

Endoglin Haploinsufficiency Promotes Fibroblast Accumulation during Wound Healing through Akt Activation

Miguel Pericacho¹, Soraya Velasco¹, Marta Prieto¹, Elena Llano¹, José M. López-Novoa^{1,2}, Alicia Rodríguez-Barbero^{1,2*}

1 Renal and Cardiovascular Physiopathology Unit, Instituto "Reina Sofía" de Investigación Nefrológica, Departamento de Fisiología y Farmacología, Universidad de Salamanca, Salamanca, Spain, **2** Biomedical Research Institute of Salamanca, Salamanca, Spain

Abstract

Accurate regulation of dermal fibroblast function plays a crucial role in wound healing. Many fibrotic diseases are characterized by a failure to conclude normal tissue repair and the persistence of fibroblasts inside lesions. In the present study we demonstrate that endoglin haploinsufficiency promotes fibroblast accumulation during wound healing. Moreover, scars from endoglin-heterozygous (*Eng*^{+/-}) mice show persisting fibroblasts 12 days after wounding, which could lead to a fibrotic scar. Endoglin haploinsufficiency results in increased proliferation and migration of primary cultured murine dermal fibroblasts (MDFs). Moreover, *Eng*^{+/-} MDF have diminished responses to apoptotic signals compared with control cells. Altogether, these modifications could explain the augmented presence of fibroblasts in *Eng*^{+/-} mice wounds. We demonstrate that endoglin expression regulates Akt phosphorylation and that PI3K inhibition abolishes the differences in proliferation between endoglin haploinsufficient and control cells. Finally, persistent fibroblasts in *Eng*^{+/-} mice wound co-localize with a greater degree of Akt phosphorylation. Thus, endoglin haploinsufficiency seems to promote fibroblast accumulation during wound healing through the activation of the PI3K/Akt pathway. These studies open new non-Smad signaling pathway for endoglin regulating fibroblast cell function during wound healing, as new therapeutic opportunities for the treatment of fibrotic wounds.

Citation: Pericacho M, Velasco S, Prieto M, Llano E, López-Novoa JM, et al. (2013) Endoglin Haploinsufficiency Promotes Fibroblast Accumulation during Wound Healing through Akt Activation. PLoS ONE 8(1): e54687. doi:10.1371/journal.pone.0054687

Editor: Johanna M. Brandner, University Hospital Hamburg-Eppendorf, Germany

Received: June 26, 2012; **Accepted:** December 17, 2012; **Published:** January 17, 2013

Copyright: © 2013 Pericacho et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from the Junta de Castilla y León (SA052A06 and SA029A09) to ARB and SA001/C05 to JMLN, and Ministerio de Ciencia e Innovación (BFU2004-00285/BFI, and SAF2007-63893 to JMLN) and the Instituto de Salud Carlos III, (RETIC RedInRen RD/0016). MP was a holder of a fellowship from the Junta de Castilla y León, and SV was a fellowship holder of the Spanish Ministerio de Educación y Ciencia. The Renal and Cardiovascular Physiology Unit is an Excellence Research Group awarded by Junta de Castilla y León. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: barberoa@usal.es

Introduction

Wound healing is a complex and highly coordinated process involving a number of interdependent stages including inflammation, proliferation and remodeling [1,2]. Impairment of wound healing represents a particularly challenging clinical problem to which no efficacious treatments currently exist. Thus, understanding the complexity of the healing process is critical to resolve patient problems. In physiological remodeling, such as during dermal wound healing, fibroblast activation finishes when tissue is repaired, and activated fibroblasts disappear by apoptosis [3,4]. However, in pathological wound healing activated fibroblasts persist and leads to fibrosis and tissue deformation, which is evident in hypertrophic scars in the fibrotic phase of scleroderma, after burn injury and in fibrosis of vital organs such as liver, heart and lung [4].

Different cells types and numerous growth factors are involved in each phase of wound healing. Among them, transforming growth factor beta (TGFβ) and its receptors, including endoglin, are essential in this process. TGFβ plays a critical role in different phases of wound healing by regulating production of extracellular

matrix (ECM), proteases, chemotaxis, migration and proliferation of different cell types which regulate scar contraction, angiogenesis, granulation tissue formation, ECM remodeling and scar maturation [5].

Endoglin (CD105) is a type III co-receptor for the TGFβ receptors: TβRII, ALK1 and ALK5. Endoglin is expressed in a number of cell types including endothelial cells, monocytes, tissue macrophages, stromal cells, fibroblast, etc... and modulates TGFβ dependent responses [6,7]. Mutations in the endoglin gene can lead to hereditary hemorrhagic telangiectasia (HHT) and defective angiogenesis [8]. Endoglin, in combination with TGFβ family members, plays an important role in regulating different cellular functions such as endothelial cell adhesion, migration and proliferation [9,10].

Several authors have described endoglin upregulation in different fibrotic processes. Thus, endoglin expression is increased in cutaneous scleroderma fibroblasts [11], liver fibrosis [12,13], fibroblasts isolated from strictures in Crohn's disease [14] or cardiac fibroblasts developing fibrosis [15]. Moreover, endoglin is upregulated in chronic progressive renal disease [16] and in several models of renal fibrosis [17,18,19]. Endoglin is mainly

Table 1. Sequences of primers.

Gene	Sense primer (5'-3')	Anti-sense primer (5'-3')
Eng	GACTTCAGATTGGAATACCTTGG	CAGTGCCGTGTCTTCTGTAAT
Collagen I α	TGTTGCTGAAAGACTACCTCGT	CCTCCCATGTAAATAGCACCT
Fibronectin	ATGTGGACCCCTCTGATAGT	GCCCAGTGATTTCAGCAAAGG
PAI-1	GCTGCACTGGTGACTCACTT	AGGAGCTGGCTGTTCTTTC
GAPDH	TGAAGGTCGGGTGAACGGATTGGC	CATGTAGGCCATGAGGTCCACCAC
RPS13	GATGCTAAATCCGCCTGAT	TAGAGCAGAGGCTGTGGATG

doi:10.1371/journal.pone.0054687.t001

considered as an antifibrotic molecule. Several studies show that endoglin counteracts TGF β 1-dependent responses, such as increased expression of extracellular matrix components, including PAI-1, collagen and fibronectin [20,21,22,23]. It has been described that endoglin could exert this antifibrotic role modulating TGF β 1 signaling through pro-proliferative ALK1-Smad1/5 pathway instead pro-fibrotic ALK5-Smad2/3 pathway [24,25,26]. These results have been confirmed in cultured fibroblasts as endoglin overexpression leads to a diminution of ECM proteins expression [14,27]. However, some controversy exists as other authors have described profibrotic effects of endoglin expression [13,28,29]. These results suggest that the specific role of endoglin depends on the cell type, environmental conditions or the fibrosis model assessed. Nevertheless the importance of the study of the role of endoglin in fibrotic processes is clear.

To evaluate whether endoglin might be involved in post-wound healing fibrosis, we used endoglin-heterozygous mice ($Eng^{+/-}$), since mice lacking endoglin ($Eng^{-/-}$) die from cardiovascular defects at mid-gestation [30,31,32]. To investigate the mechanism of action of endoglin, we assessed the response of endoglin-heterozygous mice and dermal fibroblasts derived from them to wounding both *in vivo* and *in vitro*. Our results revealed that endoglin acts as an essential component for the accurate completion of tissue repair by its ability to decrease the number of fibroblasts in wounds through its capacity to block Akt activation.

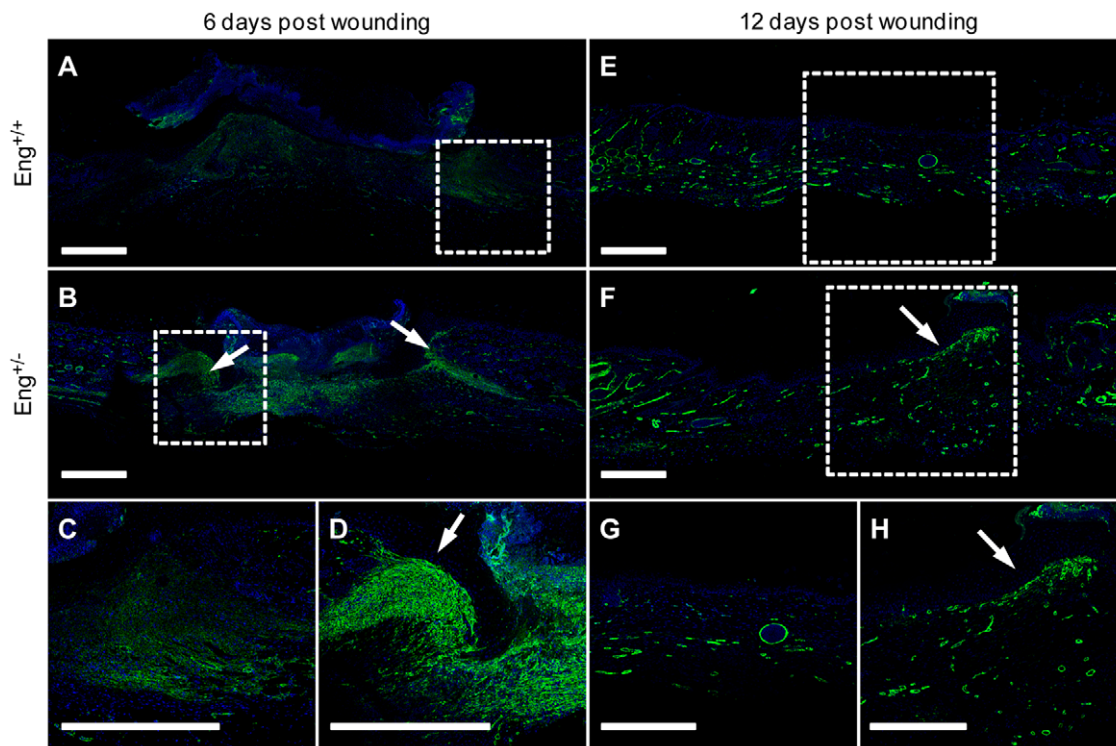


Figure 1. Histological analysis of activated fibroblasts presence during $Eng^{+/-}$ wound healing. (A and B) Immunofluorescence staining of alpha-smooth muscle actin (α -SMA) on Formalin-fixed Parafin-embedded wounds from $Eng^{+/+}$ (A) and $Eng^{+/-}$ (B) mice at day 6. (C and D) Granulation tissue on wounds edges from $Eng^{+/+}$ (C) and $Eng^{+/-}$ (D) mice in more details. (E and F) Immunofluorescence staining of α -SMA on Formalin-fixed Parafin-embedded scars from $Eng^{+/+}$ (E) and $Eng^{+/-}$ (F) mice at day 12. (G and H) $Eng^{+/+}$ (G) and $Eng^{+/-}$ (H) day-12 scars magnification. Bars = 500 μ m. Arrow = Activated fibroblasts accumulation. A representative image from four independent experiments is shown. doi:10.1371/journal.pone.0054687.g001

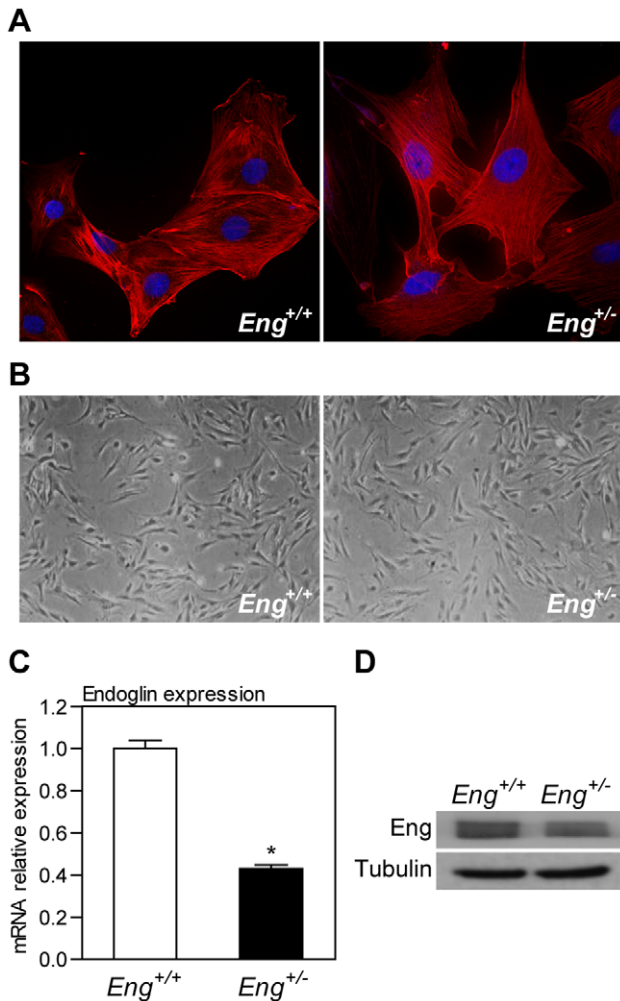


Figure 2. Characterization of primary cultured murine dermal fibroblasts (MDF) from endoglin heterozygous mice. (A) Immunofluorescence analysis of α -SMA expression in *Eng*^{+/+} and *Eng*^{+/-} MDF. (B) Phase contrast analysis of *Eng*^{+/+} and *Eng*^{+/-} MDF morphology. (C and D) Analysis of relative endoglin expression between *Eng*^{+/+} and *Eng*^{+/-} MDF by quantitative RT-PCR (C) and western blot (D). Mean+SEM is represented (n=3). * p<0.05 - significance of the difference between cells, Student's t-test. A representative blot from three independent experiments is shown. doi:10.1371/journal.pone.0054687.g002

Materials and Methods

Animals and wound model

All procedures were approved by the Committee for the Care and Use of Animals of the University of Salamanca and complied with the Guide for the Care and Use of Laboratory Animals [33]. *Eng*^{+/+} and *Eng*^{+/-} mice were obtained as previously described [31]. The animals were a generous gift from Michelle Letarte (Hospital for Sick Children, Toronto, Canada), and they were cared for and genotyped as previously described [34].

Eighteen *Eng*^{+/+} and eighteen *Eng*^{+/-} 10-week-old animals were used for the *in vivo* studies. Mice were anesthetized with isoflurane and two 5 mm of diameter excisional wound were made in the shaved middorsal skin. An aseptic technique was used for all surgical manipulations performed the mice.

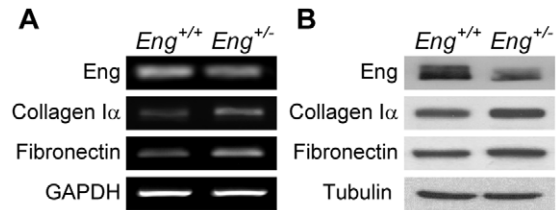


Figure 3. Effect of endoglin haploinsufficiency on extracellular matrix formation. (A) Synthesis of collagen α and fibronectin by *Eng*^{+/+} and *Eng*^{+/-} MDF were analyzed by RT-PCR. GAPDH was used as housekeeping (B) Synthesis of collagen α and fibronectin were also evaluated by western blot in total protein extracts from *Eng*^{+/+} and *Eng*^{+/-} MDF using Tubulin as loading control. A representative blot from three independent experiments is shown. doi:10.1371/journal.pone.0054687.g003

Histology and immunohistofluorescence

For histological analysis, 6-day and 12-day wounds were harvested and fixed in cold 4% buffered formalin, dehydrated, bisected, mounted in paraffin, and sectioned for histology and immunohistofluorescence. Heat-induced antigen retrieval was performed in citrate buffer (pH 9.00). The primary antibodies used were mouse monoclonal anti- α -SMA (Sigma, at 1:300 dilution) and rabbit polyclonal anti-phospho-Akt (Ser⁴⁷³) (Cell Signaling, at 1:10 dilution). Following washes in PBS, sections were incubated with fluorescent-conjugated secondary antibodies (anti-mouse FITC and anti-rabbit Cy3 respectively) at room temperature. Slides were mounted in Vectashield (Vector Laboratories) after nucleus staining with DAPI. All images were obtained using a confocal microscope (Leica) with identical parameters for intensity, pinhole aperture, etc.

Cell cultures

Primary cultured murine dermal fibroblasts (MDF) were obtained from *Eng*^{+/+} and *Eng*^{+/-} mice. Animals were euthanized and the shaved skin was placed in a sterile flask containing 0.25% trypsin (Sigma) in PBS and incubated overnight at 4°C, after which the epidermis was separated from the dermis. The dermis was subjected to further digestion with 0.25% collagenase (Sigma) in PBS for 2–3 hours at 37°C. Cells were collected by centrifugation, washed once with PBS, and plated on 100 mm Petri dishes.

MDF and NIH3T3 (ATCC) cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 100 U/ml of penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere. All experiments were performed in the presence of 10% FBS and MDF were used at passage four.

Reagents and antibodies

Purified TGF β 1 was purchased from R&D Systems. The PI3K inhibitor LY294002 was from Sigma. Antibodies against Smad1, Smad2/3, Id-1 and Akt were from SantaCruz Biotechnology. Antibodies against phosphorylated Smad1/3, phosphorylated Akt (Ser⁴⁷³) and phosphorylated Akt (Thr³⁰⁸) were from Cell Signaling. Antibodies against fibronectin and collagen I were from Chemicon. The antibody against PCNA was from BD Pharmingen. The antibody against α -SMA was from Sigma. The antibody against phosphorylated Smad2 was generated as described previously [35]. The antibodies against endoglin have been described previously and were MJ7/18 for murine endoglin [36] and TEA1/58-1 for human endoglin [37].

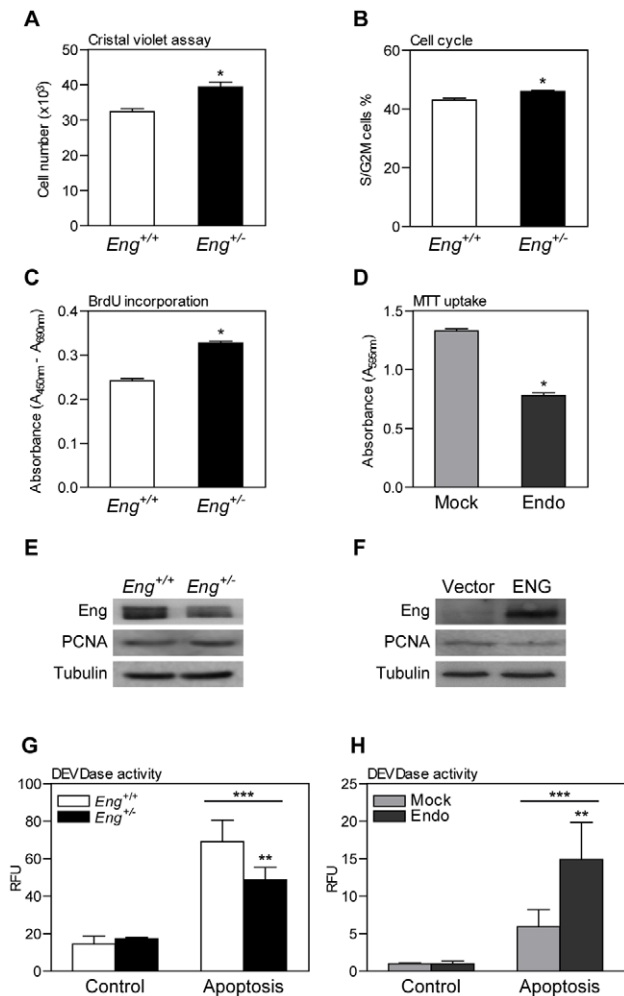


Figure 4. Effect of endoglin expression on fibroblast proliferation and apoptosis. (A–C) Endoglin haploinsufficiency effect on MDF proliferation was estimated by crystal violet assay (A), cell cycle analysis (B) and BrdU incorporation (C) in proliferative conditions (10% FCS). NIH3T3 fibroblasts overexpressing human endoglin (Endo) and infected with empty vector (Mock) were used to confirm the effect of endoglin expression on fibroblast proliferation. NIH3T3 proliferation was assessed by MTT (D). (E and F) PCNA expression analyzed by western blot was compared between *Eng*^{+/+} and *Eng*^{+/-} MDF (E) and on *Eng*^{+/-} MDF transfected with endoglin (ENG) or the empty vector (F). (G and H) *Eng*^{+/+} and *Eng*^{+/-} MDF (G) and Mock and Endo NIH3T3 (H) *in vitro* responses to apoptotic stimulus were determined by measuring DEVDase (caspase) activity after treatment with a mix of 2.5 μ g/ml anti-Fas antibody plus 250 μ g/ml cycloheximide. Mean+SEM is represented (n = 3). * $p < 0.05$ -significance of the difference between cells, Student's *t*-test. ** $p < 0.05$ -significance of the difference between cells in apoptosis conditions, *** $p < 0.05$ -significance of the difference between apoptosis treatment and control conditions, Two-way ANOVA. A representative blot from three independent experiments is shown. doi:10.1371/journal.pone.0054687.g004

Retroviral transduction

293T cells (3×10^6) were plated on a 10 cm dish, incubated overnight, and then co-transfected according to the calcium phosphate precipitation method with 10 μ g of pCL-Eco plasmid containing the gag, pol and env viral proteins and 10 μ g of a pBabe-puro retroviral vector containing the human endoglin gene (15 hr at 37°C). After 48 hr, the virus-containing medium was filtered (0.45 μ m filter, Millipore) and supplemented with 4 μ g/ml polybrene (Sigma) (first supernatant). Viral supernatants were

collected for an additional 8 hr, as before (second supernatant). NIH3T3 cells were plated at 8×10^5 cells per 100 mm dish and incubated overnight. For infection, the culture medium was replaced with the appropriate first supernatant and incubated for 8 hr at 37°C. The infection process was repeated using the second supernatant. Twenty-four hours after infection, infected cells were selected for 3 days in the presence of 2 μ g/ml puromycin, and plated on the 5th day post-infection for the corresponding assays.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described [23]. Cells were plated onto glass coverslips, fixed, permeabilized, and incubated with primary antibodies for 1 hour. After washing, the cells were incubated with the appropriate fluorescence-conjugated secondary antibodies (Molecular Probes) for 1 hour. Slides only incubated with the secondary antibodies were used as controls for non-specific signal. Cells were washed in 0.2% BSA-PBS, briefly rinsed in 2 μ M Hoechst 33258 reagent (Invitrogen) to stain nuclei, and mounted with ProLong[®] antifade (Invitrogen). Stained cells were photographed with a Nikon Eclipse TE 2000-U confocal microscope.

Western blot analyses

Western blot analyses were essentially performed as described previously [23]. Cells were lysed in ice-cold lysis buffer and protein concentrations were determined (Bradford, Bio-Rad). Protein samples were separated by SDS-PAGE, blotted onto PVDF membranes, and incubated with the primary antibody. Following incubation with horseradish peroxidase-conjugated secondary antibody, the bands were visualized with a luminol-based detection system with p-iodophenol enhancement. Anti-tubulin antibody was used to confirm equal loading of protein in each lane. Some membranes were re-probed with several antibodies using a stripping solution (Chemicon) and following the manufacturer's instructions.

RT-PCR analysis

Total RNA was isolated using Nucleospin RNAII (Macherey-Nagel), according to the manufacturer's instructions. Single-strand cDNA was generated from 2 μ g of total RNA using poly-dT as primer with M-MLV reverse transcriptase (Promega). For RT-PCR, 1 μ l of cDNA was used in a standard 50- μ l PCR mixture with 400 nM of each primer and 2 U of FastStart Taq DNA polymerase (Roche). The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by SybrSafe (Invitrogen) staining.

Quantitative RT-PCR was performed in triplicate. Each 20 μ l reaction contained 1 μ l of cDNA, 400 nM of each primer, and 1x iQ SybrGreen Supermix (Bio-Rad). Standard curves were run for each transcript to ensure exponential amplification and to rule out non-specific amplification. Gene expression was normalized to RPS13 expression. The reactions were run on an iQ5 Real-time PCR detection system (Bio-Rad). The specific primers used for PCR are described in Table 1.

Plasmids, transfection, and luciferase reporter assays

The expression plasmids for human endoglin (*ENG*) have been described previously [38,39]. ON-TARGETplus SMARTpool siRNA against *ENG* and control siRNA were obtained from Dharmacon. The TGF β -responsive vectors used as reporters were the ALK5-Smad3-specific (CAGA)₁₂-Luc [40], the specific Smad2-responsive Fast/pAre-Luc [41], and p(Bre)₂-Luc, which contains ALK1-specific response elements [42]. In the luciferase

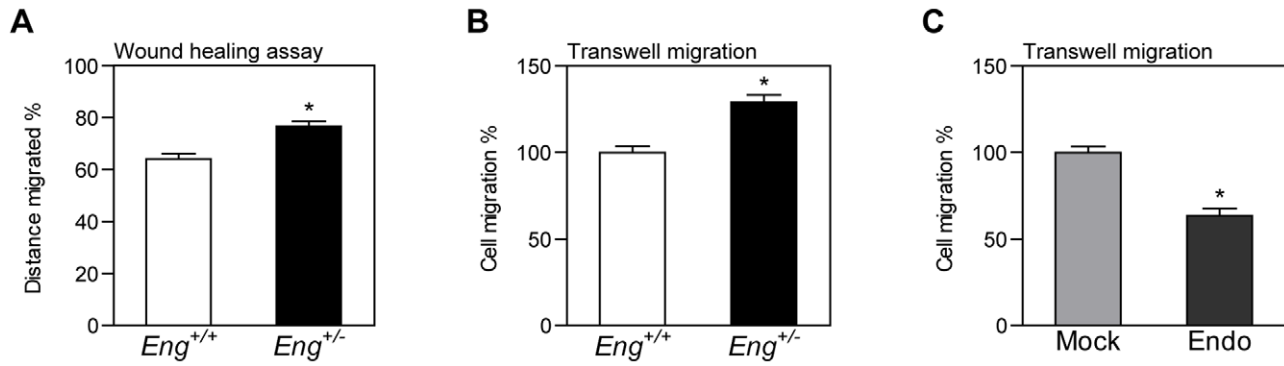


Figure 5. Effect of endoglin expression on fibroblast migration. (A and B) *Eng*^{+/+} and *Eng*^{+/-} MDFA *in vitro* migration was analyzed by wound healing assay (A) and 8-mm pore transwell migration (B). (C) Migration of NIH3T3 fibroblasts overexpressing human endoglin (Endo) and infected with empty vector (Mock) was assessed by transwell assay. * $p < 0.05$ -significance of the difference between cells, Student's *t*-test. doi:10.1371/journal.pone.0054687.g005

assays, the expression plasmid pRL-TK vector containing the Renilla luciferase gene (Promega) served as an internal control to correct transfection efficiency. Cells were transfected using Lipofectamine 2000 (Invitrogen) for 5 hours, according to the

manufacturer's instructions. Luciferase and Renilla activities were measured using a dual-reporter assay kit (Promega).

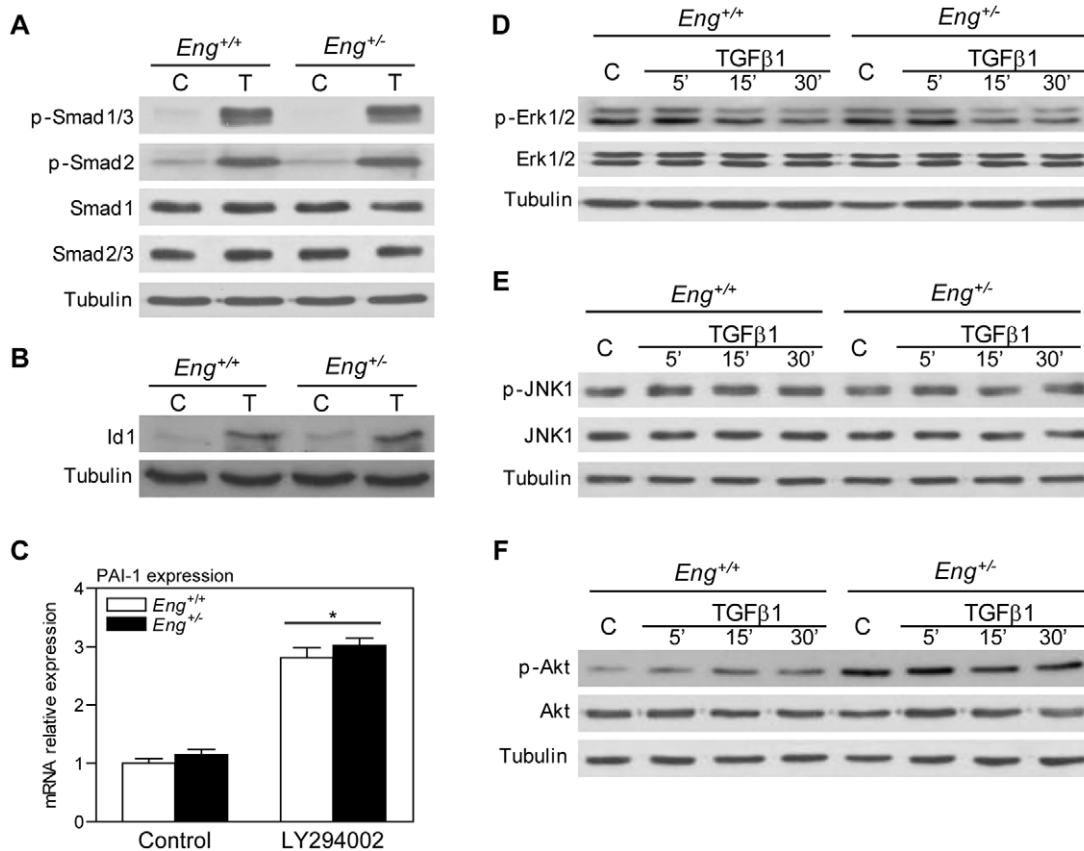


Figure 6. Effect of endoglin haploinsufficiency on TGF β 1 signaling pathways. (A) *Eng*^{+/+} and *Eng*^{+/-} MDFA were stimulated with 1 ng/ml TGF β 1 for 30 minutes. Total protein extracts were analyzed by western blot with anti-phospho-Smad1/3, anti-phospho-Smad2, anti-Smad1 and anti-Smad2/3 (B). Id1 expression was assessed by western blot after 1 hour TGF β 1 treatment. (C) TGF β 1-induced PAI-1 expression increment was analyzed by quantitative RT-PCR. A representative experiment of three independent experiments using triplicate samples is shown. (D-F) MAPKs and PI3K/Akt pathways were analyzed after stimulation with 1 ng/ml TGF β 1 for 5, 15 and 30 minutes. Total proteins extracts were resolved by western blot and incubated with anti-phospho-Erk1/2 and anti-Erk1/2 (D), anti-phospho-JNK1 and anti-JNK1 (E) and anti-phospho-Akt and anti-Akt (F) respectively. Tubulin was used as loading control. Mean+SEM is represented (n=3). * $p < 0.05$ -significance of the difference between TGF β 1 treatment and control conditions, Two-way ANOVA. A representative blot from three independent experiments is shown. doi:10.1371/journal.pone.0054687.g006

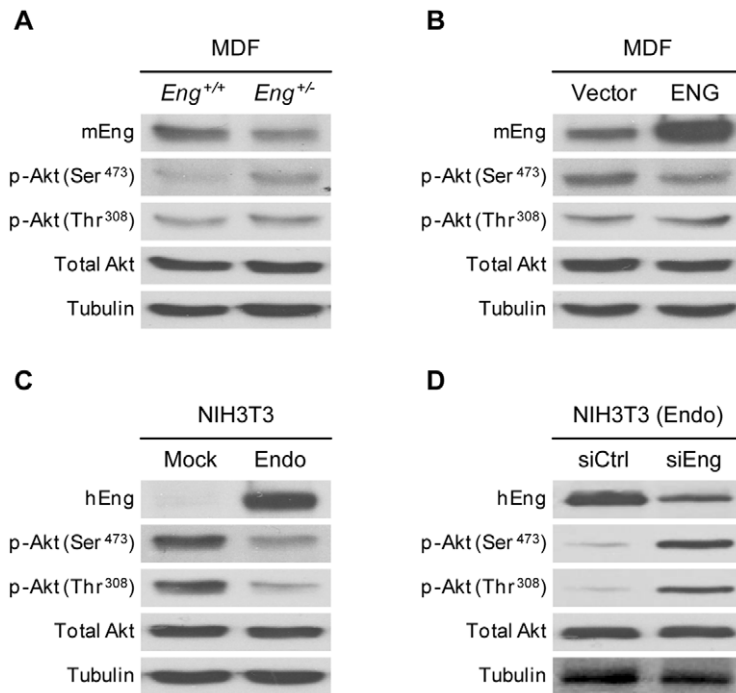


Figure 7. Effect of endoglin expression on Akt phosphorylation. (A) Ser⁴⁷³ and Thr³⁰⁸ Akt phosphorylation and Akt expression were analyzed by western blot in total protein extracts from *Eng*^{+/+} and *Eng*^{+/-} MDF. (B–D) Similar analysis were carried on in *Eng*^{+/-} MDF transiently transfected with ENG or empty vector (B); NIH3T3 overexpressing by retroviral infection endoglin (Endo) and its controls (Mock) (C); and Endo fibroblasts transfected with anti-Eng siRNA (D). Tubulin was used as loading control. A representative blot from three independent experiments is shown. doi:10.1371/journal.pone.0054687.g007

Proliferation assays

For crystal-violet assays, 5000 cells were seeded on a 24-well plate and incubated in 10% FCS medium with or without additional agonists. After the indicated times, cells were stained with crystal-violet and, upon solubilization, the amount of dye taken up by the cells was quantitated in a plate reader. Cell number was estimated based on absorbance at 595 nm. MDF proliferation was also determined by a colorimetric Cell Proliferation ELISA (Roche) following the recommendations of the manufacturer. This ELISA is based on the measurement of BrdU incorporation during DNA synthesis in replicating (cycling) cells. NIH3T3 cell proliferation was analyzed with the MTT-based assay (Sigma). The cell cycle profile was analyzed by flow cytometry.

Migration assays

For wound healing assays, confluent monolayers were wounded using a sterile pipette tip and the extent of wound closure was determined along 24 hours by calculating the migrated distance/total wound distance. Furthermore, cell invasion was assessed through an 8.0 μ m-pore membrane. MDF cells were seeded in the upper chamber of the insert in 2% FCS medium and allowed to migrate to 10% FCS medium, placed in the lower chamber, for 24 hours. Migrated cells were determined by crystal-violet assay.

Caspase activity

To determine DEVDase activity, 20000 cells were seeded on black 96-wells plates for 24 h. Cells were treated for 3 h with vehicle (Control) or a mix of 2.5 μ g/ml anti-Fas antibody (Upstate-Millipore) plus 250 μ g/ml cycloheximide (Apoptosis). DEVDase activity was determined with SensoLyteTM Homo-

geneus Rh110 Caspase-3/7 Assay Kit (Anaspec) following the recommendations of the manufacturer.

Statistical analyses

All numerical data are presented as means \pm S.E.M and were analyzed by two-way ANOVA and Student's *t*-test. All statistical tests were performed using GraphPad Prism 5 software.

Results

Activated fibroblast persistence in the wounds of *Eng*^{+/-} mice

Immunohistological analyses of 6-day post-wounding skin sections revealed the presence of activated fibroblasts (α -SMA positive cells) in the granulation tissue of both genotypes. However, a higher number of activated fibroblasts were observed in granulation tissues from *Eng*^{+/-} mice than in the control animals (Figure 1A–D). In 12-day post-wounding skin sections from *Eng*^{+/+} mice, α -SMA expression was limited to a few resident fibroblasts, similar to normal skin (Figure 1E and 1G). However, in wounds of *Eng*^{+/-} mice activated fibroblasts persisted close to the epidermis (Figure 1F and 1H). In both 6-day and 12-day skin sections we can also detect blood vessels because of the staining of the smooth muscle cells. In order to perform an in-depth analysis of these endoglin-mediated differences in activated fibroblast accumulation, we developed a primary culture of murine dermal fibroblasts (MDF) from *Eng*^{+/+} and *Eng*^{+/-} mice.

Endoglin heterozygosity promotes extracellular matrix proteins synthesis

In order to evaluate the effect of endoglin deficiency in extracellular matrix (ECM) deposition, we analyzed the expression

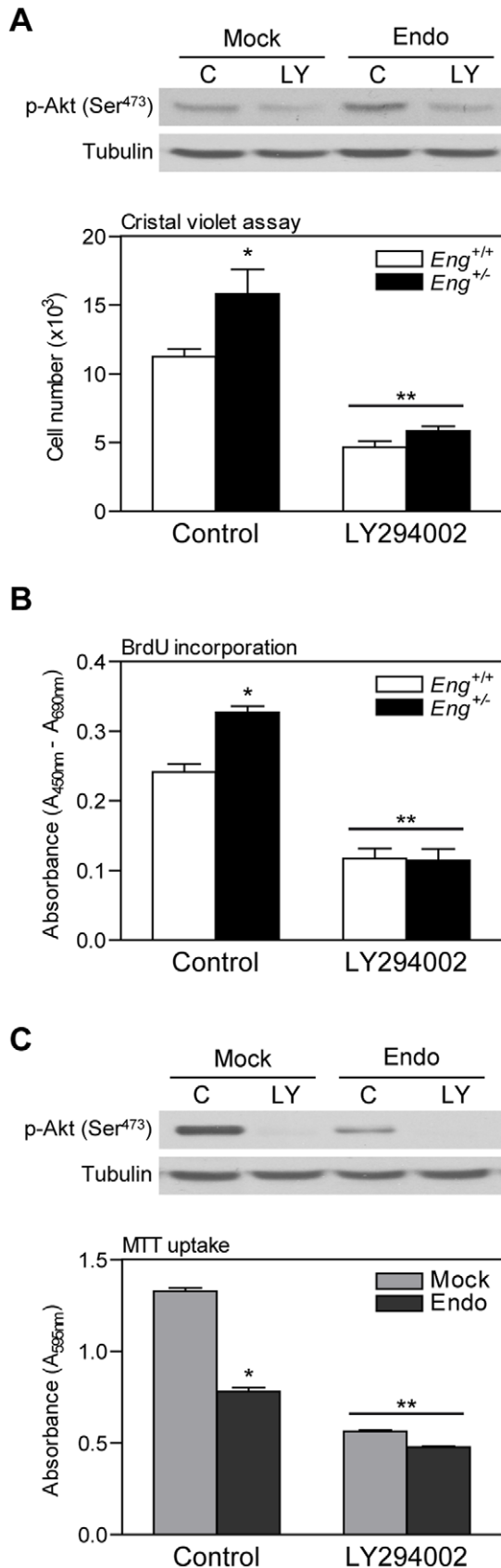


Figure 8. Effect of PI3K inhibition on endoglin-mediated different cell proliferation. (A) Cell number of MDF was analyzed by crystal violet assay after 10 μ M LY294002 treatment for 4 days.

Inhibition of Akt activation after LY294002 treatment was analyzed by western blot (A, upper panel). (B) BrdU incorporation of *Eng*^{+/+} and *Eng*^{+/-} MDF after LY294002 treatment was also analyzed. (C) Endo and Mock fibroblasts proliferation was assessed by MTT after 10 μ M LY294002 treatment. Inhibition of Akt activation after LY294002 treatment was analyzed by western blot (C, upper panel). Mean+SEM is represented (n=3). * p<0.05-significance of the difference between cells in control conditions, ** p<0.05-significance of the difference between LY294002 treatment and control conditions, Two-way ANOVA. Tubulin was used as loading control. A representative blot from three independent experiments is shown. doi:10.1371/journal.pone.0054687.g008

of ECM-related genes in primary cultured Murine Dermal Fibroblasts (MDF).

MDF cultures were established using dermis from either control or endoglin-heterozygous mice. After two passages in culture, we obtained a homogeneous monolayer without significant morphological differences between *Eng*^{+/+} and *Eng*^{+/-} MDF in phase-contrast microscopy studies (Figure 2B). We found no differences between both genotypes in expression or disposition of α -SMA, a defining characteristic of activated fibroblasts (Figure 2A). Quantitative RT-PCR and western blot analyses revealed reduced endoglin mRNA and protein levels in *Eng*^{+/-} as compared to *Eng*^{+/+} MDF, respectively (Figure 2C and D). RT-PCR analysis showed that *Eng*^{+/-}-derived MDF presents higher expression of collagen I α and fibronectin than *Eng*^{+/+} MDF (Figure 3A). We confirmed by western blot analysis that this difference occurs also at the protein level (Figure 3B).

Endoglin haploinsufficiency increases proliferation of MDF

Since proliferation, persistence and migration of activated fibroblasts are key events for fibroblast accumulation in granulation tissue, we analyzed whether endoglin might be modulating these properties in primary cultured dermal fibroblasts. We and others have previously reported the effect of endoglin expression on proliferation and migration in several cell types [24,26,43,44].

To investigate how affect the reduce levels of endoglin in proliferation of MDF cells, we determined cellular proliferation rates using crystal-violet staining. This analysis showed that *Eng*^{+/-} cells proliferated significantly faster than wild-type MDF (Figure 4A). In addition, analysis of the cell cycle by flow cytometry revealed a greater percentage of *Eng*^{+/-} cells in S+G2/M phases as compared to *Eng*^{+/+} (Figure 4B). We also analyzed the cell proliferation measuring incorporation of BrdU during DNA synthesis in cycling cells. *Eng*^{+/-} cells proliferate significantly more than wild-type MDF (Figure 4C). Western blot analyses showed that *Eng*^{+/-} cells had a higher expression of proliferating cell nuclear antigen (PCNA) than *Eng*^{+/+} cells (Figure 4E, left panel). These effects on cellular proliferation were directly dependent on a low endoglin expression, since transfection of *Eng*^{+/-} MDF with endoglin (ENG) resulted in a decreased expression of PCNA (Figure 4F, right panel).

To further assess the effect of endoglin expression on fibroblasts behavior, we expressed human endoglin (hENG) in NIH3T3 fibroblasts using retroviral infection. This model allowed us to investigate whether this effect was directly dependent on endoglin expression and whether it was consistent in other fibroblast cell line. Consistent with our previous results, MTT assays revealed that endoglin overexpression inhibited NIH3T3 cell proliferation (Figure 4D).

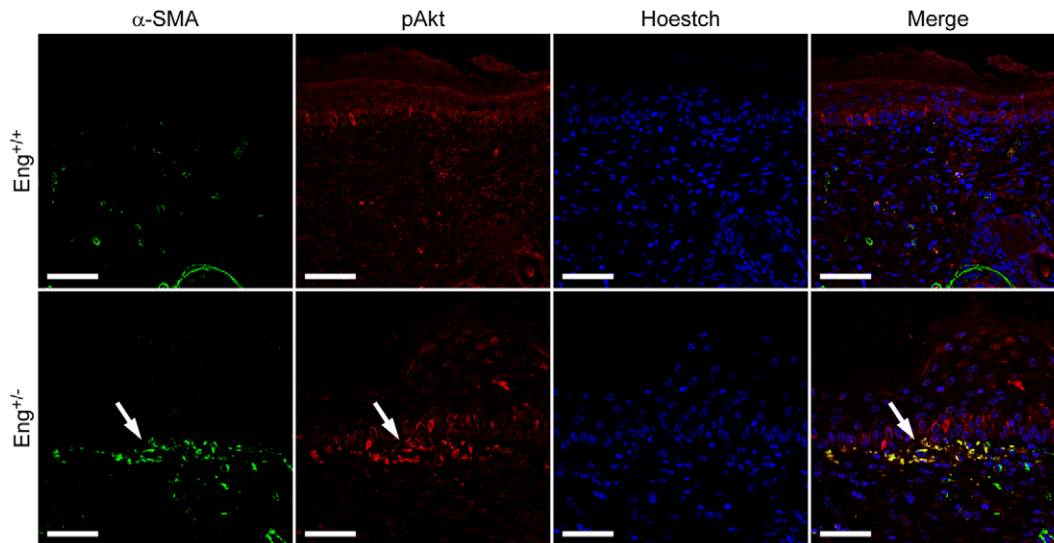


Figure 9. Histological analysis of Akt phosphorylation after wound healing. Immunofluorescence staining of alpha-smooth muscle actin (α -SMA) and phospho-Akt (pAkt) on Formalin-fixed Paraffin-embedded wounds from $Eng^{+/+}$ and $Eng^{+/-}$ mice at day 12. Bars = 50 μ m. Arrow = Colocalization of α -SMA and pAkt. A representative image from four independent experiments is shown. doi:10.1371/journal.pone.0054687.g009

Endoglin haploinsufficiency MDF presents diminished apoptosis response

After wound closure, activated fibroblasts should disappear by apoptosis. However, we have previously described a persistence of activated fibroblasts in wounds of $Eng^{+/-}$ mice that could lead to fibrosis and tissue deformation. This persistence could be explained whether $Eng^{+/-}$ activated fibroblasts have diminished responses to apoptotic signals compared with control cells. To test this hypothesis we treated MDF with a combination of an activator of the extrinsic (anti-Fas antibody) and an activator of the intrinsic pathway (cycloheximide) of apoptosis and analyzed Caspase-3/7 activity. $Eng^{+/-}$ cells present significantly less DEVDase activity than $Eng^{+/+}$ cells after apoptotic treatment (Figure 4G). Endoglin overexpression in NIH3T3 fibroblasts result in a significant increase of Caspase-3/7 activity in response to apoptotic stimulus.

Endoglin haploinsufficiency increases migration of MDF

Dermal fibroblasts migration was analyzed with two independent techniques, which afforded similar results. Confluent monolayers were damaged by a scratch-wound assay and the cells were allowed to migrate for 24 hours. $Eng^{+/-}$ cells migrated significantly farther than $Eng^{+/+}$ cells (Figure 5A). Time-lapse videos revealed that monolayer repair occurred as a result of fibroblast motility instead of proliferation (data not shown). Moreover, similar results were obtained in transwell migration assays (Figure 5B). Endoglin overexpression also inhibited NIH3T3 cell migration analyzed with transwell migration assays (Figure 5C).

TGF β 1 signaling through the Smad and MAPK pathways in fibroblasts are unaffected by endoglin haploinsufficiency

It has been proposed that endoglin modifies cellular properties such as proliferation and migration by modulating TGF β signaling through the ALK1-Smad1, ALK5-Smad2/3 or MAPKs pathways [24,45,46,47,48,49,50]. Thus, first we analyzed the effect of endoglin haploinsufficiency on ALK1-Smad1 and ALK5-Smad2/

3 signaling pathway activation in response to TGF β 1 treatment in dermal fibroblasts. Stimulation of MDF with TGF β 1 for 30 minutes resulted in Smad1, Smad2 and Smad3 phosphorylation (Figure 6A) without significant differences between $Eng^{+/+}$ and $Eng^{+/-}$ MDF. Moreover, TGF β 1-induced Id1 protein and PAI-1 mRNA expression was equal in endoglin-haploinsufficient and wild-type cells (Figure 6B and C). These findings suggest that endoglin expression does not modulate TGF β 1 signaling through the Smad pathways in dermal fibroblasts.

We also analyzed the effect of endoglin expression in other signaling pathways such as MAPKs or PI3K/Akt, which have been shown to be activated by TGF β 1. Stimulation with TGF β 1 resulted in an increased phosphorylation of Erk1 and Erk2 after 5 minutes of treatment (Figure 6D). TGF β 1 treatment also resulted in JNK1 activation (Figure 6E). However, there were no differences between $Eng^{+/+}$ and $Eng^{+/-}$ MDF in TGF β 1-mediated activation of these pathways. We failed to detect p38 phosphorylation in MDF after TGF β 1 treatment (data not shown).

Endoglin expression modifies Akt activation

Under basal conditions (10% FCS), $Eng^{+/-}$ cells showed a higher degree of Akt activation than control cells. TGF β 1 treatment stimulated Akt phosphorylation in both cell types, maintaining the previously observed differences between $Eng^{+/+}$ and $Eng^{+/-}$ MDF (Figure 6F). As stated, western blot analyses of MDF revealed that basal Akt activation was increased in endoglin-heterozygous cells. Akt activation is determined by multisite phosphorylation, with two main phosphorylation sites: Thr³⁰⁸ and Ser⁴⁷³ [51]. We found differences in both Akt phosphorylation sites, although greatest increase was seen in Ser⁴⁷³ phosphorylation (Figure 7A). In order to assess the direct relationship between endoglin expression and Akt activation, we overexpressed endoglin in MDFs (Figure 7B, upper panel) and analyzed Akt activation. MDF transiently transfected with endoglin showed a lower degree of Akt activation than cells transfected with empty vector (Figure 7B). Furthermore, we analyzed Akt activation in endoglin-infected NIH3T3 fibroblasts. Endoglin overexpression resulted in an important decrease in the Akt phosphorylation of

both Thr³⁰⁸ and Ser⁴⁷³ (Figure 7C). Transient transfection of these cells with a siRNA for endoglin decreased endoglin expression and increased Akt phosphorylation (Figure 7D). Thus, the amount of endoglin expression seems to modulate basal Akt activation in fibroblasts.

Akt mediates differential cell proliferation due to endoglin expression

In order to determine the relationship between endoglin haploinsufficiency-induced proliferation and endoglin haploinsufficiency-induced Akt activation, we analyzed the effect of the PI3K inhibitor LY294002 on fibroblast proliferation. LY294002 has been shown to act as a highly selective inhibitor of PI3K, being an important tool for elucidating the biological role of the PI3K/Akt pathway [52,53]. Whether the differences in cell proliferation found in endoglin-deficient cells will be a direct consequence of Akt activation, then, the treatment of these cells with the PI3K inhibitor, LY294002, would prevent the differences in endoglin-dependent cell proliferation. Consistent with the above results, in the absence of LY294002, the number of cells was higher in *Eng*^{+/-} than in wild-type cells. Incubation with LY294002 resulted in an inhibition of both *Eng*^{+/+} and *Eng*^{+/-} cell proliferation and the number of cells was similar in both *Eng*^{+/+} and *Eng*^{+/-} cells (Figure 8A). Similar results were obtained after analysis of cell proliferation by BrdU incorporation (Figure 8B).

Furthermore, we studied the effect of LY294002 treatment on NIH3T3 cell proliferation in the presence or absence of endoglin overexpression. In the absence of LY294002, the number of cells, four days after plating, was lower in NIH3T3 cells overexpressing endoglin than in control cells. LY294002 treatment inhibited proliferation in both cell lines and the differences in proliferation disappeared (Figure 8C). Western blot analyses revealed that LY294002 at a dose of 10 μ M was sufficient for the inhibition of Akt phosphorylation in both MDF and NIH3T3 cells (Figure 8A–C).

Activated fibroblast accumulation in *Eng*^{+/-} wounds is related to increased Akt phosphorylation

In order to study in more detail the involvement of the PI3K/Akt pathway in the accumulation of activated fibroblasts *in vivo*, we analyzed Akt phosphorylation in 12-day-post-wounding *Eng*^{+/+} and *Eng*^{+/-} skin sections (Figure 9). Control skin sections did not display any particular distribution of pAkt, although the degree of Akt phosphorylation seemed to be higher in the basal epidermis than in the dermis. Interestingly, the distribution of Akt phosphorylation in the upper layer of the dermis of the *Eng*^{+/-} scars was focused at the sites of α -SMA expression, coincident with the location of activated fibroblasts. Thus, α -SMA staining co-localized with Akt phosphorylation in *Eng*^{+/-} but not in *Eng*^{+/+} scars, indicating the direct relationship between endoglin haploinsufficiency, Akt phosphorylation and the maintenance of activated fibroblasts in mice skin.

Discussion

The importance of endoglin in fibrosis has been widely accredited [11,12,13,14,15,16,17,18,19,28,29]. Fibrosis is the end result of a succession of events that occur after mechanical damage to the epithelium and/or endothelium [54]. Fibrotic diseases may be attributable to a variety of causes, but it is generally thought that an initiating injury event activates repair processes that aim to restore the original tissue architecture, and a failure to finely tune the repair process leads to persistent fibroblast activation and tissue destruction [55]. During physiological dermal

wound healing, the activity of activated fibroblasts is terminated when the tissue is repaired. In pathological wound healing, however, activated fibroblasts persist and lead to tissue deformation and fibrosis [3,4,56]. The potential role of endoglin in fibroplasia during wound healing has been proposed [5] since a significant elevation in fibroblast-associated endoglin levels was observed between days 4 and 10 of wound healing [57]. To investigate the functional role of endoglin in wound healing induced fibrosis we have used mice heterozygous for endoglin (*Eng*^{+/-}) and their wild-type littermates (*Eng*^{+/+}) [31]. Endoglin knockout mice die at midgestation because of defective angiogenesis [7]. *Eng*^{+/-} mice have allowed to analyze the involvement of endoglin in different processes such as angiogenesis [43], cardiovascular function [34] and tumor development [58,59]. In this work, we show that *Eng*^{+/-} mice exhibit persistence of activated fibroblasts in the wounds, as detected by α -SMA staining. In addition, primary cultured fibroblasts from these mice display higher expression of ECM-related molecules, increased proliferation and migration rates and diminished responses to apoptotic signals, whereas endoglin over-expression results in an inhibition of these processes. According to these results endoglin deficiency would lead to an increased fibrosis post-wounding. Although some studies suggest a profibrotic role for endoglin [13,28,29], our results are consistent with many previous works that demonstrate that endoglin acts as an antifibrotic molecule [14,20,21,22,23,24,25,26,27].

This antifibrotic effect of endoglin seems to be mediated by PI3K/Akt signaling pathway. Thus, under basal conditions Akt phosphorylation was increased in *Eng*^{+/-} dermal fibroblasts and this effect was inhibited after endoglin overexpression. Akt/PKB is an intermediate signaling component of the PI3K pathway that is activated by phosphorylation in Thr and Ser residues and that is involved in several cellular processes, including growth, metabolism, reproduction, and life span [60,61,62]. In particular, Akt has been described as a fibroblast proliferation promoter [53,63]. Here, we show that PI3K pathway inhibition reduced the faster proliferation of endoglin-haploinsufficient dermal fibroblasts to levels similar to those of control cells. Moreover, the differences in cell proliferation due to endoglin expression in NIH3T3 fibroblasts were abolished after PI3K inhibition. These results suggest that the difference in proliferation found in endoglin-haploinsufficient cells is a direct consequence of different Akt activation. Recently, the role of GSK-3 β in wound healing has been analyzed [64]; GSK-3 β is a downstream member of the PI3K pathway that is degraded after Akt-mediated phosphorylation. Interestingly, GSK-3 β knock-down results in increased *in vivo* activated fibroblasts accumulation in the wound area and enhanced proliferation and migration *in vitro*. Our work, together with that of the above authors, highlights the relevance of PI3K/Akt pathway as an important mediator during wound healing and its importance in the regulation of activated fibroblasts accumulation after repair.

Whatever the mechanism of endoglin regulating Akt activation this effect seems to be independent of TGF β 1 signaling. It is widely accepted that endoglin has an important role regulating TGF β signaling. Endoglin expression promotes TGF β -mediated ALK1-Smad1/5 activation, in contrast with the classical ALK5-Smad2/3 activation. This model has mainly been described in endothelial cells [24,45], and myoblasts [25,26]. However, our study supports a previously proposed idea that endoglin has cellular effects independent of TGF β [9,49,65,66]. There are only a few works in the literature that relates endoglin and PI3K-Akt pathway and almost all of them refer a regulation of endoglin expression by PI3K-Akt pathway [67,68,69,70,71]. According with our results, very recently, Lee *et al.* shown the PI3K/Akt pathway as an

endoglin target in regulating the stabilization of capillary sprouts and endothelial cell survival. They proposed a model in which GIPC could be the mediator coupling endoglin to the PI3K subunits and Akt at the plasma membrane and could be implicated in a differential response to TGF β 1 and BMP-9. In the presence of endoglin, TGF β 1 inhibits Akt phosphorylation with a modest enhancement by BMP-9 treatment [72]. Our data support these results as we also find a regulation of Akt phosphorylation depending on endoglin expression. Moreover, endoglin regulated PI3K/Akt signaling pathway could be a broad mechanism involved in the regulation of further physiologic processes and in different cell types.

In summary, our work analyzing the involvement of endoglin in activated fibroblasts accumulation may contribute to a better understanding of the pathophysiological processes of wound healing that give rise to fibrogenesis. Moreover, we proposed the PI3K/Akt pathway as the mechanism of action of endoglin on the regulation of post-wound healing fibrosis. Therefore, according to our results, endoglin and the PI3K/Akt pathway may be therapeutic targets for the treatment of fibrotic wounds.

References

- Sorrell JM, Caplan AI (2004) Fibroblast heterogeneity: more than skin deep. *J Cell Sci* 117: 667–675.
- Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. *Nature* 453: 314–321.
- Desmouliere A, Redard M, Darby I, Gabbiani G (1995) Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* 146: 56–66.
- Hinz B (2007) Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 127: 526–537.
- Valluru M, Staton CA, Reed MW, Brown NJ (2011) Transforming Growth Factor-beta and Endoglin Signaling Orchestrate Wound Healing. *Front Physiol* 2: 89.
- Gougos A, Letarte M (1990) Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem* 265: 8361–8364.
- Lopez-Novoa JM, Bernabeu C (2010) The physiological role of endoglin in the cardiovascular system. *Am J Physiol Heart Circ Physiol* 299: H959–974.
- ten Dijke P, Goumans MJ, Pardali E (2008) Endoglin in angiogenesis and vascular diseases. *Angiogenesis* 11: 79–89.
- Conley BA, Koleva R, Smith JD, Kacer D, Zhang D, et al. (2004) Endoglin controls cell migration and composition of focal adhesions: function of the cytosolic domain. *J Biol Chem* 279: 27440–27449.
- Bernabeu C, Conley BA, Vary CP (2007) Novel biochemical pathways of endoglin in vascular cell physiology. *J Cell Biochem* 102: 1375–1388.
- Leask A, Abraham DJ, Finlay DR, Holmes A, Pennington D, et al. (2002) Dysregulation of transforming growth factor beta signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts. *Arthritis Rheum* 46: 1857–1865.
- Clemente M, Nunez O, Lorente R, Rincon D, Matilla A, et al. (2006) Increased intrahepatic and circulating levels of endoglin, a TGF-beta1 co-receptor, in patients with chronic hepatitis C virus infection: relationship to histological and serum markers of hepatic fibrosis. *J Viral Hepat* 13: 625–632.
- Meurer SK, Tihaa L, Borkham-Kamphorst E, Weiskirchen R (2011) Expression and functional analysis of endoglin in isolated liver cells and its involvement in fibrogenic Smad signalling. *Cell Signal* 23: 683–699.
- Burke JP, Watson RW, Mulsow JJ, Docherty NG, Coffey JC, et al. (2010) Endoglin negatively regulates transforming growth factor beta1-induced profibrotic responses in intestinal fibroblasts. *Br J Surg* 97: 892–901.
- Chen K, Mehta JL, Li D, Joseph L, Joseph J (2004) Transforming growth factor beta receptor endoglin is expressed in cardiac fibroblasts and modulates profibrogenic actions of angiotensin II. *Circ Res* 95: 1167–1173.
- Roy-Chaudhury P, Simpson JG, Power DA (1997) Endoglin, a transforming growth factor-beta-binding protein, is upregulated in chronic progressive renal disease. *Exp Nephrol* 5: 55–60.
- Rodriguez-Pena A, Prieto M, Duwel A, Rivas JV, Eleno N, et al. (2001) Up-regulation of endoglin, a TGF-beta-binding protein, in rats with experimental renal fibrosis induced by renal mass reduction. *Nephrol Dial Transplant* 16 Suppl 1: 34–39.
- Rodriguez-Pena A, Eleno N, Duwel A, Arevalo M, Perez-Barriocanal F, et al. (2002) Endoglin upregulation during experimental renal interstitial fibrosis in mice. *Hypertension* 40: 713–720.
- Docherty NG, Lopez-Novoa JM, Arevalo M, Duwel A, Rodriguez-Pena A, et al. (2006) Endoglin regulates renal ischaemia-reperfusion injury. *Nephrol Dial Transplant* 21: 2106–2119.

Acknowledgments

The technical assistance of Annette Düwel and Lucía Martín is greatly appreciated. We thank Dr. Michelle Letarte (Hospital for Sick Kids, Toronto, Canada) for the kind gift of *Eng*^{+/-} mice, Dr. Miguel Quintanilla (Instituto Alberto Sols de Investigaciones Biomedicas CSIC, Madrid, Spain) for the kind gift the mouse-endoglin plasmid, Dr. Peter ten Dijke (Leiden University Medical Center, Leiden, The Netherlands) for the Smads antibodies and promoters, and Dr. Carmelo Bernabeu (Centro de Investigaciones Biológicas, CSIC, Madrid, Spain) for the endoglin antibodies and endoglin expression plasmid. We thank Dr. Paul Oh for his valuable discussion and advice.

Author Contributions

Conceived and designed the experiments: ARB M. Pericacho JMLN. Performed the experiments: M. Pericacho SV M. Prieto ELL ARB. Analyzed the data: M. Pericacho SV M. Prieto ARB JMLN. Contributed reagents/materials/analysis tools: M. Pericacho SV M. Prieto ARB JMLN ELL. Wrote the paper: ARB M. Pericacho SV M. Prieto JMLN ELL.

- Diez-Marques L, Ortega-Velazquez R, Langa C, Rodriguez-Barbero A, Lopez-Novoa JM, et al. (2002) Expression of endoglin in human mesangial cells: modulation of extracellular matrix synthesis. *Biochim Biophys Acta* 1587: 36–44.
- Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, et al. (1996) Endoglin modulates cellular responses to TGF-beta 1. *J Cell Biol* 133: 1109–1121.
- Obreo J, Diez-Marques L, Lamas S, Duwel A, Eleno N, et al. (2004) Endoglin expression regulates basal and TGF-beta1-induced extracellular matrix synthesis in cultured L6E9 myoblasts. *Cell Physiol Biochem* 14: 301–310.
- Rodriguez-Barbero A, Obreo J, Eleno N, Rodriguez-Pena A, Duwel A, et al. (2001) Endoglin expression in human and rat mesangial cells and its upregulation by TGF-beta1. *Biochem Biophys Res Commun* 282: 142–147.
- Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, et al. (2004) Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *Embo J* 23: 4018–4028.
- Schnerer O, Meurer SK, Tihaa L, Gressner AM, Weiskirchen R (2007) Endoglin differentially modulates antagonistic transforming growth factor-beta1 and BMP-7 signaling. *J Biol Chem* 282: 13934–13943.
- Velasco S, Alvarez-Munoz P, Pericacho M, Dijke PT, Bernabeu C, et al. (2008) L- and S-endoglin differentially modulate TGFbeta1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts. *J Cell Sci* 121: 913–919.
- Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C (2002) Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. *J Biol Chem* 277: 29197–29209.
- Morris E, Chrobak I, Bujor A, Hant F, Mummery C, et al. (2011) Endoglin promotes TGF-beta/Smad1 signaling in scleroderma fibroblasts. *J Cell Physiol* 226: 3340–3348.
- Scharpfenecker M, Floot B, Russell NS, Ten Dijke P, Stewart FA (2009) Endoglin haploinsufficiency reduces radiation-induced fibrosis and telangiectasia formation in mouse kidneys. *Radiother Oncol* 92: 484–491.
- Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, et al. (2000) Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol* 217: 42–53.
- Bourdeau A, Dumont DJ, Letarte M (1999) A murine model of hereditary hemorrhagic telangiectasia. *J Clin Invest* 104: 1343–1351.
- Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, et al. (1999) Defective angiogenesis in mice lacking endoglin. *Science* 284: 1534–1537.
- Institute of Laboratory Animal Resources (U.S.) (1996) Guide for the care and use of laboratory animals. [7th ed. Washington, D.C.: National Academy Press. xii, 125 p.
- Jerkic M, Rivas-Elena JV, Prieto M, Carron R, Sanz-Rodriguez F, et al. (2004) Endoglin regulates nitric oxide-dependent vasodilatation. *Faseb J* 18: 609–611.
- Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, et al. (1998) The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett* 434: 83–87.
- Ge AZ, Butcher EC (1994) Cloning and expression of a cDNA encoding mouse endoglin, an endothelial cell TGF-beta ligand. *Gene* 138: 201–206.
- Robledo MM, Ursa MA, Sanchez-Madrid F, Teixido J (1998) Associations between TGF-beta receptors in human bone marrow stromal cells. *Br J Haematol* 102: 804–811.
- Perez-Gomez E, Eleno N, Lopez-Novoa JM, Ramirez JR, Velasco B, et al. (2005) Characterization of murine S-endoglin isoform and its effects on tumor development. *Oncogene* 24: 4450–4461.

39. Letamendia A, Lastres P, Botella LM, Raab U, Langa C, et al. (1998) Role of endoglin in cellular responses to transforming growth factor-beta. A comparative study with betaglycan. *J Biol Chem* 273: 33011–33019.
40. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, et al. (1998) Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *Embo J* 17: 3091–3100.
41. Santibanez JF, Letamendia A, Perez-Barriocanal F, Silvestri C, Saura M, et al. (2007) Endoglin increases eNOS expression by modulating Smad2 protein levels and Smad2-dependent TGF-beta signaling. *J Cell Physiol* 210: 456–468.
42. Korchynskiy O, ten Dijke P (2002) Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* 277: 4883–4891.
43. Jerkic M, Rodriguez-Barbero A, Prieto M, Toporsian M, Pericacho M, et al. (2006) Reduced angiogenic responses in adult Endoglin heterozygous mice. *Cardiovasc Res* 69: 845–854.
44. Jerkic M, Rivas-Elena JV, Santibanez JF, Prieto M, Rodriguez-Barbero A, et al. (2006) Endoglin regulates cyclooxygenase-2 expression and activity. *Circ Res* 99: 248–256.
45. Blanco FJ, Santibanez JF, Guerrero-Estero M, Langa C, Vary CP, et al. (2005) Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-beta receptor complex. *J Cell Physiol* 204: 574–584.
46. David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S (2007) Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* 109: 1953–1961.
47. Koleva RI, Conley BA, Romero D, Riley KS, Marto JA, et al. (2006) Endoglin structure and function: Determinants of endoglin phosphorylation by transforming growth factor-beta receptors. *J Biol Chem* 281: 25110–25123.
48. Pece-Barbara N, Vera S, Kathirkamathamby K, Liebner S, Di Guglielmo GM, et al. (2005) Endoglin null endothelial cells proliferate faster and are more responsive to transforming growth factor beta1 with higher affinity receptors and an activated Alk1 pathway. *J Biol Chem* 280: 27800–27808.
49. Lee NY, Blobe GC (2007) The interaction of endoglin with beta-arrestin2 regulates transforming growth factor-beta-mediated ERK activation and migration in endothelial cells. *J Biol Chem* 282: 21507–21517.
50. Rodriguez-Barbero A, Obreo J, Alvarez-Munoz P, Pandiella A, Bernabeu C, et al. (2006) Endoglin modulation of TGF-beta1-induced collagen synthesis is dependent on ERK1/2 MAPK activation. *Cell Physiol Biochem* 18: 135–142.
51. Scheid MP, Woodgett JR (2003) Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett* 546: 108–112.
52. Vlahos CJ, Matter WF, Hui KY, Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 269: 5241–5248.
53. Collado M, Medema RH, Garcia-Cao I, Dubuisson ML, Barradas M, et al. (2000) Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by p27Kip1. *J Biol Chem* 275: 21960–21968.
54. Kisseleva T, Brenner DA (2008) Mechanisms of fibrogenesis. *Exp Biol Med* (Maywood) 233: 109–122.
55. Lam AP, Gottardi CJ (2011) beta-catenin signaling: a novel mediator of fibrosis and potential therapeutic target. *Curr Opin Rheumatol* 23: 562–567.
56. McAnulty RJ (2007) Fibroblasts and myofibroblasts: their source, function and role in disease. *Int J Biochem Cell Biol* 39: 666–671.
57. Torsney E, Charlton R, Parums D, Collis M, Arthur HM (2002) Inducible expression of human endoglin during inflammation and wound healing in vivo. *Inflamm Res* 51: 464–470.
58. Duwel A, Eleno N, Jerkic M, Arevalo M, Bolanos JP, et al. (2007) Reduced tumor growth and angiogenesis in endoglin-haploinsufficient mice. *Tumour Biol* 28: 1–8.
59. Perez-Gomez E, Villa-Morales M, Santos J, Fernandez-Piqueras J, Gamallo C, et al. (2007) A role for endoglin as a suppressor of malignancy during mouse skin carcinogenesis. *Cancer Res* 67: 10268–10277.
60. Osaki M, Oshimura M, Ito H (2004) PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis* 9: 667–676.
61. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, et al. (2004) PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* 30: 193–204.
62. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2: 489–501.
63. Feutz AC, Barrandon Y, Monard D (2008) Control of thrombin signaling through PI3K is a mechanism underlying plasticity between hair follicle dermal sheath and papilla cells. *J Cell Sci* 121: 1435–1443.
64. Kapoor M, Liu S, Shi-wen X, Huh K, McCann M, et al. (2008) GSK-3beta in mouse fibroblasts controls wound healing and fibrosis through an endothelin-1-dependent mechanism. *J Clin Invest* 118: 3279–3290.
65. Sanz-Rodriguez F, Guerrero-Estero M, Botella LM, Banville D, Vary CP, et al. (2004) Endoglin regulates cytoskeletal organization through binding to ZRP-1, a member of the Lim family of proteins. *J Biol Chem* 279: 32858–32868.
66. Toporsian M, Gros R, Kabir MG, Vera S, Govindaraju K, et al. (2005) A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. *Circ Res* 96: 684–692.
67. Mouta-Bellum C, Kirov A, Miceli-Libby L, Mancini ML, Petrova TV, et al. (2009) Organ-specific lymphangiectasia, arrested lymphatic sprouting, and maturation defects resulting from gene-targeting of the PI3K regulatory isoforms p85alpha, p55alpha, and p50alpha. *Dev Dyn* 238: 2670–2679.
68. Fujita D, Tanabe A, Sekijima T, Soen H, Narahara K, et al. (2010) Role of extracellular signal-regulated kinase and AKT cascades in regulating hypoxia-induced angiogenic factors produced by a trophoblast-derived cell line. *J Endocrinol* 206: 131–140.
69. Shyu KG, Wang BW, Chen WJ, Kuan P, Hung CR (2010) Mechanism of the inhibitory effect of atorvastatin on endoglin expression induced by transforming growth factor-beta1 in cultured cardiac fibroblasts. *Eur J Heart Fail* 12: 219–226.
70. Cudmore MJ, Ahmad S, Sissaoui S, Ramma W, Ma B, et al. (2010) Loss of Akt activity increases circulating soluble endoglin release in preeclampsia: identification of inter-dependency between Akt-1 and heme oxygenase-1. *Eur Heart J* 33: 1150–1158.
71. Subramaniam V, Chakrabarti R, Prud'homme GJ, Jothy S (2010) Tranilast inhibits cell proliferation and migration and promotes apoptosis in murine breast cancer. *Anticancer Drugs* 21: 351–361.
72. Lee NY, Golzio C, Gatzka CE, Sharma A, Katsanis N, et al. (2012) Endoglin Regulates PI3-Kinase/Akt Trafficking and Signaling to Alter Endothelial Capillary Stability During Angiogenesis. *Mol Biol Cell*.