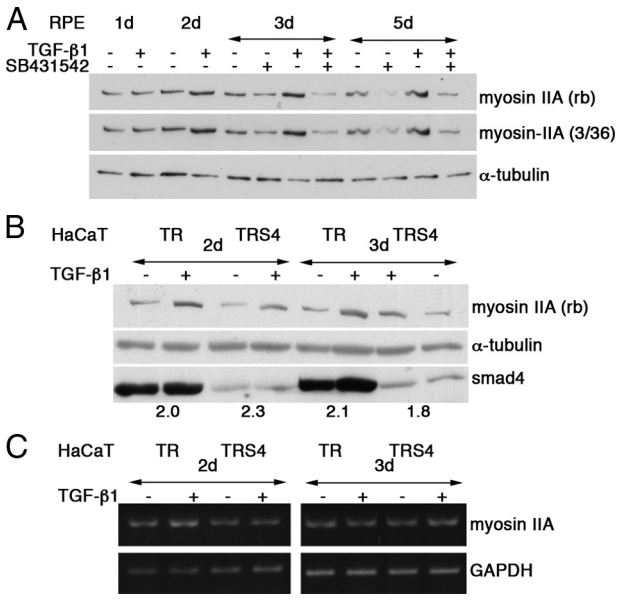

Figure 4. TGF-β1–induced myosin-IIA up-regulation is Smad4 independent. (A) RPE cultures in the absence or presence of the ALK45 kinase inhibitor SB431542 were stimulated TGF-β1 as indicated. Immunoblots of total cell extracts are shown that were probed sequentially for myosin-IIA by using two different antibodies, a rabbit antibody (Sigma-Aldrich) or monoclonal (mouse, 3/36); α-tubulin was used as loading control. By densitometry, myosin-II was up-regulated by at least 55%. (B) HaCaT-TR-S4, a stable clone for inducible depletion of Smad4, and the parental cell line HaCaT-TR were treated with tetracycline for 2 d to reduce Smad4 expression and then stimulated with TGF-β1 for the indicated times. Total cell extracts were probed for myosin-IIA (rabbit; Sigma-Aldrich) and Smad4. The numbers indicate the ratio between TGF-β–treated and control samples for myosin-II. (C) RT-PCR analysis for myosin-IIA in control and TGF-β1–treated HaCaT-TR and HaCaT-TR-S4 cells; GAPDH served as a control to monitor RNA input. By densitometry, no significant differences were observed between control and TGF-β–treated samples.

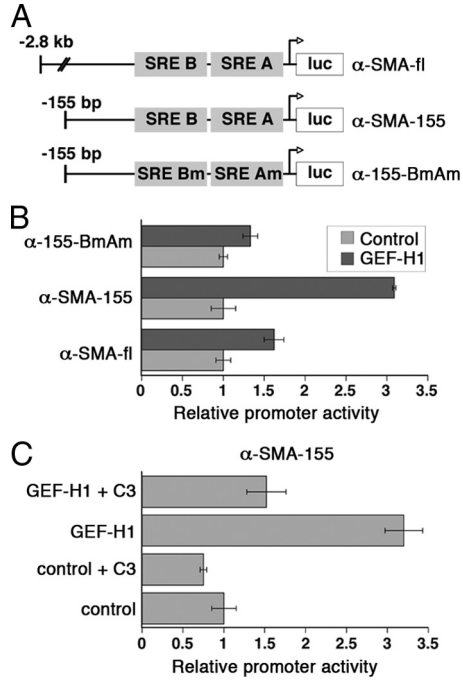

Figure 5. GEF-H1 regulates the α-SMA promoter in a Rho-dependent manner. (A) Schematic representation of the α-SMA promoter constructs used. α-SMA-fl is a 2.8-kb full-length α-SMA promoter; α-SMA-155 is a minimal promoter (155-base pairs) containing the two SREs and lacking upstream inhibitory elements; and α-155-BmAm is an identical minimal region promoter in which the SREs had been mutated. The relative position of the two SREs (SRE A and SRE B) is indicated. (B) RPE cells were cotransfected with a α-SMA firefly luciferase reporter construct and a control *Renilla* luciferase promoter plasmid together with either expression (GEF-H1) or empty (control) vector. The results are expressed as percentage of control transfections (shown are means $\pm$ 1 SD of 4 determinations). Note that the full-length promoter contains additional upstream regulatory elements that suppress full activation in response to Rho stimulation. (C) The α-SMA-155 reporter construct was used and cells were incubated with TAT-C3, a membrane-permeable C3 transferase.

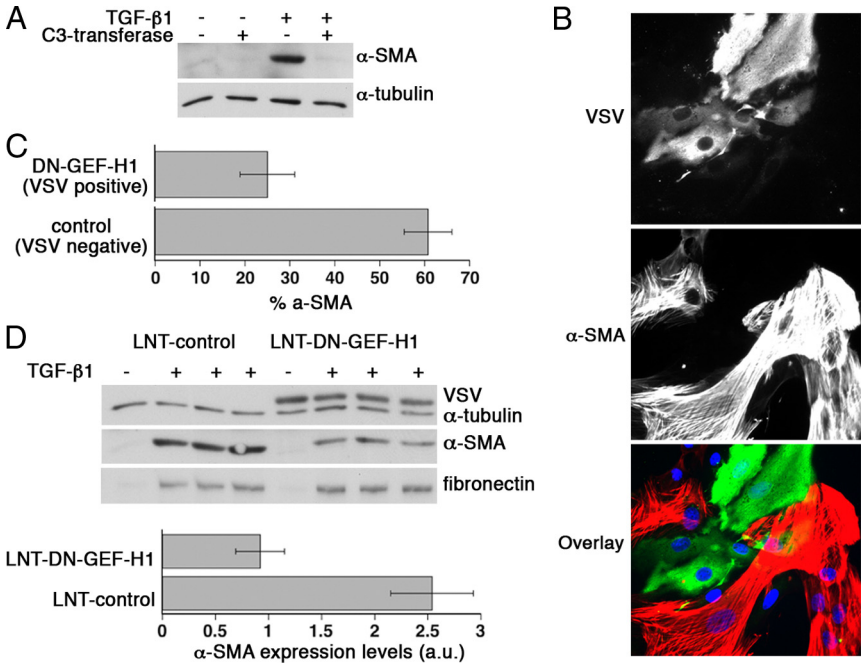

Figure 6. Rho signaling and GEF-H1 regulate α-SMA expression induced by TGF-β1. (A) RPE cells were incubated for 3 d with or without TGF-β1. During the last 2 d, membrane-permeable C3 transferase was added as indicated. Expression of GEF-H1 and α-tubulin was then analyzed by immunoblotting. (B and C) RPE cells were transiently transfected with DN-GEF-H1 and treated with TGF-β1 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-β1 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-β1–induced α-SMA expression.

up-regulation (Figure 4A), depletion of Smad4 only partially counteracted the increase (Figure 4B) and mRNA levels did not significantly change in response to TGF-β (Figure 4C), indicating posttranscriptional regulation by a mechanism at least partially distinct from the one that targets GEF-H1.

GEF-H1 Regulates α-SMA Expression Induced by TGF-β1

Induction of α-SMA expression has been suggested to be of functional relevance for the pathologies of retinopathies such as retinal detachments or proliferative vitreoretinal disorders (Grisanti and Guidry, 1995). Transcription of α-SMA is regulated by actin reorganization induced by Rho activation through serum response factor (SRF) (Hill *et al.*, 1995; Wamhoff *et al.*, 2006). Because GEF-H1 is an activator of RhoA and can activate an SRE-specific reporter gene construct in MDCK (Aijaz *et al.*, 2005) and RPE cells (Supplemental Figure 1), we next asked whether GEF-H1 stimulates α-SMA expression in response to TGF-β.

We first used a reporter gene assay to determine whether GEF-H1 is able to stimulate transcription of the α-SMA promoter and, if yes, whether this involves the SREs, the binding sites of the transcription factor SRF (Mack and Owens, 1999; Miano *et al.*, 2007). Figure 5A shows that cotransfection of GEF-H1 stimulated the full-length promoter (α-SMA-fl) and a shorter promoter (α-SMA-155) in a manner that depended on the two SRE elements (α-155-BmAm) (Figure 5B). As expected, the short promoter responded more strongly to Rho activation as it lacks the repressing upstream region of the promoter. Inhibition of Rho with C3 transferase counteracted stimulation of the α-SMA promoter by GEF-H1 (Figure 5C), confirming the Rho dependence. Thus, GEF-H1 regulates α-SMA promoter activity in an SRE- and Rho-dependent manner.

We next tested whether GEF-H1 and Rho signaling regulate α-SMA expression during TGF-β stimulation. First, we incubated control and TGF-β–treated RPE cells with membrane-permeable C3 transferase. Figure 6A shows that this resulted in an efficient repression of α-SMA induction, indicating up-regulation requires Rho signaling.

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-H1and 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-β1. 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-β1. 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 (Tsuchida *et al.*, 2003), no inhibition of up-regulation was observed. These results show that expression of DN-GEF-H1 counteracts the TGF-β1–induced increase in α-SMA expression.

Treatment of cultures plated at low-density (0.5×10^4 cells/cm^2) 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 (6×10^4 cells/cm^2) 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-

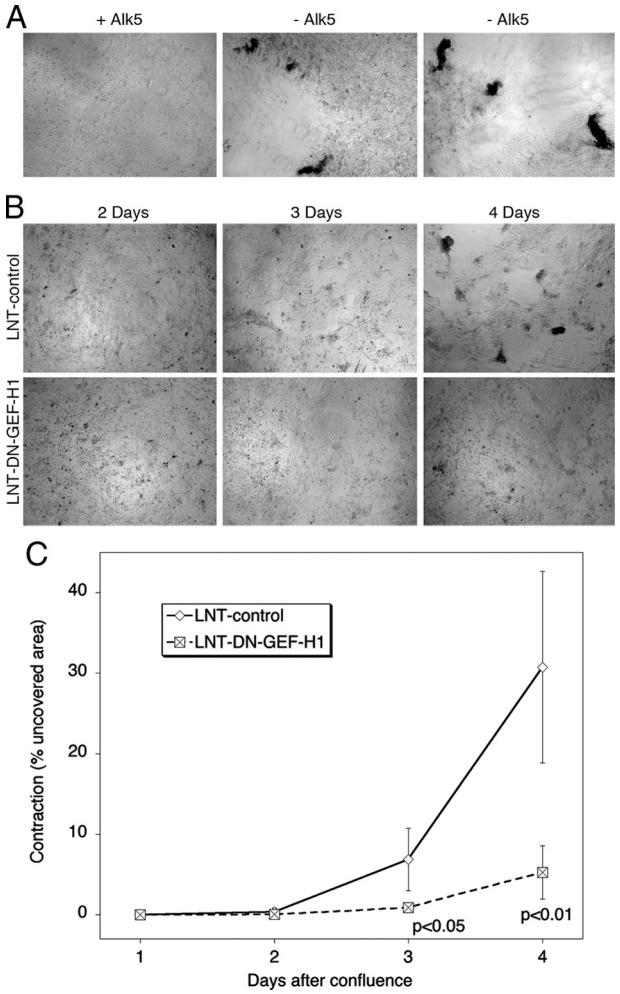

Figure 7. GEF-H1 inhibition counteracts TGF-β–dependent monolayer contraction and detachment. (A) Three-week-old primary cultures of porcine RPE cells were plated at high density (6×10^4 cells/cm^2) on fibronectin-coated coverslips. The confluent monolayers were then incubated with or without the ALK5 inhibitor SB431542. Note, monolayers in which TGF-β signaling was not inhibited contracted and started to detach from the substrate. (B and C) Three-week-old primary cultures of porcine RPE cells infected with a control lentivirus (LNT-control) or a virus encoding dominant negative GEF-H1 (LNT-DN-GEF-H1) were cultured as in panel A without the ALK5 inhibitor and inspected daily. Cell-free areas were then quantified and expressed as percentage of total area. The quantification in C is based on the analysis of four-independent cultures per condition. Indicated are p values derived from a *t* test.

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

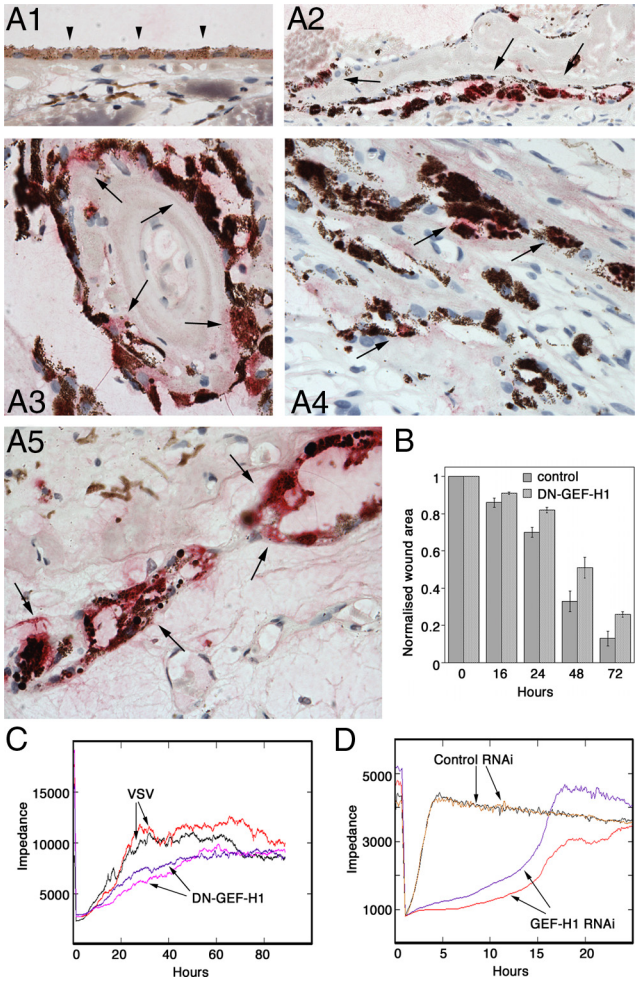

Figure 8. GEF-H1 is up-regulated in migratory RPE cells in vivo and regulates cell migration. (A1) Negative GEF-H1 staining was observed in RPE cells from a normal-looking area adjacent to a choroidal malignant melanoma (case 1, 40×). (A2) Positive GEF-H1 staining in elongated migratory RPE configured as a monolayer (case 2, trauma, 40×). (A3) Positive GEF-H1 staining in migratory RPE around vessels from a case of posterior Uveitis (case 3, 40×). (A4) Positive GEF-H1 staining in migratory pigmented RPE within an area of subretinal scaring from a case of corneal infection (case 4, 40×). (A5) Positive GEF-H1 staining in apex to apex islands of RPE cells from a case of retinal detachment (case 5, 40×). In all instances. a red chromogen was used and sections were counterstained with hematoxylin. Note that RPE cells can be recognized by their strong pigmentation and are classified as migratory when they are displaced form their normal location at the back of the retina and have moved into the neural retina. In A1, the RPE is indicated with arrow heads, and the arrows in A2–A5 point to groups of RPE cells positive for GEF-H1. (B) RPE cells infected with LNT-control or LNT-DN-GEF-H1 were treated with TGF-β1 for 3 d, and then a wound was manually inflicted with a pipette tip. Pictures were then taken after 16, 24, 48, and 72 h, and the wound area was quantified. The wound areas were normalized to the areas obtained at 0 h that are referred as 1, and all other areas were then expressed as fractions of the initial wound, the graphs represent normalized wound areas at different times (shown are averages ± 1 SD; n = 4). (C) RPE cells infected with LNT-control or LNT-DN-GEF-H1 were treated with TGF-β1 for 3 d and then subjected to a high electric field to induce a wound in the center of each monolayer. The graphs represent wound closure as measured by recovery of impedance along time (2 separate measurements for each condition are shown that had been analyzed in parallel and correspond to a representative experiment). (D) MDCK cells, control cells or cells permitting tetracycline-induced GEF-H1 depletion by RNA interference (Benais-Pont *et al.*, 2003), 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.

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; Thuault *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; Samarin *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

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 NET1 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 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.

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