

# Immunosuppressor FK506 Increases Endoglin and Activin Receptor-Like Kinase 1 Expression and Modulates Transforming Growth Factor- $\beta$ 1 Signaling in Endothelial Cells

Virginia Albiñana, Francisco Sanz-Rodríguez, Lucía Recio-Poveda, Carmelo Bernabéu, and Luisa M. Botella

Centro de Investigaciones Biológicas (CIB), Consejo Superior de Investigaciones Científicas (CSIC) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER) (V.A., L.R.P., C.B., L.M.B.); and Departamento de Biología Celular, Facultad de Biología, Universidad Autónoma de Madrid (F.S.R.), Madrid, Spain

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## ABSTRACT

Hereditary hemorrhagic telangiectasia (HHT), or Rendu-Osler-Weber syndrome, is an autosomal-dominant vascular disease. The clinical manifestations are epistaxis, mucocutaneous and gastrointestinal telangiectases, and arteriovenous malformations in internal organs. Patients show severe epistaxis, and/or gastrointestinal bleeding, both of which notably interfere with their quality of life. There are two predominant types of HHT caused by mutations in *endoglin* (*ENG*) and *ACVRL1/activin receptor-like kinase 1* (*ALK1*) genes, named HHT1 and HHT2, respectively. *ENG* and *ALK1* code for proteins involved in the transforming growth factor (TGF)- $\beta$ 1 signaling pathway, and it is widely accepted that HHT pathogenicity results from haploinsufficiency. No cure for HHT has been found, so identification of drugs able to increase the expression of these genes is essential when proposing new therapies. We report the efficacy

of tacrolimus (FK506) in increasing *ENG* and *ALK1* expression. The rationale comes from a case report of a patient with HHT who received a liver transplantation after hepatic failure due to a liver arteriovenous malformation. The liver was transplanted, and the immunosuppressor FK506 was used to prevent the rejection. After the first month of FK506 treatment, the internal and external telangiectases, epistaxes, and anemia disappeared. Here, we find that the immunosuppressor FK506 increases the protein and mRNA expression of *ENG* and *ALK1* in cultured endothelial cells and enhances the TGF- $\beta$ 1/*ALK1* signaling pathway and endothelial cell functions like tubulogenesis and migration. These results suggest that the mechanism of action of FK506 involves a partial correction of endoglin and *ALK1* haploinsufficiency and may therefore be an interesting drug for use in patients with HHT who undergo transplantation.

## Introduction

Hereditary hemorrhagic telangiectasia (HHT), or Rendu-Osler-Weber syndrome, is an autosomal-dominant vascular disease with incomplete penetrance characterized by localized angiodysplasia. This is manifested as epistaxis, mucocutaneous and gastrointestinal telangiectases, and arterio-

venous malformations in the pulmonary, cerebral, or hepatic circulation (Shovlin and Letarte, 1999). HHT prevalence is on average between 1:5000/8000, although it is higher in specific areas, such as the Jura region in France, Funen Island in Denmark and certain Caribbean islands in the Netherlands Antilles (Jessurun et al., 1993; Kjeldsen et al., 1999). Its prevalence in Spain has been calculated to be around one in 8000, according to HHT studies in progress since 2003 (Fernandez et al., 2006; Fontalba et al., 2008). There are two main HHT types, types 1 and 2, that are caused by mutations in *endoglin* (*ENG*) and *ACVRL1* (*ALK1*) genes, respectively (McAllister et al., 1994; Johnson et al., 1996). In approximately 2% of all patients with HHT, the origin of the disease is a mutation in the *MADH4* gene, which

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**ABBREVIATIONS:** HHT, hereditary hemorrhagic telangiectasia; *ENG*, endoglin; *ACVRL/ALK1*, activin receptor-like kinase 1; FKBP12, FK binding protein 12; Id1, inhibitor differentiation 1; PAI-1, plasminogen activator inhibitor-1; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; Smad, similar mother against decapentaplegic; FK506, tacrolimus; RI, receptor I; HMEC-1, human microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione transferase; ChIP, chromatin immunoprecipitation; SBE, similar mother against decapentaplegic-binding element; VSMC, vascular smooth muscle cell; NFAT, nuclear factor of activated T cells; BRE, bone morphogenic protein-responsive element.

codes for Smad4 coactivator, leading to the combined syndrome of Juvenile Polyposis and HHT (Marchuk et al., 1995). A common property of all of these genes is that they code for proteins involved in the TGF- $\beta$ 1 signaling pathway, critical for the proper development of the blood vessels. TGF- $\beta$ 1 binds to receptor II and the resulting complex recruits and phosphorylates receptor I (RI). In endothelial cells ALK1 is the specific RI, whereas ALK5 is the ubiquitous RI in most of the cell types. The receptor complex also contains the auxiliary receptor endoglin. Activated RI transmits the signal to the nucleus through the Smad family of activators. First, RI phosphorylates R-Smads, which then associate with CoSmads, namely Smad4. The R-Smad/CoSmad complex translocates to the nucleus to regulate the target genes by binding TGF- $\beta$ 1-responsive elements in their promoter regions.

In addition to *ENG* and *ALK1*, a third and fourth HHT loci for unknown genes have been described in chromosomes 5 (HHT3) and 7 (HHT4), respectively (Cole et al., 2005; Bayrak-Toydemir et al., 2006). It is generally accepted that HHT1 and HHT2 pathogenicity is triggered by endoglin or ALK1 haploinsufficiency (Abdalla and Letarte, 2006). Therefore, the identification of drugs capable of increasing the expression of these genes is essential when considering new treatments for HHT.

The most frequent clinical manifestation of HHT is epistaxis (nose bleeds), normally from light to moderate in intensity (Plauchu et al., 1989; Morales-Angulo and del Valle-Zapico, 1998; Shovlin, 2010). However, some patients show severe epistaxis, which significantly interferes with their quality of life. The origin of this epistaxis is due to the existence of telangiectases on the nasal mucosa. As a consequence of vascular alterations, telangiectases are very sensitive to slight traumata and even to the air when breathing, which gives rise to nose bleeds. Many different therapies have been assayed, but none of them with conclusive results, such as the antibrinolytic agents  $\epsilon$ -aminocaproic or tranexamic acids (Fernandez et al., 2007; Morales-Angulo et al., 2007) or therapy based in hormones. Estrogens like raloxifene, a selective estrogen receptor modulator, at doses used for oral contraception may eliminate bleeding in women symptomatic for HHT, as we have published previously (Albinana et al., 2010).

There is a reported case of a woman with HHT who received a liver transplant who was then treated with the immunosuppressor agent tacrolimus [monohydrate,  $C_{44}H_{69}NO_{12} \cdot H_2O$  (FK506)] (Kino et al., 1987a,b); her HHT symptoms improved after transplantation. Extensive necrosis and multiple large arteriovenous malformations were found in the excised native liver. The patient regained normal health without experiencing rejection of the transplanted liver or thrombosis of hepatic vessels. She had no mucosal bleeding during the year after transplantation and was no longer anemic (hemoglobin level, 110 g/l). Telangiectases disappeared from her skin along with buccal mucosa within the first 2 months after transplantation and did not recur. Endoscopies at 1 month and at 1 year showed a complete resolution of telangiectases from the upper gastrointestinal tract (Skaro et al., 2006).

Based on these promising clinical results, an in vitro study of the effects of FK506 in cultured endothelial cells was carried out to unravel the molecular mechanisms involved in these putative therapeutic effects of FK506. This compound

is a naturally occurring product that is approved for clinical use based on the merit of its immunosuppressive activity (McConnell and Wadzinski, 2009). As a result of our experiments, we can conclude that this drug actually increases the expression of endoglin and ALK1 at the endothelial cell surface, thus partially counteracting the haploinsufficiency of these HHT genes.

## Materials and Methods

**Cell Culture.** Human microvascular endothelial cells (HMEC-1) and human umbilical vein endothelial cells (HUVECs) were cultured in endothelial basal medium (Lonza Walkersville Inc., Walkersville, MD) supplemented with 10% bovine fetal serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin and EGM-2 SingleQuots (Lonza Walkersville). Plates were coated previously with 0.2% gelatin in PBS (Sigma-Aldrich, St. Louis, MO). Endothelial cells were treated with different concentrations of FK506 (Sigma-Aldrich) ranging from 0 to 100 ng/ml and TGF- $\beta$ 1 1 or 10 ng/ml (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK).

**Flow Cytometry.** Cells were incubated with anti-endoglin (P4A4; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and anti-ALK1 (MAB370; R&D Systems, Minneapolis MN) mouse antibodies for 60 min at 4°C. After 2 washes with PBS, Alexa Fluor 488 anti-mouse IgG (Invitrogen) was added and incubated for 30 min at 4°C. Finally, cells were washed twice, and the fluorescence was measured in a Coulter Epics XL flow cytometer (Beckman Coulter, High Wycombe, UK). The results obtained were expressed as expression indexes, resulting of the product of positive cell percentage multiplied by the medium fluorescence intensity of the total population.

**Real-Time Reverse Transcription-Polymerase Chain Reaction.** Total cellular RNA was extracted from HMEC-1 cells using a commercial kit RNeasy (QIAGEN, Valencia, CA). One microgram of total RNA was reverse-transcribed in a final volume of 20  $\mu$ l with the First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) using random primers. SYBR Green PCR system (Bio-Rad Laboratories, Hercules, CA) was used to carry out the real-time PCR. The sequence of the oligonucleotides used for *ENG*, *ALK1*, and *18S*, as endogenous control, were the following: *ENG*: forward, 5'-AGCCT-CAGCCCCACAAGT-3'; reverse, 5'-GTCACCTCGTCCCTCTCG-3'; *ALK1*: forward, 5'-ATCTGAGCAGGGCGACAC-3'; reverse, 5'-ACTC-CCTGTGGTGCAGTCA-3'; and *18S*: forward, 5'-CTCAACAC-GGGAAACCTCAC-3'; reverse, 5'-CGCTCCACCACTAAGAACG-3'. The samples were used in triplicate, and the experiment was repeated twice.

**Western Blot Analysis.** Cells were lysed on ice for 30 min in 1% SDS, and lysates were centrifuged at 14,000g for 5 min. Similar amounts of proteins from aliquots of cleared cell lysates were boiled in SDS sample buffer and analyzed by 7.5% SDS-PAGE under nonreducing conditions. For GST pull-downs, GST fusion constructs of full-length FK binding protein 12 (FKBP12) (kindly provided by Dr. J. M. Redondo, Centro de Investigaciones Cardiovasculares, Madrid, Spain) were purified using glutathione-Sepharose 4B beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). As negative control, a pull-down with GST protein alone was included. GST pull-downs with total lysates of untreated or FK506-treated HUVECs were conducted as described previously (Botella et al., 2001). Basically, total lysates were incubated with glutathione-Sepharose-bound fusion protein (FKBP12) overnight at 4°C. Beads were washed five times in wash buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 10% glycerol). Specifically bound proteins and total extracts were detected by SDS-PAGE (10% for ALK1 and 12% acrylamide for FKBP12 detection). For Western blot analysis, proteins from gels were electrotransferred to nitrocellulose membranes, followed by immunodetection with anti-p-Smad1/5/8 (Cell Signaling Technology, Barcelona, Spain), anti-FKBP12 (Santa

Cruz Biotechnology, Santa Cruz, CA), and anti- $\beta$ -actin (Sigma-Aldrich). Secondary antibodies were horseradish peroxidase conjugates from Dako Denmark A/S (Glostrup, Denmark). Membranes were developed by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific).

**Cell Transfections and Reporter Assays.** Transient transfections of HMEC-1 cells were carried out in P-24 plates using 1  $\mu$ g of reporters for the *ENG* promoter pCD105 (–350/+350) in pXP2 (pENG/pXP2), the *ALK1* promoter pALK1 (–1035/+209) in pGL2 (pALK1/pGL2), and the BRE-luc, CAGA-luc, pId1-luc, and PAI1-luc p800-luc constructs (Sánchez-Elsner et al., 2002; Garrido-Martin et al., 2010) in the presence of a commercial transfection reagent (Superfect; QIAGEN). After transfection, cells were incubated in the absence or presence of 100 ng/ml FK506 for 24 h. Relative luciferase units were measured in a TD20/20 luminometer (Promega, Madison, WI). Samples were cotransfected with 20 ng/ml simian virus 40- $\beta$ -galactosidase expression vector to correct for transfection efficiency.  $\beta$ -Galactosidase activity was measured by using Galacto-light (Tropix, Bedford, MA). Transfections were made in triplicate and repeated at least in three independent experiments. Representative experiments are shown in the figures. Treatments with TGF- $\beta$ 1 (R&D) were performed at 1 ng/ml for 3 h to stimulate the ALK1/endoglin pathway (BRE-luc, pId1-luc) and 10 ng/ml for 24 h to stimulate ALK5 (CAGA-luc, PAI-1/p800luc). Treatments with the immunosuppressor FK506 were performed at 100 ng/ml.

**Electrophoretic Mobility Shift Assay.** To obtain nuclear extracts, subconfluent HUVEC cell cultures were either untreated or treated with 100 ng/ml FK506 for 24 h and then collected. Cells were washed, harvested in ice-cold PBS and lysed to collect the nuclear extracts. Complementary oligonucleotides, encompassing the region –50/24 base pairs of the *ENG* promoter, were annealed followed by 5' end-labeling using [ $^{32}$ P]ATP and T4 polynucleotide kinase. Approximately 5 ng ( $10^5$  cpm) of the probe was incubated with 10  $\mu$ g of nuclear extract and 2.5  $\mu$ g/reaction of poly(dI-dC) as unspecific competitor for 30 min on ice. The following oligonucleotides were used: –50/–24, wild-type 5'-GCAGGCGGCTGGGCCCCAGCCCCCTTCTC-3'; and –50/–24, Sp1-Mut 5'-GCAGGCGGCTGGGTTTAGCCCCCTTCTC-3'. For competition experiments, a 100-fold excess of unlabeled double-stranded oligonucleotide was added. In experiments with antibodies, 10  $\mu$ g of protein extract and 1  $\mu$ g of commercial antibody were preincubated for 60 min on ice before the addition of the remaining components of the binding reaction. Anti-Sp1 rabbit polyclonal antibody and anti-Smad4 (both from Santa Cruz Biotechnology) were used. Binding reactions were separated by nondenaturing 6% PAGE in 0.5 $\times$  Tris-Borate-EDTA buffer at 4°C, dried, and visualized by autoradiography. Electrophoretic mobility shift assays were repeated three times with similar results, and representative experiments are shown in the corresponding figures.

**Immunofluorescent Microscopy.** Cells grown onto glass coverslips, previously coated with 0.2% gelatin, were fixed with 3.5% formaldehyde in PBS, washed, and blocked with 2% bovine serum albumin in PBS for 1 h at 4°C. Cells were then incubated for 1 h at 4°C with anti-ALK1 (R&D), anti-FKBP12, and anti-Smad4 antibodies. For anti-FKBP12 staining, cells were permeabilized with 100  $\mu$ g/ml L- $\alpha$ -lysophosphatidylcholine, followed by incubation with Alexa Fluor 488- and Alexa Fluor 547-conjugated anti-rabbit/mouse IgG antibodies (Invitrogen). Coverslips were mounted with Prolong Gold with 4,6-diamidino-2-phenylindole (Invitrogen) and observed with a spectral confocal microscope Leica TCS SP2 (Leica Microsystems, Inc., Nussloch, Germany). Images were processed with ImageJ 1.43u (<http://rsbweb.nih.gov/ij/>).

**Chromatin Immunoprecipitation.** HUVEC subconfluent cell cultures were either treated with 100 ng/ml FK506 or untreated and then collected. Chromatin immunoprecipitation (ChIP) was performed with the kit ChIP-IT Express (Active Motif, Rixensart, Belgium) following the manufacturer's instructions. In brief, HUVECs were grown in three 15-cm<sup>2</sup> plates per condition until confluence. Then, cells were fixed with 3.5% formaldehyde in Dulbecco's modi-

fied Eagle's medium (Invitrogen). Fixation solution was poured off, and cells were washed in ice-cold PBS. The fixation reaction was stopped by adding glycine stop-fix solution and then washing. Cells were scraped in the presence of phenylmethylsulfonyl fluoride and lysed. Nuclei were separated by using a Dounce homogenizer and digested with enzymatic shearing cocktail for 10 min. One aliquot of sheared chromatin was used as "input chromatin," and the other aliquots were incubated with protein G magnetic beads and mouse antibody against human polymerase II as positive control, anti-human IgG as negative control, or anti-Smad4 (Santa Cruz Biotechnology). After overnight incubation in the cold, immunocomplexes bound to Protein G magnetic beads were pelleted, washed, and eluted with elution buffer provided with the kit. Then, cross-linking was reversed, proteins were removed by proteinase K treatment, and the remaining DNA was used for PCR. The sequences of the primers used for PCR were as follows: inhibitor differentiation 1 (Id1): forward, 5'-CGACCGCGCCCCGCAAGAAACG-3'; reverse, 5'-CAAAGACCTCAGAGCAGGGTC-3' for the region (–1043/–730) of Id1 promoter; and plasminogen activator inhibitor-1 (PAI-1): forward, 5'-GCTTTACCATGGTAACCCCTGG-3'; reverse, 5'-CCGGTGCTCTGGACCACCTCC-3' for the region (–798/–477) of PAI-1 promoter. The amplified fragments encompass Smad binding elements (SBE) of *Id1* and *PAI-1* promoters.

**Wound Healing and Tube Formation Assay.** In vitro scratched wounds were created by scraping confluent HMEC-1 monolayers in a P-24 wells plate with sterile disposable pipette tips. The remaining cells were washed with PBS and incubated with EGM-2 in the absence or presence of FK506 or TGF- $\beta$ 1 for up to 24 h. Endothelial cell migration into the denuded area was monitored by photographing of the plates at different times. For tube formation assays, HMEC-1 cells were plated in EGM-2 culture medium in the absence or presence of FK506 on P-6 well Matrigel plates (BD Biosciences Discovery, Bedford, MA) and incubated at 37°C, as indicated by the manufacturer. Tube formation was monitored for 24 h.

**Statistics.** Data represent the mean  $\pm$  S.D. Differences in mean values were analyzed using the Student's *t* test. *P* values <0.05 were considered to be statistically significant. In the figures, the statistically significant values are marked with asterisks.

## Results

**FK506 Increases ALK1 and Endoglin Protein Levels at the Surface of Endothelial Cells.** Because HHT1 and HHT2 are caused by haploinsufficiency of endoglin and ALK1, respectively, we assessed the effect of FK506 on endoglin and ALK1, by observing the levels of both proteins in in vitro cultures of endothelial cells, after 24 h of FK506 treatment, and at different doses (0–100 ng/ml). In Fig. 1A, the fold induction of endoglin and ALK1 relative to untreated cells (control) are shown. The amount of endoglin increased 1.8-fold in cells treated with 100 ng/ml FK506, whereas in the case of ALK1, the increase was 1.6-fold (Fig. 1A). Doses lower than 100 ng/ml did not show significant differences between untreated and treated cells (data not shown). In summary, FK506 gave rise to an increase in the expression of both proteins at the cell surface, the optimal concentration being 100 ng/ml. Because the optimal effect at the protein level was observed at 100 ng/ml FK506, this concentration was selected for further experiments. As positive controls, cells were treated with TGF- $\beta$ 1 (10 ng/ml) or raloxifene (0.2 nM) for 24 h, giving rise to an increase in endoglin in the case of TGF- $\beta$ 1 and endoglin and ALK1 in the case of raloxifene of approximately the same magnitude as FK506. Induction of endoglin by TGF- $\beta$ 1 in the same way as induction of endoglin and ALK1 by raloxifene has been reported previously (Rius et al., 1998; Albiñana et al., 2010).



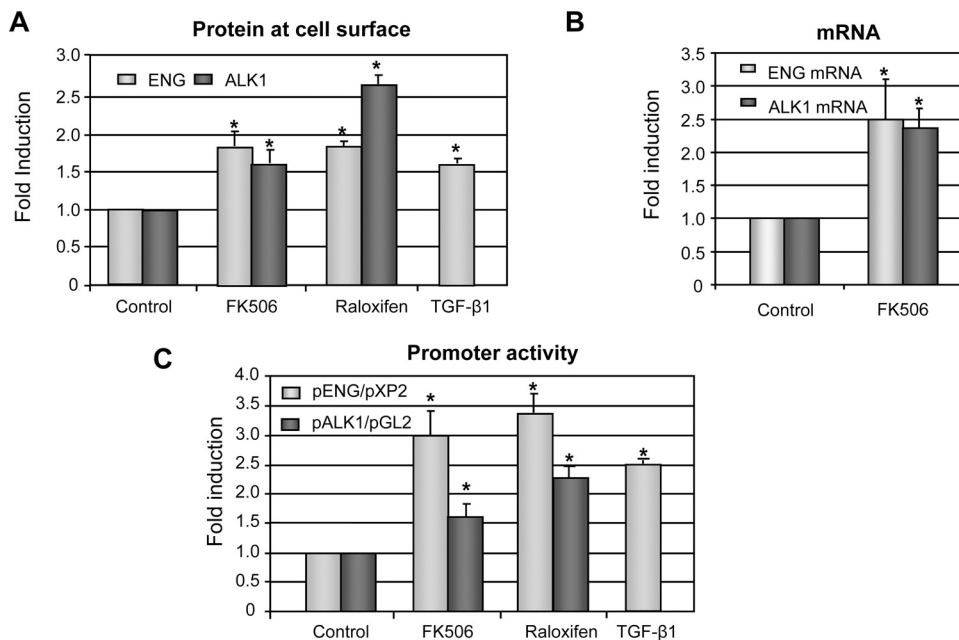
**FK506 Increases the Expression of ENG and ALK1 mRNA.** Given that FK506 increased endoglin and ALK1 protein levels at the surface, we carried out experiments to determine whether this effect was due to an increase in the messenger RNA levels from these genes. Real-time reverse transcription-polymerase chain reaction analysis revealed that the levels of ENG and ALK1 mRNA were stimulated up to a maximum of 2.5- and 2.4-fold, respectively, when cells were treated with 100 ng/ml FK506 (Fig. 1B).

**FK506 Treatment Increased ENG and ALK1 Promoter Activity.** Because FK506 not only increased the levels of protein, but also those of mRNA, next we proceeded to investigate whether these effects were due to an increase in promoter activity of *ENG* and *ALK1*. Cells were transfected with two reporters driven by both promoters and treated in the absence or presence of FK506. As shown in Fig. 1C, cells treated with the immunosuppressor showed promoter stimulation in both genes, 3- and 1.6-fold for *ENG* and *ALK1*, respectively. As positive controls, treatments with TGF- $\beta$ 1 or raloxifene showed a significant stimulation of the endoglin promoter construct (Sánchez-Elsner et al., 2002; Albiñana et al., 2010).

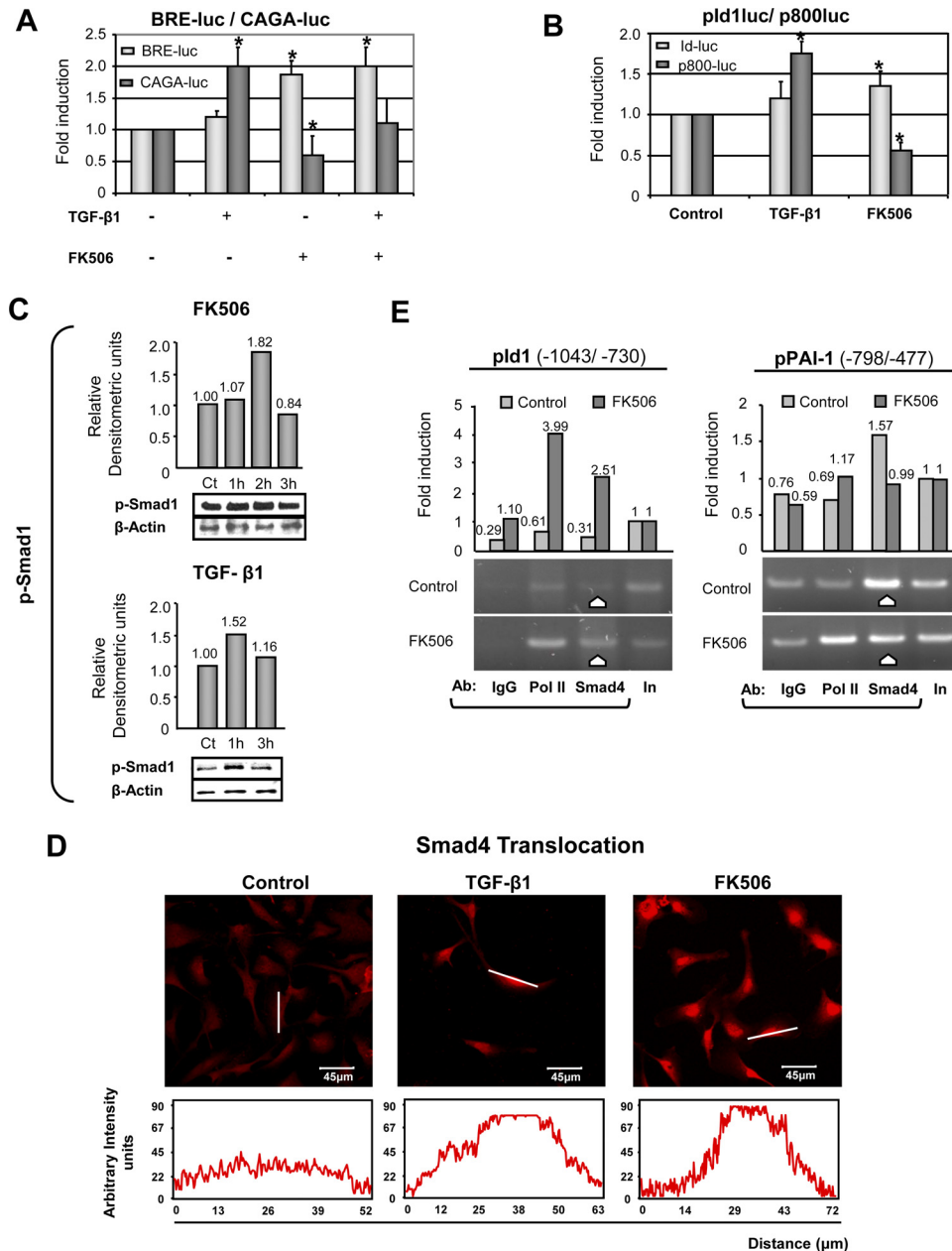
**FK506 Modulates TGF- $\beta$ 1 Signaling in Endothelial Cells.** Giordano et al., (2008) have shown how FK506 can activate the TGF- $\beta$ 1/ALK5 pathway in vascular smooth muscle cells (VSMCs) by increasing the phosphorylation of Smad 2/3. This effect was explained by the binding of FK506 to the nucleophilin FKBP12, a TGF- $\beta$ 1 receptor type I inhibitor. Once FK506 binds FKBP12, the TGF- $\beta$ 1 receptor type I is

released, and its phosphorylation activities increase. Because we have observed that the mRNA and protein levels of endoglin and ALK1 increase in HMEC-1 after FK506 treatment, the hypothesis is that the FK506 mechanism of action may be similar in both endothelial cells and smooth muscle cells. The immunosuppressor would then be modulating the TGF- $\beta$ 1 pathway. BRE-luc and CAGA-luc are reporter vectors that contain artificial promoters consisting of repeated Smad binding consensus sequences; in particular, these are reporters for TGF- $\beta$ 1/ALK1 and TGF- $\beta$ 1/ALK5 pathways, respectively. BRE-luc consists of two bone morphogenic protein-responsive element (BRE) sites (GTCT) upstream of the pGL3 luciferase reporter, which can be bound by Smad1/5/8 after activation of the TGF- $\beta$ 1/ALK1 pathway, specific for endothelial cells. CAGA-luc contains 12 CAGA Smad binding motifs upstream the pGL3 luciferase reporter. These sequences are binding sites for Smad2/3 transmitting the signal from activated TGF- $\beta$ 1/ALK5.

HMEC-1 cells were transfected with BRE-luc or CAGA-luc and treated with 100 ng/ml FK506 or/and TGF- $\beta$ 1, the latter as a positive control for the TGF- $\beta$ 1 reporters. FK506 treatment increased the promoter activity of BRE-luc, but decreased the activity in CAGA-luc (Fig. 2A). As positive controls for TGF- $\beta$ 1 pathway activation, two doses of TGF- $\beta$ 1 were used: 1 ng/ml for 3 h (a condition for preferential ALK1 activation), and 10 ng/ml TGF- $\beta$ 1 for 24 h (a condition for ALK5 activation) for BRE-luc and CAGA-luc, respectively. As shown in Fig. 2A, both TGF- $\beta$ 1 treatments increased the corresponding reporter activities. These results suggest that



**Fig. 1.** Effect of FK506 on ALK1 and endoglin expression. A, effect on protein expression. HMEC-1 cells were cultured in the absence or presence of FK506 (100 ng/ml) for 24 h. Treatments with TGF- $\beta$ 1 (10 ng/ml) or raloxifene (0.2 nM) were performed as positive controls. Levels of endoglin and ALK1 were measured by flow cytometry. Experiments were performed in duplicate, and the same type of experiment was repeated at least three times. The figure shows a representative experiment. Asterisks mark the points that are statistically significant with respect to the untreated control. \*,  $P < 0.05$ . B, effect on mRNA levels. Endothelial cells were treated for 24 h with FK506 (100 ng/ml). Real-time PCR experiments were carried out, extracting total RNA that was retro-transcribed and amplified. Endoglin and ALK1 transcription levels were compared with the endogenous control of 18S ribosomal RNA. Samples were in triplicate in each experiment, and the experiment was repeated three times. The figure shows a representative experiment. \*,  $P < 0.05$ . C, effect on the promoter activity. HMEC-1 cells were transiently transfected with the pCD105 (–350/+350) *ENG* promoter reporter vector or with the pGL2 (–1035/+209) *ALK1* promoter construct. Luciferase activity was measured in cells treated with FK506 (100 ng/ml) and in untreated cells. The activity of *ENG* and *ALK1* promoters increased 3- and 1.6-fold, respectively. As a positive control, cells were treated with TGF- $\beta$ 1 (10 ng/ml) or raloxifene (0.2 nM). Samples were in triplicate in each experiment, and the experiment was repeated three times. The figure shows a representative experiment. Asterisks mark data of statistical significance in relation to control; \*,  $P < 0.05$ .



**Fig. 2.** Effect of FK506 on TGF- $\beta$ 1 pathway reporters. A, effect of FK506 on BRE-luc and CAGA-luc reporter vector activity. The BRE-luc vector contains binding sites for p-Smad proteins that signal via ALK1 pathway and the CAGA-luc construct contains binding sites for p-Smad that signal via the ALK5 pathway. Both reporters increased their activity when cells were treated with TGF- $\beta$ 1 (1 ng/ml), but only the activity of BRE-luc increased when endothelial cells were treated with FK506 (100 ng/ml). The activity of the CAGA-luc reporter vector in treated cells decreased 0.5-fold. B, effect of FK506 on pId1-luc and p800-luc. Activity of both reporters increased when cells were treated with TGF- $\beta$ 1 (1 ng/ml), but after FK506 (100 ng/ml) treatment, the increment only happened in Id-luc. The p800 expression decreased, as in the case of the CAGA-luc reporter vector. Asterisks mark data of statistical significance in relation to control; \*,  $P < 0.05$ . Experiments were repeated at least three times, with triplicates in each experiment. The figure shows a representative experiment. C, FK506 increases the phosphorylation of Smad 1/5/8 (TGF- $\beta$ 1/ALK1). Endothelial cells were treated with TGF- $\beta$ 1 (1 ng/ml) and FK506 (100 ng/ml) for the indicated time. A Western blot assay was performed with the lysates. When cells were treated with FK506, the phosphorylation of Smad1 increased, as well as that of endothelial cells treated with TGF- $\beta$ 1. The histograms are the densitometry of the p-Smad1 band relative to  $\beta$ -actin used as loading control. D, FK506 induces nuclear translocation of Smad4. When the TGF- $\beta$ 1 pathway is activated, Smad proteins are phosphorylated by receptor type I. p-Smads bind to Smad4, and the complexes are translocated to the nucleus to activate the expression of the target genes. HMEC-1 cells, both untreated and treated with FK506 (100 ng/ml) or TGF- $\beta$ 1 (10 ng/ml), were used to perform an immunofluorescent microscopy assay to localize Smad4 (top). In untreated cells, Smad4 is distributed in the cytoplasm and when cells are treated with TGF- $\beta$ 1, the pathway is activated, and Smad4 translocates to the nucleus. The same occurs when HMEC-1 cells are treated with FK506. Bottom, the intensity graph scanned along the white line of a representative cell. The graph quantifies the distribution of Smad4 signal in the cell. These results show that FK506 promotes the TGF- $\beta$ 1 pathway. The experiment was repeated three times. A representative experiment is shown. E, FK506 modulates the binding of Smad4 to the *Id1* and *PAI-1* gene promoters. FK506-treated or untreated HUVECs were subjected to immunoprecipitation with mouse antibody against human polymerase II (pol II) as positive control, anti-human IgG as negative control, or anti-Smad4. The immunoprecipitated DNA was used for PCR in the presence of specific primers for *Id1* and *PAI-1* promoters containing SBE-responsive elements. Bands were subjected to densitometry, and the results are represented relative to the input bands (In). White arrowheads mark Smad4 immunoprecipitated PCR bands from *Id1* and *PAI-1* promoters of FK506 treated or untreated HUVECs.

FK506 modulates TGF- $\beta$ 1 signaling, stimulating the specific endothelial ALK1/Smad1/5 pathway and repressing the ALK5/Smad2/3 pathway.

These results were further confirmed using pId1-luc and p800-luc luciferase reporters driven by fragments of natural promoters. They contain the *Id1* and plasminogen activator inhibitor 1 (*PAI-1*) promoters, activated by TGF- $\beta$ 1/ALK1 and TGF- $\beta$ 1/ALK5, respectively. The transfection results in HMEC-1 cells with these two natural TGF- $\beta$ 1-responsive promoters (Fig. 2B) were similar to those of their artificial counterparts shown in Fig. 2A, thus supporting the activation of the TGF- $\beta$ 1/ALK1/Smad1/5/8 pathway by FK506.

**FK506 Increases the Phosphorylation of Smad1 (TGF- $\beta$ 1/ALK1) and the Translocation of Smad4 to the Nucleus.** Endothelial cells treated with TGF- $\beta$ 1 (1 ng/ml) showed an increase in the amount of phosphorylated Smad1 after 1 h, as expected (Fig. 2C). In parallel, cells treated with the immunosuppressor FK506 displayed maximal levels of phosphorylated Smad1 after 2 h. However, when we looked for p-Smad2 or 3 in the same extracts, no phosphorylation was detected after FK506 treatment (data not shown). This behavior is at variance with that of mouse VSMCs, in which FK506 increases the phosphorylation of Smad2/3, sequestering FKBP12, a RI inhibitor. This differential behavior is explained by the type of TGF- $\beta$ 1 receptors involved. Whereas in VSMCs, the activation is through the TGF- $\beta$ 1/ALK5 pathway (Giordano et al., 2008), in endothelial cells, FK506 acts through the endothelial-specific receptor type I, ALK1. Thus, according to Fig. 2A and the results of Fig. 2B, FK506 triggers the TGF- $\beta$ 1/ALK1 pathway in endothelial cells. To reinforce this idea, we next performed microscopy studies to visualize the nuclear translocation of Smad signaling. When the TGF- $\beta$ 1 pathway is active, Smad proteins are phosphorylated by receptor I. The p-Smad proteins bind Smad4 and translocate to the nucleus to activate the target gene expression. HMEC-1 cells, with and without treatment of FK506 (100 ng/ml) and TGF- $\beta$ 1 (1 ng/ml), were used to perform an immunofluorescent microscopy assay to immunolocalize Smad4. In untreated cells, Smad4 is distributed in the cytoplasm, and when cells are treated with TGF- $\beta$ 1, Smad4 translocates to the nucleus (Fig. 2D). The same happens when HMEC-1 cells are treated with FK506, showing an even higher nuclear intensity of Smad4 than TGF- $\beta$ 1-treated cells. These results suggest that FK506 promotes the TGF- $\beta$ 1/ALK1/Smad1 pathway (Fig. 2, C and D).

**FK506 Modulates the Binding of Smad4 to the Id1 and PAI1 Gene Promoters.** As shown in Fig. 2D, FK506 treatment in HMEC-1 cells, leads to an increase in the translocation of Smad4 to the nucleus. Smad4 is the CoSmad that translocates the TGF- $\beta$  signal to the nucleus, either depending on ALK1/pSmad1/5 or ALK5/pSmad2/3. To further assess the specificity of the FK506-induced signaling route documented in Fig. 2, A and B, using reporters of the ALK1/pSmad1/5 (*Id1* promoter) and the ALK5/pSmad2/3 (*PAI-1* promoter) pathways, ChIP assays were carried out. Chromatin immunoprecipitation of Smad4 followed by PCR with primers for *Id1* and *PAI-1* natural promoters encompassing SBE containing regions were performed. As shown in Fig. 2E, the amount of *Id1* DNA promoter immunoprecipitated with Smad4 is higher in FK506-treated HUVECs than in untreated cells (2.5 versus 0.31). On the contrary, in *PAI-1*, DNA immunoprecipitated with Smad4 decreases after FK506 treat-

ment, compared with untreated cells (0.99 versus 1.57). These results support our hypothesis that FK506 is modulating the TGF- $\beta$  signaling, activating the ALK1/pSmad1/5 pathway, and inhibiting the ALK5/pSmad2/3.

**FK506 Increases Binding of Sp1/Smad Complex to a Region of *ENG* Proximal Promoter.** The proximal promoter of *ENG*, contains a functional Sp1 binding site (−50/−24 encompassing region) essential for *ENG* basal transcriptional activity. This Sp1 site is adjacent to Smad consensus elements, and a synergistic functional cooperation between these Sp1 and Smad sites on the stimulation of *ENG* promoter activity has been reported (Botella et al., 2001). Accordingly, it seems that FK506 activates the TGF- $\beta$ 1/ALK1 pathway increasing the levels of Smad1 phosphorylation. To assess whether the FK506 treatment was also facilitating the binding of the Sp1/Smad complex in this region (−50/−24) of the *ENG* proximal promoter, thus increasing its transcriptional activity, HUVECs were treated with and without FK506, and then nuclear extracts were used for electrophoretic mobility shift assay studies. Figure 3 shows that the complexes formed with and without FK506 are essentially the same (lanes 2 and 3, Fig. 3A) and that there is no increase in the intensity of the retardation bands after 24 h of FK506 treatment (lane 3, Fig. 3A). These bands are specific, as shown after competition with an excess of nonradioactive wild-type and mutated oligonucleotides (lanes 4 and 5, Fig. 3A). The main complex (top/bottom bands) consists of Sp1 and Smads as revealed by specific antibodies and corresponds to the transcriptional complex already reported by our group using the same probe (Botella et al., 2001). Because no differences were found in the pattern or intensity of binding after 24 h or FK506 treatment, the binding was followed in a time course treatment. As seen in Fig. 3B, the bands representing specific binding to the *ENG* promoter sequence increased almost twice after 3 h of FK506 treatment, as quantified by densitometry, and decreasing afterward, especially between 6 and 12 h. Altogether, the results show that Sp1-Smad binding efficiency to the *ENG* proximal promoter is increased in the presence of the immunosuppressor FK506. These results correspond to HUVECs, a cell type that renders higher concentrations of nuclear extracts than HMEC-1. Similar results were obtained with HMEC-1 cells, although with fainter bands due to the lower yield of nuclear proteins (data not shown).

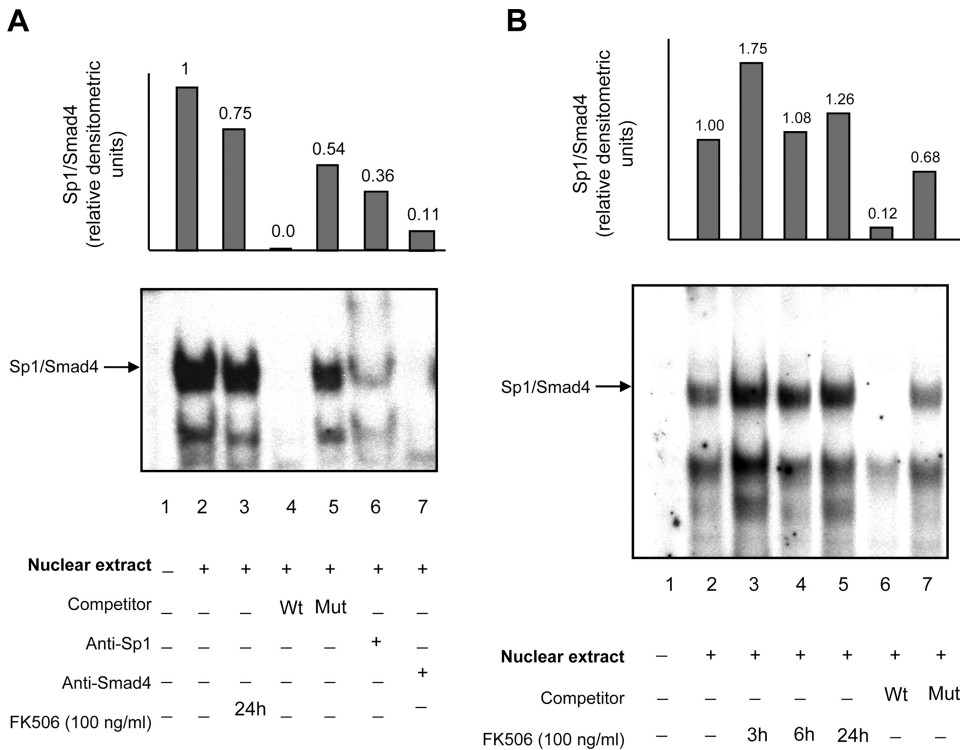
**FKBP12 Colocalizes and Coprecipitates with ALK1.** An immunofluorescent assay for colocalization of ALK1 and FKBP12 was performed in endothelial cells (HUVECs). As shown in Fig. 4, top row, cells without FK506 treatment show a certain degree of ALK1/FKBP12 colocalization, a similar result as reported by Giordano et al. (2008), in vascular smooth muscle cells with FKBP12 and the ubiquitous type I receptor ALK5. As shown by these authors, FKBP12 is an inhibitor of TGF- $\beta$ 1 receptor type I ALK5 in myoblasts. Likewise, our experiments suggest that FKBP12 is close to ALK1, and therefore FKBP12 may interfere with ALK1 signaling. However, when cells were treated with TGF- $\beta$ 1 or FK506, this colocalization disappeared because ALK1 was then preferentially bound to TGF- $\beta$ 1, and/or FK506 was sequestering FKBP12, leading to an active TGF- $\beta$ 1 signaling through the free ALK1 receptor (Fig. 4A).

To elucidate whether there is a physical interaction/association between FKBP12 and ALK1, HUVEC protein lysates

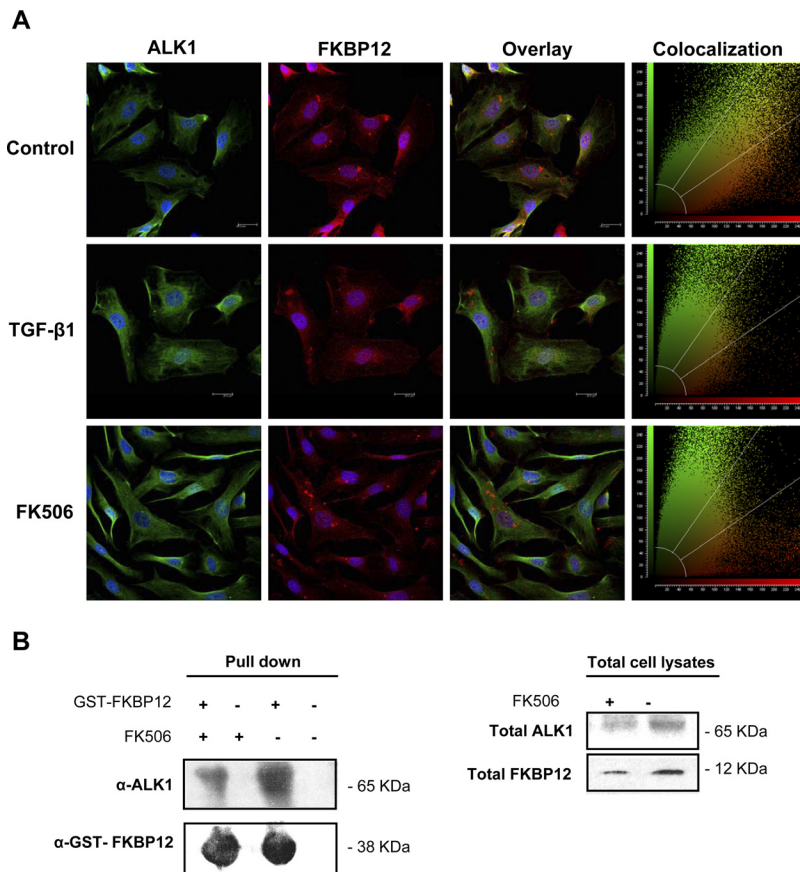


were subjected to GST-FKBP12 pull-downs. As shown in Fig. 4B, ALK1 was clearly coprecipitated with the recombinant FKBP12. In addition, the amount of ALK1 pulled down by GST-FKBP12 was decreased when cells were treated with

FK506, in line with the experiment of colocalization by immunofluorescent microscopy (Fig. 4A). Altogether, these results support the direct interaction between ALK1 and FKBP12.



**Fig. 3.** FK506 increases binding of Sp1/Smad complex to a region of *ENG* proximal promoter. HUVECs were treated with and without FK506 (100 ng/ml), and then nuclear extracts were used to bind a radiolabeled fragment of *ENG* proximal promoter. Nuclear extracts of untreated cells were incubated as negative controls in the presence of 100-fold excess of unlabeled wild-type and mutant oligonucleotides. A, characterization of the Sp1/Smad complex. To characterize the bands, specific antibodies anti-Sp1 and anti-Smad4 were used. The intensity of the complex was markedly decreased in the presence of this antibody, and a supershifted band appeared at the same time. When the complexes were incubated in the presence of anti-Smad4, the bands disappeared. B, time course of FK506 treatment. The amount of components binding to the *ENG* promoter is markedly increased after 3 h of treatment. Altogether, the results show that the efficiency of Sp1-Smad binding, and therefore basal *ENG* transcription is increased in the presence of the immunosuppressor FK506. Experiments were repeated at least three times. The figure shows a representative experiment.



**Fig. 4.** Colocalization and association between FKBP12 and ALK1. A, FKBP12 colocalizes with ALK1. An immunofluorescent assay was performed for the colocalization of ALK1 and FKBP12 in HUVECs. ALK1 and FKBP12 were stained with Alexa Fluor 488 and Alexa Fluor 547, respectively, conjugated with anti-rabbit/mouse IgG antibodies. Nuclei are stained in blue with 4,6-diamidino-2-phenylindole. The fourth column is the colocalization graph. y-Axis shows green fluorescence (ALK1), and x-axis shows red fluorescence (FKBP12). The colocalization area is shown in the middle of the graph. In untreated cells, FKBP12 acts like an ALK1 inhibitor because FKBP12 is bound to ALK1; therefore, colocalization is observed (top row). When cells are treated with TGF- $\beta$ 1 or FK506, colocalization disappears because TGF- $\beta$ 1 binds to ALK1 or FK506 binds to FKBP12; in this way, TGF- $\beta$ 1 signaling is activated. Experiments were repeated at least three times. A representative experiment is shown. B, pull-down of ALK1 by GST-FKBP12 protein. HUVEC protein lysates were subjected to GST-FKBP12 pull-downs. Total cell lysates and GST-FKBP12 pull-downs were subjected to SDS-PAGE followed by immunodetection with specific antibodies. ALK1 coprecipitates with FKBP12, supporting the direct interaction ALK1-FKBP12. Negative control was done with a GST alone, pull-down.

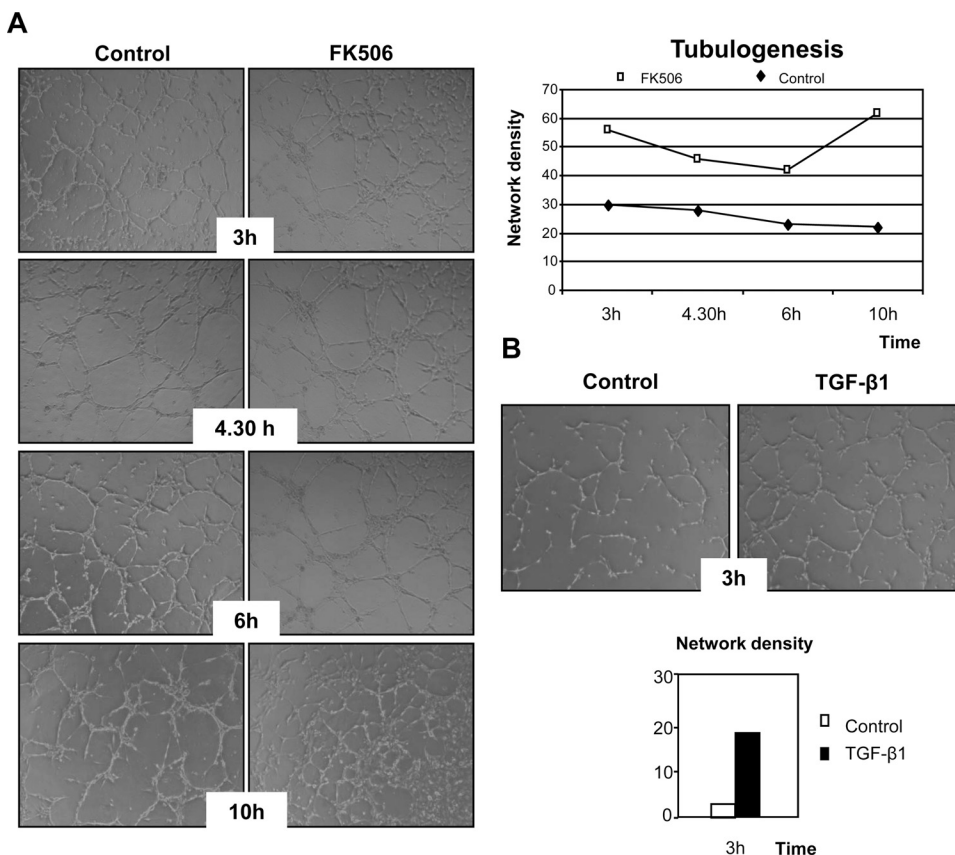
**Effects of FK506 on Wound Healing and Tube Formation.** We performed two types of experiments to measure the in vitro effects of FK506 on endoglin and ALK1 function, namely, tube formation as an in vitro angiogenesis test, and wound healing as an in vitro cell-migration assay. The proangiogenic and migration promoting effects of FK506 as functional consequences of increases in endoglin and ALK1 were evaluated by tubulogenesis and wound healing assays. Figure 5A shows cord formation in HMEC-1 cells in a time course experiment comparing FK506-treated cells with untreated ones. Tubes developed faster in FK506-treated endothelial cells, and the cord network was completely developed in FK506 treated cells after 4 h, whereas it required approximately 8 h in untreated cells. On the other hand, FK506 maintained the stability of the tube network longer (compare control and FK506-treated cells after 10 h) (Fig. 5A). In addition, when the confluent HMEC-1 monolayer was disrupted, the treatment with FK506 promoted a faster migration of endothelial cells. Thus, whereas the wound is closed between 20 and 24 h in FK506-treated cells, in control cells the closure occurred later, at approximately 40 to 44 h (Fig. 6A). As positive controls of these functional tests, cells were treated with TGF- $\beta$ 1 (10 ng/ml), which increases the cell migration and the tube formation (Fig. 5B and 6B, respectively). The results of these two functional experiments are in agreement with the observed increases in ALK1 and endoglin protein levels and therefore with the angiogenic, and migratory stimulation effect derived from the functionality of these proteins in endothelial cells.

## Discussion

Rendu-Osler-Weber disease (or HHT) is the result of endoglin and ALK1 haploinsufficiency. These genes are affected in 90% of patients with HHT. Therefore, one of the therapeutic aims in the investigation of this disease is to find drugs capable of increasing the transcriptional activity of these genes. Different transcription factors that regulate the *ENG* promoter are known, such as TGF- $\beta$ 1, hypoxia-inducible factor-1 $\alpha$ , vascular injury, Sp1, and KLF6 (Botella et al., 2001; Sánchez-Elsner et al., 2002; Bernabeu et al., 2010). Although ALK1 transcription is less well known than that of endoglin, a recent study has begun to unravel its regulation (Garrido-Martin et al., 2010).

Whereas TGF- $\beta$ 1 could be an interesting cytokine to potentiate the promoters of both genes, the multiple actions triggered in the different types of cells in the organism prevent its direct use in HHT therapies, although the stimulation of its pathway by other effectors might be an ideal strategy in therapeutical approaches.

So far, several drugs are being used with a certain degree of success to control epistaxis and gastrointestinal bleeding. Anti-fibrinolytic agents, such as  $\epsilon$ -aminocaproic or tranexamic acids, systemically administered have produced satisfactory results with an improvement in epistaxis and the associated anemia and with an increase in the transcriptional activity of *ENG* and *ALK1* promoters (Fernandez et al., 2007; Morales-Angulo et al., 2007). However, contraindications appear in those patients prone to suffer thrombosis; hence, it is necessary to screen the HHT population for high levels of coagulant factors VIII and V and von Willebrand factor before



**Fig. 5.** Tubulogenesis assay. A, cells were incubated on Matrigel (P-24)-coated plates at 37°C in the absence or presence of FK506 (100 ng/ml). The cord network formation was measured taking photos at different times up to 10 h after cell plating. The appearance of a complete network is achieved in 3 h in the case of FK506 treated cells, whereas untreated controls cells remain partially in tubes with patches of disorganized, sparse cells. The picture shown is representative of the whole plate. B, treatment with TGF- $\beta$ 1 (10 ng/ml) was performed as a positive control. As shown, more cord formation is observed in treated cells.

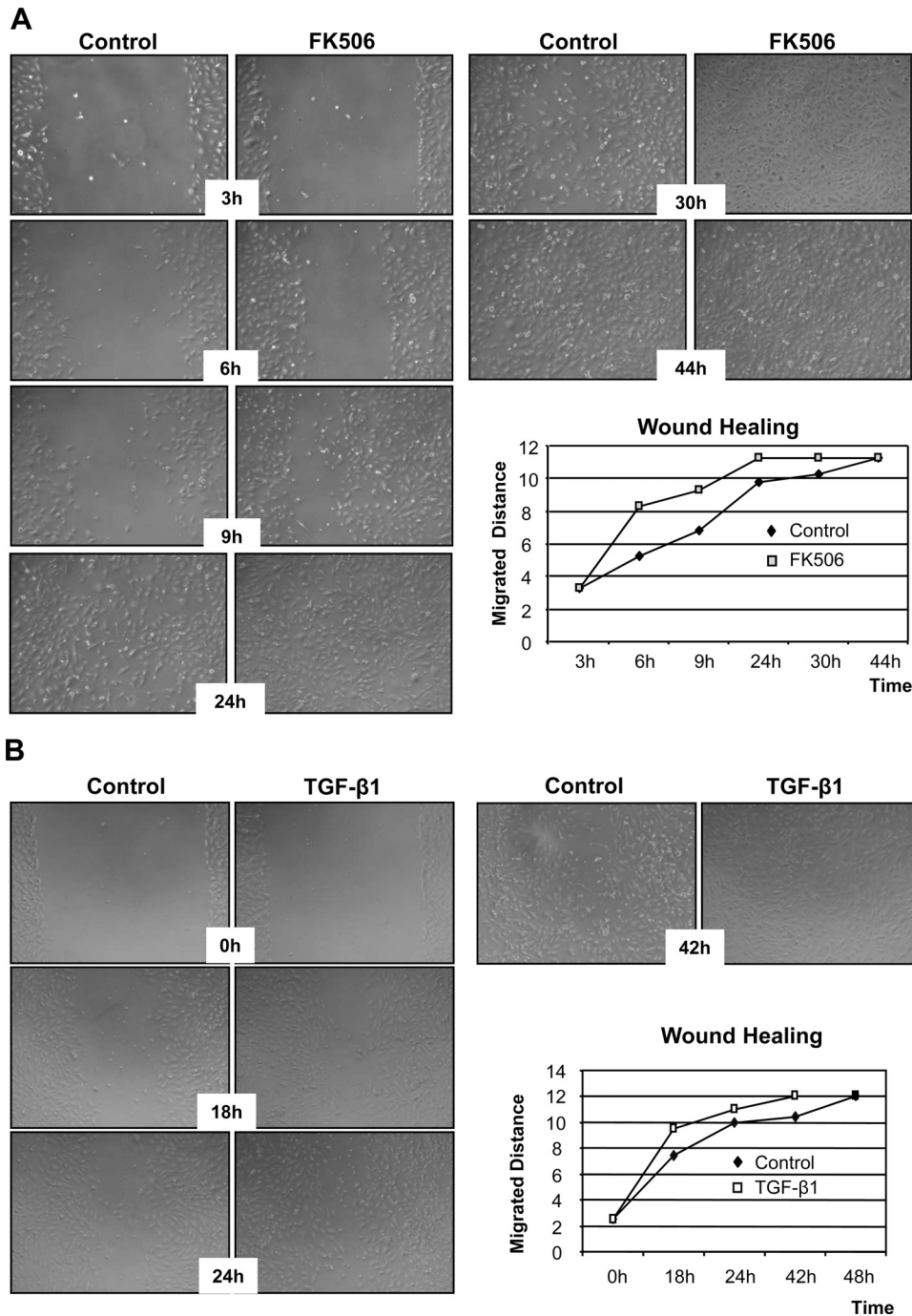


starting an antifibrinolytic treatment (Shovlin et al., 2007). In patients with high levels of coagulation factors, antifibrinolytic therapies may increase the risk of deep venous thromboembolism (Ghosh and Ghosh, 2008). Therefore, we need to look for alternative therapeutic sources to counteract HHT epistaxis.

Another therapy source is based on hormones in which the use of estradiol/norethindrone for epistaxis and gastrointestinal management of HHT has resulted in a variable degree of efficacy depending on the patient. Zacharski et al. (2001) described a case-based report with long-term cessation of epistaxis using tamoxifen in a postmenopausal woman. The conclusion is that systemic estrogens at doses used for oral contraception may eliminate bleeding in women with symp-

tomatic HHT. In line with this, we have published the positive effects of raloxifene, a selective estrogen receptor modulator, on patients with HHT together with the biochemical rationale behind this (Albiñana et al., 2010).

The immunosuppressor FK506, which prevents the rejection of transplants, was efficient in a case report of a 53-year-old woman with HHT who underwent a hepatic transplant. The important observation was that after FK506 treatment subsequent to the transplant, her HHT symptoms improved, including anemia, epistaxes, and skin/mucosa telangiectases (Skaro et al., 2006). Although liver transplants may cause regression of spider nevi, transplantation alone is not known to induce HHT angiodyplasia regression or to reduce mucosal hemorrhages. The report of telangiectasia regression af-



**Fig. 6.** Wound healing. A, confluent HMEC-1 monolayers treated with or without 100 ng/ml FK506 were disrupted with a pipette tip to test the speed of migration with which cells were able to close the wound. Photos were taken at different times, and the speed of migration was quantified by densitometry of the filled space in the wound at each time point. Experiments were repeated twice, and a representative picture is shown. The completed closure was between 20 and 24 h in treated cells and between 40 and 44 h in untreated cells. Immunosuppressor FK506 at concentrations of 100 ng/ml speeds up the closure of the wound in endothelial cells. B, treatment with TGF- $\beta$ 1 (10 ng/ml) was performed as a positive control. As shown, the wound is closed at 42 h after injury in treated cells, whereas the wound remained unclosed in untreated cells.

ter liver transplantation suggests that the immunosuppressive regimen may have contributed to the regression, possibly through the vascular endothelial growth factor pathway. However, as reported previously, FK506 does not inhibit vascular endothelial growth factor production (Rajnoch et al., 2005). Another possibility is the activation of NFAT pathway by FK506 in endothelial cells; however, our results demonstrated that tacrolimus at the assayed concentrations from 0 to 100 ng/ml did not affect NFAT activity, as measured by a reporter of this pathway (data not shown).

Immunophilins are a family of proteins known mainly as receptors of the immunosuppressant drugs such as cyclosporin A and FK506, although they do serve several other functions, including regulation of mitochondrial permeability, modulation of ion channel stability, and functioning as chaperones for a variety of proteins (Udina and Navarro, 2002). The development of new agents that selectively bind to immunophilins, such as FK506, opens up new and interesting perspectives for the treatment of diseases affecting the TGF- $\beta$ 1 signaling network, including cancer and HHT, as discussed in this work.

It is noteworthy that FK506 and other derivatives inhibit the function of immunophilins. In particular, FKBP12 is a natural inhibitor of TGF- $\beta$ 1-mediated signaling; therefore, ligands, such as FK506, act as TGF- $\beta$ 1 activators, competing with the FKBP12 for the TGF- $\beta$ 1 receptors (Udina and Navarro, 2002). In fact, in a previous report, Giordano et al. (2008) demonstrated that FK506 increases the phosphorylation of Smad2/3, sequestering FKBP12, a receptor type I inhibitor, in mouse VSMCs. In this work, we have described the action of FK506 in endothelial cells, in which it triggers activation through the endothelial-specific receptor type I, ALK1/Smad1/5/8, as demonstrated by ALK1-specific reporter activation of this pathway (BRE-luc), and the natural promoter regulated by this way, *Id1*. As a consequence, the ALK5 pathway is inhibited as shown by CAGA-luc and PAI-1-luc reporters, whose activity is decreased by FK506 treatment. This is in agreement with the ALK1/ALK5 balance in endothelial cells (Lebrin et al., 2004; Blanco et al., 2005; Shovlin, 2010).

The results of our work support the hypothesis that the decrease in the HHT symptoms may be due to an increase in promoter activity of the genes *ALK1* and *ENG*, giving rise to haploinsufficiency, as shown by using reporter constructs of both promoters and obtaining an increase in the transcription of both genes. The increase in transcription leads to an approximate 1.5- to 2-fold rise in messenger levels for both genes and the resulting increase in endoglin and ALK1 protein levels on the surface of endothelial cells. Because the haploinsufficiency of both proteins in HHT1 and 2 is the origin of the disease, the increasing expression of these proteins after FK506 treatment would counteract, at least partially, this haploinsufficiency. We have shown in this article that FK506 is able to stimulate the TGF- $\beta$ 1 pathway by binding immunophilin FKBP12, which sequesters the endothelial receptor type I, ALK1, partially inhibiting the TGF- $\beta$ 1 signaling. By binding FK506 to FKBP12, the receptor type I ALK1 is released, and TGF- $\beta$ 1/ALK1/Smad1/5/8 is activated through phosphorylation. Evidences found in endothelial cells from patients with HHT showed that there is a coordinated relationship at a transcriptional level between TGF- $\beta$ 1, *ALK5*, *ALK1*, and *ENG* promoters (Fernandez et al.,

2005). Hence, the stimulation of the TGF- $\beta$ 1 pathway increases the expression of these components, especially endoglin and ALK1. Moreover, all of the other functions dependent on this pathway, including endoglin activation (Botella et al., 2001), cell proliferation, and migration (Lebrin et al., 2004; Blanco et al., 2005), would also be stimulated. As a consequence, faster migration of endothelial cells (wound healing) and proper angiogenesis (tube formation) would be promoted.

At this point, it is worth noting that the results presented here have been obtained in normal (non-HHT) endothelial cells. The lack of experimental data on HHT cells is a limitation of this study. Therefore, the extrapolation of the conclusions to HHT endothelial cells remains hypothetical. However, because endoglin and ALK1 haploinsufficiency is accepted as the origin of HHT pathogenicity, HHT endothelial cells express at least half of endoglin- or ALK1-active proteins. Because the mechanism of action proposed involves the stimulation of TGF- $\beta$ 1/ALK1/endoglin signaling by FK506, it can be postulated that HHT endothelial cells may respond to the drug through the wild-type alleles expressed by them.

In conclusion, all of the results shown in this article support the use of tacrolimus rather than other immunosuppressors for patients with HHT subjected to organ transplants, because its use may contribute not only to preventing rejection but also to alleviating the HHT-derived symptoms. In fact, the advantage of FK506 is that, in addition to its immunosuppressor properties, it is able to activate the TGF- $\beta$ 1/ALK1 pathway in endothelial cells, thus increasing the levels of endoglin and ALK1-haploinsufficient proteins in patients with HHT. At the same time, the stimulation of the TGF- $\beta$ 1 signaling by FK506 opens an alternative immunosuppressor pathway in endothelial cells, in addition to the classic immunophilin-NFAT/interleukin inhibition.

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#### Authorship Contributions

*Participated in research design:* Albiñana, Bernabéu, and Botella.  
*Conducted experiments:* Albiñana, Sanz-Rodríguez, and Recio-Poveda.  
*Performed data analysis:* Albiñana and Sanz-Rodríguez.  
*Wrote or contributed to the writing of the manuscript:* Albiñana, Bernabéu, and Botella.  
*Other:* Bernabéu and Botella acquired funding for the research.

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**Address correspondence to:** Dr. Luisa María Botella, Centro de Investigaciones Biológicas, CSIC, C/Ramiro de Maeztu, 9 Madrid 28040, Spain. E-mail: cibluisa@cib.csic.es

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