Original Research Article

Genome-wide transcriptional and functional analysis of endoglin isoforms in the human promonocytic cell line U937[†]

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Running head: Endoglin isoforms in myeloid cells

Keywords:
- Monocytes
- Endoglin
- Gene expression
- Integrins
- Activin A
- Cell adhesion

Contract grant sponsor: *Ministerio de Economia y Competitividad* of Spain; **Contract grant numbers:** SAF2010-19222 and SAF2013-43421-R
Contract grant sponsor: Genoma España (MEICA)
Contract grant sponsor: Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Instituto de Salud Carlos III (ISCIII)

[†]This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jcp.24827]

Additional Supporting Information may be found in the online version of this article.

Received 11 February 2014; Revised 22 July 2014; Accepted 5 September 2014
Journal of Cellular Physiology

DOI 10.1002/jcp.24827

ABSTRACT

Endoglin is an auxiliary cell surface receptor for TGF-β family members. Two different alternatively spliced isoforms, long (L)-endoglin and short (S)-endoglin, have been reported. S-endoglin and L-endoglin proteins vary from each other in their cytoplasmic tails that contain 14 and 47 amino acids, respectively. A critical role for endoglin in vascular development has primarily been studied in endothelial cells. In addition, endoglin expression is upregulated during monocyte-to-macrophage differentiation, however, little is known about its role in this myeloid context. To investigate the function of endoglin in monocytes, stable transfectants expressing the two endoglin isoforms in the promonocytic human cell line U937 were generated. The differential gene expression fingerprinting of these endoglin transfectants using DNA microarrays and further bioinformatics analysis showed a clear alteration in essential biological functions, mainly those related to "Cellular Movement", including cell adhesion and transmigration. Interestingly, these cellular functions are highly dependent on adhesion molecules, including integrins α1 (CD49a, *ITGA1* gene), αL (CD11a, *ITGAL* gene), αM (CD11b, *ITGAM* gene) and β2 (CD18, *ITGB2* gene) and the chemokine receptor CCR2 (CD192, *CCR2* gene), which are downregulated in endoglin transfectants. Moreover, activin A (*INHBA* gene), a TGF-β superfamily member involved in macrophage polarization, was distinctly affected in each endoglin transfectant, and may contribute to the regulated expression of integrins. These data were confirmed by quantitative PCR, flow cytometry and functional tests. Taken together, these results provide new insight into endoglin function in monocytes.

INTRODUCTION

Endoglin is a TGF-β co-receptor involved in the regulation of many physiological processes, among which angiogenesis is the best characterised (Lopez-Novoa and Bernabeu, 2010; Mahmoud et al., 2011). The association between endoglin and angiogenesis is so close that the null endoglin mouse model is unviable and dies *in utero* at E10.5 due to severe defects in cardiovascular system development (Mahmoud et al., 2011). In humans, heterozygous mutations in the *endoglin* gene are the underlying cause of the autosomal dominant vascular disorder called hereditary hemorrhagic telangiectasia type 1 (HHT-1 or Osler-Weber-Rendu disease type 1), which is characterised by dilated vessels and arteriovenous malformations that lead to recurrent bleedings and shunting in several internal organs (McDonald et al., 2011; Shovlin, 2010). Nevertheless, endoglin is not exclusively expressed in blood vessels but also in a number of cell types and tissues, including smooth muscle cells, fibroblasts, syncytiotrophoblasts and macrophages where its expression has been poorly studied. In the case of myeloid cells, endoglin is upregulated in activated monocytes in culture (Lastres et al., 1992; O'Connell et al., 1992). Under inflammatory conditions *in vivo*, increased endoglin levels are associated with an inflammatory infiltrate concurrent with the transition to macrophages (Torsney et al., 2002). Also, endoglin induction during embryoid body differentiation in an overexpression-inducible model leads to a considerable increase in haematopoietic progenitors (Baik et al., 2012), and peripheral blood mononuclear cells from HHT-1 patients show a dysfunctional homing (Post et al., 2010). Overall, the role of endoglin in the monocyte-macrophage transition, and in the myeloid lineage in general, is poorly understood.

Most of the functions of endoglin have been focused on its predominantly expressed isoform, L-endoglin (L-Eng), and very little is known about its alternatively spliced isoform, S-endoglin (S-Eng) (Lopez-Novoa and Bernabeu, 2010). Both isoforms differ exclusively in the cytoplasmic long and short region of 47 and 14 residues, respectively, being specific for S-

endoglin only a sequence of 7 residues. This small structural difference explains the lack of appropriate tools (antibodies) to clearly distinguish between L-endoglin and S-endoglin proteins. The common extracellular domain of L-endoglin and S-endoglin is able to bind several TGF-beta family members, including TGF-β1, activin A or BMP9 (Bellon et al., 1993; Barbara et al., 1999; Alt et al., 2012) and it is assumed that the structural differences of their cytoplasmic domain might account for the distinct functional effects of each isoform. Thus, within the TGF-β signaling context of endothelial cells, L-endoglin promotes ALK1-Smad1 route, whereas S-endoglin activates the ALK5-Smad3 pathway (Blanco et al., 2005; 2008; Velasco et al., 2008). Also, the cytoplasmic region of human L-endoglin, but not S-endoglin, acts as a docking site for proteins involved in cytoskeleton organization (Koleva et al., 2006; Sanz-Rodriguez et al; 2004; Conley et al., 2004). L- and S-endoglin arise from an alternative splicing mechanism were the splicing factor SRSF1 (ASF/SF2) plays a regulatory role promoting S-endoglin expression in an intron retention process associated with endothelial senescence (Blanco and Bernabeu, 2011). Thus, additional analyses of S-endoglin are necessary for a better understanding of the role of this alternative isoform out of the endothelial context. In this sense, Lastres et al. (Lastres et al., 1996) demonstrated the regulatory role of both endoglin isoforms in the promonocytic cell line U937. Thus, growth inhibition induced by the TGF-β treatment was only counteracted by the expression of L-endoglin, but both isoforms decreased fibronectin production and cellular adhesion, among other functions.

Leucocyte trafficking from the peripheral blood to an inflammatory focus involves mainly the processes of cellular adhesion and further transmigration through the blood vessel wall. Cellular adhesion basically consists of three sequential steps: rolling, activation and arrest, each characterised and mediated by specific molecules such as selectins, chemokines and integrins, respectively (Ley et al., 2007). In this context, chemokines trigger the activation signal for leucocyte adhesion due to their chemoattractant properties and their ability to

orchestrate a wide array of leucocyte functions during inflammation and immunity (Zlotnik and Yoshie, 2012). Chemokines constitute a large family of small, mostly secreted proteins comprising more than 50 members, which elicit their functions through a set of 20 different transmembrane G-proteincoupled receptors (GPCRs) responsible not only for triggering intracellular signals, but also for contributing to gene expression (Zlotnik and Yoshie, 2012). In addition, chemokines are the most powerful physiological activators of integrin-mediated cellular arrest (Dixit and Simon, 2012). In particular, chemokines can rapidly regulate integrin avidity in a cell-specific manner by increasing both integrin affinity and valency (Laudanna et al., 2002). Thus, integrins are GPCRs-dependent for leucocyte arrest. The most relevant integrins involved in leucocyte arrest belong to the β1 and β2 subfamilies, in association with an α-chain, e.g., α4β1 (VLA-4), α1β1 (VLA-1), αLβ2 (LFA-1), among others.

Activin A is another relevant cytokine belonging to the TGF-β superfamily closely related to the myeloid lineage. It has a central role in innate immunity and is considered nowadays to be a crucial modulator of inflammatory responses due to its proinflammatory and regulatory activities (Ogawa et al., 2006). Indeed, activin A modulates the release of quite a few mediators, such as the early proinflammatory cytokine interleukin-1β (IL1β) (Ohguchi et al., 1998) among others, and the expression of many surface proteins (de Kretser et al., 2012; Hedger et al., 2011). Structurally, mature and circulating activin A is a disulfide-linked homodimer composed of two βA chains encoded by the *INHBA* gene. Activin A elicits its cellular responses mainly through the Smad2/Smad3 pathway, but also through MAP kinase signaling, and is involved in inflammatory pathologies including rheumatoid arthritis and inflammatory bowel disease. Moreover, activin A contributes to the maintenance of pluripotency of embryonic stem cells, and it exerts antitumourigenic effects. Accordingly, the role of activin A in macrophage polarization has recently been reported, where activin A is considered as an M1 cytokine that promotes a pro-inflammatory phenotype and precludes the

acquisition of anti-inflammatory and tumour-associated M2 macrophage markers (Puig-Kroger et al., 2009).

Here, we report that endoglin expression in myeloid cells impacts essential integrin-mediated biological functions, including cell adhesion and transmigration.

MATERIALS AND METHODS

Cell culture

The human promonocytic cell line U937 and its stable transfectants expressing either human L- or S-endoglin and a control Mock condition of cells transfected with an empty expression vector, were described previously (Lastres et al., 1996), and maintained in RPMI 1640 supplemented with 10% heat-inactivated faetal calf serum (FCS), 2 mM L-glutamine, and penicillin (100 U/mL). Pooled clones with a similar endoglin expression index were maintained with 1 mg/mL G418 (Gibco) and used for biochemical and functional characterisations. When required, cells were labelled with 2.5 μg/mL of the fluorescent probe CFSE (Invitrogen) in PBS for 10 minutes at room temperature 24 hours before the respective assay. This labelling process neither alters nor impairs cell viability. To induce differentiation, U937 transfectants were handled under dim lighting conditions and incubated with 100 nM 1α,25-Dihydroxyvitamin-D3 or vehicle (<0.1% ethanol) for 72 hours. Human peripheral blood mononuclear cells were isolated from whole venous blood of HHT-1 patients (n=4) and normal donors (n=4) over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient according to standard procedures. Mononuclear cells were incubated in 6-cm wells of P-6 Falcon plates DMEM (Gibco) supplemented with 10% FCS. After 48 hr, total RNA from adherent macrophages was extracted and subjected to quantitative PCR. The mutations in the *Endoglin* gene of HHT-1 patients are summarized in Supplementary Material Table S1. The human umbilical vein endothelial cells (HUVECs) were cultured in EBM-2 medium plus EGM-2 SingleQuots supplement (Lonza, Walkersville, MD, USA) in

early passages to maintain the endothelial phenotype and avoid cell senescence. Cells were maintained in a NAPCO incubator in a humidified and 5% CO_2 atmosphere at 37°C.

Differential gene expression by DNA microarrays

Total RNA was isolated from U937 transfectants using a hybrid method TRIzol/RNeasy kit (Invitrogen and Qiagen, respectively). Then, a One-Color Microarray-Based Gene Expression Analysis Protocol (Agilent Technologies, Palo Alto, CA, USA) was used to amplify and label RNA. Briefly, 1 μg of total RNA was reverse transcribed using the T7 promoter primer and the Moloney murine leukaemia virus (MMLV) reverse transcriptase (RT). cDNA was then converted to anti-sense RNA (aRNA) by using T7 RNA polymerase to amplify target material and incorporate cyanine 3 (Cy3)-labelled CTP simultaneously.

Samples were hybridised to a Whole Human Genome Microarray 4x44K (G4112F, Agilent Technologies). Thus, 1.65 μg of Cy3-labelled aRNA was hybridised for 17 hours at 65ºC in an Agilent hybridization oven (G2545A, Agilent Technologies) set to 10 rpm in a final concentration of 1x GEx Hybridization Buffer HI-RPM (Agilent Technologies). Arrays were washed and dried out using a centrifuge according to the manufacturer's instructions (One-Color Microarray-Based Gene Expression Analysis, Agilent Technologies). Arrays were scanned at 5-μm resolution on an Agilent DNA Microarray Scanner (G2565BA, Agilent Technologies) using the default settings for 4x44k format one-color arrays. Images provided by the scanner were analysed using Feature Extraction software v9.5.3.1 (Agilent Technologies).

Data were analysed with affylmGUI R software (Wettenhall et al., 2006), and the robust Multi-array Analysis (RMA) algorithm was used for background correction, normalisation and expression level summarisation. Differential expression analysis was performed using limma (Smyth, 2004) included in the affylmGUI package. P-values were corrected for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Functional, network and pathway analyses were conducted with Ingenuity Pathway Analysis software (Ingenuity Systems©, www.ingenuity.com).

Nucleofection

Nucleofections with silencing vectors were performed following the manufacturer's instructions with Lonza nucleofector kits for U937 cells (VCA-1004) and HUVECs (VPB-1002), using the Nucleofector I (Amaxa, Germany). Silencing experiments were carried out by simultaneously nucleofecting two endoglin siRNAs (s4677 and s4679), using scrambled siRNAs (AM4611 and AM4613) as a negative control (Ambion, Life Technology). Cells were centrifuged at 1,200 rpm for 10 min and resuspended in nucleofector solution to a final concentration of $1x10^6$/100 µL. Then, 100 µL cell suspension was combined with 100 nM siRNA, transferred to the cuvette, and nucleofected using the W-01 (U937) or A-34 (HUVECs) program. Then, cells were seeded out in 12-well plates containing prewarmed medium. After 48 hours incubation at 37ºC and 5% of CO_2, cells were analyzed by flow cytometry or quantitative RT-PCR.

Quantitative PCR

Total RNA was isolated as described previously and 1 µg was retrotranscribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Then, a qPCR was set up using the specific primers designed by the ProbeFinder website (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp, Roche Applied Science) for each target gene (Supplementary material Table S2). Reactions were carried out using the SYBR Green reagent in an iQ5 system (Bio-Rad).

Flow cytometry

Cells were collected and washed in PBS by soft centrifugation at 160xg. Then, cells were blocked with 2% human AB^+ serum at 4ºC. Expression of surface markers was determined using the mAbs P4A4 (anti-Endoglin), TS2/7 (anti-α1), TS1/11 (anti-αL), TS1/18 (anti-β2), Bear1 (anti-αM) and OX63 (IgG-control) for 30 min, washed, and then incubated with a

1:500 dilution of Alexa Fluor 488-anti-mouse IgG (Molecular Probes, Eugene, OR, USA) for an additional 30 min. Samples were washed and resuspended in PBS with propidium iodide and analysed in a FC500 Beckman Coulter flow cytometer.

Cellular adhesion to immobilised ligand and endothelial monolayer

U937 stable transfectants were CFSE-labelled as described above. Then, $5x10^4$ cells in 100 µL were added to 96-well pre-coated plates with either human collagen type 1 or the chimeric protein ICAM-1/Fc, a generous gift from Dr Carlos Cabañas (Centro de Biología Molecular Severo Ochoa, CSIC, Madrid). Plates were incubated at 37ºC for the indicated time-lapses and rinsed three times with PBS to remove unbound cells.

To assay the adhesion properties of U937 transfectants to an endothelial monolayer, HUVECs were cultured on gelatin pre-coated 12-well dishes and allowed to reach confluency. Then, CFSE-labelled U937 transfectants were added at 10^5 cells/well for and incubated for 1 hour. When required, the HUVEC monolayer was activated with TNFα (10 ng/mL) 24 hours before the assay. Alternatively, the HUVEC monolayer was likewise activated by means of scratching with a yellow tip and then allowed to heal for 5 hours. In parallel, U937 transfectants were treated or not with 200 µM $MnCl_2$ for 30 minutes before the adhesion assays, as indicated.

After the adhesion process, wells were rinsed with PBS and adherent cells were lysed with the appropriate volume of passive lysis buffer, PLB (25 mM Tris-Phosphate pH 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, and 1% Triton X-100). The fluorescence was measured using excitation/emission wavelengths of 492/517 nm, respectively, in a Varioskan Flash spectral scanning multimode reader (Thermo Scientific).

Chemotaxis and transmigration assays

Chemotaxis assays were carried out in 6.5-mm diameter/8.0-µm pore size 24-well transwells (Costar, Corning, NY, USA). Control Mock, L-Eng and S-Eng U937 transfectants were CFSE-labelled and serum-starved 24 hours before the assay. Then, $5x10^4$ cells in 100 µL

were loaded into the upper chamber and chemotaxis was carried out for 3 hours at 37ºC to the lower chamber with or without 100 ng/mL recombinant human MCP-1 (CCL2). As a positive control, recombinant SDF-1α (CXCL12) was assayed at 100 ng/mL.

For transmigration assays through an endothelial monolayer, $5x10^4$ HUVECs were seeded onto a gelatin pre-coated upper chamber and allowed to form a complete monolayer on the porous membrane of 6.5-mm diameter/5.0-μm pore size 24-well transwells (Costar). U937 transfectants were CFSE-labelled and assayed by adding or not recombinant human MCP-1 to the lower chamber as a chemoattractant at 100 ng/mL for 4 hours. Viable migrated cells in the lower chambers were collected, washed with PBS and counted in the flow cytometer by analysing each sample in the same predetermined time and flow conditions.

Gene reporter assays

To analyse the CCR2 signalling pathway, an indirect approach was established based on its negative crosstalk with the TNFα/NF-κB pathway (Jura et al., 2012). Thus, the pKBF-Luc reporter construct bearing three tandem NF-κB-responsive elements was nucleofected in U937 transfectants using the Amaxa Cell Line Nucleofector Kit® C (Lonza) following the manufacturer's protocol. Twenty-four hours post-nucleofection, TNFα (10 ng/mL) and MCP-1 were added either separately or in combination for an additional 24 hours.

The Smad-responsive promoter construct $p(CAGA)_{12}$-Luc was provided by Dr. Peter ten Dijke (Dennler. et al., 1998). In this vector, the Smad binding CAGA boxes confer TGF-β and activin stimulation to a heterologous promoter that drives the expression of luciferase as a reporter. The biological activity of activin A present in culture supernatants was tested by transfecting Mv1Lu cells, a well-established cellular model used to study signalling in the TGF-β superfamily (Puig-Kroger et al., 2009), with the $p(CAGA)_{12}$-Luc reporter using Lipofectamine 2000 (Invitrogen). After transfections, cells were treated with undiluted conditioned medium from U937 transfectants for 24 hours. When required, cells were preincubated for 30 minutes with 10 μM SB431542 (Sigma-Aldrich) before treatment.

Likewise, a blocking monoclonal antibody was added to the culture supernatants at 0.1 μg/mL to neutralize activin A activity (Clone 69403, R&D Systems).

The luciferase-based reporter constructs of the integrin α1 proximal promoter pCD11A-Luc (-1360/+83) and pCD11A170-Luc (-170/+83) were kindly provided by Dr. Angel Corbí (Centro de Investigaciones Biologicas, Madrid, Spain) and previously described (Nueda et al., 1993). These constructs were co-transfected with L-Eng, S-Eng or empty vector in Mv1Lu cells and then treated or not with 50 ng/mL HumanKineTM Activin A (Miltenyi Biotec, Auburn, CA, USA) for 24 hours.

Samples were co-transfected with the pSV40/β-Gal expression plasmid as an internal control to correct for transfection efficiency. Measurement of β-galactosidase activity was performed using the Galacto-Ligth kit (Tropix), according to the manufacturer's protocol. The experiments were performed in triplicate at least three times and measured in a GloMax Microplate Luminometer (Promega). Representative experiments are shown in the figures.

Activin A quantification by ELISA

To determine the concentrations of human activin A in conditioned culture supernatants from U937 transfectants, a Quantikine Human Activin A kit (R&D Systems) was used following the manufacturer's instructions. All immunoassays were read at 450 nm and background corrected at 540 nm in a Varioskan Flash spectral scanning multimode reader (Thermo Scientific).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) of the data obtained from representative experiments performed in triplicate and repeated at least three times, unless otherwise stated. Groups were compared using the paired Student's t-test. Significant differences are indicated as $*p<0.05$ and $**p<0.01$ (ns, non-significant).

RESULTS

Endoglin-induced differentially expressed genes

To determine the effect of endoglin on the gene signature in the myeloid lineage, previously well characterised U937 cells stably transfected with endoglin isoforms, termed Mock (control), L-Eng and S-Eng, were used (Lastres et al., 1996). Both endoglin transfectants expressed similar mRNA and protein levels of L-endoglin and S-endoglin as revealed by RT-PCR, western blot and immunofluorescence flow cytometry analyses (Supplementary Figure S1). To compare their overall expression patterns, four cultures of each cell transfectant were used as RNA probes in a whole-genome expression microarray hybridization. After statistical analysis comparing each condition versus the control, we considered differentially expressed genes to those with a $\log_2$ fold change (FC) of greater than 1 and false discovery rate (FDR) of less than 0.05. As an expected internal control, *ENG* was one of the most highly induced genes. Thus, 158 and 152 genes were upregulated in L-Eng and S-Eng, respectively, of which 66 genes were in common. On the other hand, 295 and 222 genes were downregulated in L-Eng and S-Eng, respectively, with 137 common in both transfectants (Fig. 1A; representative genes are shown in Figure 1B; see Supplementary material Table S3 for the complete list). In addition, an interesting group was generated including those genes with an opposite behaviour regarding endoglin isoform expression (Fig. 1B, black arrows). For example, the inhibin βA chain gene (*INHBA*) that forms the activin A homodimer is highly induced in L-Eng transfectants, while it is repressed in S-Eng transfectants. Conversely, the interleukin 1β gene (*IL1B*) is downregulated in L-Eng cells but is increased in S-Eng cells.

Ingenuity pathways analysis (IPA) of the microarray data grouped the differentially expressed genes according to biological function (Table 1). Thus, we focussed on particular genes in common to the top functions of the list. This gene set was characterised by a high proportion of downregulated genes, from which integrins constituted an important part. This fact prompted us to explore leukocyte recruitment to an inflammatory focus as the scenario

where endoglin may play a critical role in affecting chemotaxis, adhesion and transmigration. Therefore, we first validated some representative genes involved in these cellular processes by qPCR, effectively corroborating the regulation level observed in the microarray assay (Fig. 2). Next, some of the genes were validated by functional experiments.

Impairment of CCR2-dependent cell chemotaxis and MCP1 signalling

One of the genes affected by the overexpression of endoglin isoforms was *CCR2*, which encodes the receptor for the chemokine CCL2, or MCP-1. It was markedly repressed in both endoglin transfectants, but the strongest effect was observed in the L-Eng cells (Fig. 2). Because technical approaches addressed to validate CCR2 at the protein level failed, we set up several functional experiments to confirm this result using the properties of its ligand MCP-1. Thus, we assayed the chemotaxis of U937 transfectants in Boyden chamber-based transwells. The endoglin overexpression clearly inhibited the percentage of migrated cells in response to MCP-1 present in the lower chamber (Fig. 3A). As a chemoattractant positive control, both L-Eng and S-Eng U937 cells maintained similar transmigration abilities as control cells in response to the chemokine SDF-1α, demonstrating the specificity for the impaired CCR2/MCP-1-mediated chemoattraction. Moreover, endoglin transfectants significantly failed in their transmigration properties when an endothelial monolayer was grown on the porous membrane between the transwell chambers (Fig. 3B).

Another approach to functionally validate CCR2 repression in U937 transfectants was to analyse the negative crosstalk described between the MCP-1-induced protein-1 (MCPIP encoded by the *ZC3H12A* gene) and the TNFα/NFκB pathway (Jura et al., 2012; Liang et al., 2010). According to the microarray data, *ZC3H12A* expression was not affected by endoglin isoform expression (log_2FCM/L-Eng: 0.33, FDR: 0.02; log_2FCM/S-Eng: 0.09, FDR: 0.61) and this was verified by qPCR (Fig. 3C). Interestingly, *ZC3H12A* expression was increased upon MCP-1 treatment in control cells and this increase was partially inhibited in endoglin

transfectants compared to Mock cells (Fig. 3C). Next, we observed that TNFα treatment induced the transcriptional activity of the NFκB reporter pKBF-Luc vector, which was inhibited by the MCP-1 stimulus in mock cells. However, this inhibition was lower and statistically significant in both L-Eng and S-Eng U937 transfectants (Fig. 3D).

Endoglin isoforms inhibit integrin expression

Since endoglin transfectants showed a repression in integrin mRNA expression, we wanted to assess integrin protein expression and functionality in Mock versus endoglin transfectants. First, immunofluorescence flow cytometry analysis showed a decrease in the expression levels of integrins α1 (CD49a, *ITGA1* gene), β2 (CD18, *ITGB2* gene), αL (CD11a, *ITGAL* gene) and αM (CD11b, *ITGAM* gene) of endoglin transfectants compared to mock cells. As a control, CD14 and HLA-I were not affected by either L-Eng or S-Eng expression, in agreement with the microarray data (Fig. 4A).

In order to assess whether the endoglin-induced effects on integrin expression were reversed upon endoglin suppression, endoglin U937 transfectants were nucleofected with siRNA specific for endoglin. Endoglin suppression reversed most of the integrins´ downregulation (*ITGA1*, *ITGB2*, *ITGAL*, *ITGAM*), compared to the negative control with scrambled siRNA (Fig. 4B). The unresponsiveness of *IGAL* gene to endoglin suppression in L-Eng cells is likely due to methodological issues, including the degree of endoglin supression or the fact that these are transient transfectants, as compared to the endoglin stable transfectants. A further proof for the link between endoglin and integrins´ expression was obtained using blood-derived macrophages from patients with HHT-1, a disease characterized by endoglin haploinsufficiency. HHT-1 macrophages showed higher transcript levels of *ITGA1* and *ITGAL* genes than cells from control subjects, whereas *ITGB2* and *ITGAM* genes were not significantly altered (Fig. 4C). Moreover, endoglin suppression in human umbilical vein endothelial cells (HUVECs) led to an increase in the transcript levels of *ITGA1*, *ITGAL*, and

ITGB2 respect to HUVECs transfected with scrambled siRNA (Fig. 4D). Together these results support the link between endoglin and integrins´ expression.

Endoglin isoforms reduce integrin-mediated cell adhesion

Next, we assessed the functional impact of the altered integrin protein expression in endoglin U937 transfectants. On one hand, integrin α1 heterodimerises with the β1 chain to form the very late activation antigen VLA-1, a well-characterised collagen receptor. Therefore, an adhesion assay using a collagen type 1-coated surface was carried out. We found that U937 transfectants were able to bind to collagen at different time points (assayed from 5 to 60 minutes), but both L-Eng and S-Eng transfectants showed a significant reduction in their adhesion abilities in comparison with mock cells (Fig. 4B). Similar results were obtained in cell adhesion studies to gelatin; also, as a negative control, cellular adhesion to albumin (BSA) showed background levels (data not shown to simplify the graph).

On the other hand, integrin β2 may heterodimerise with several integrins including αL and αM. Thus, the association αLβ2 forms the leucocyte function-associated antigen LFA-1, which acts mainly as the receptor for the adhesion molecule ICAM-1. In agreement with the reduced protein expression of LFA-1, a significant inhibition in the adhesion ability of both endoglin transfectants to the immobilised chimeric version of the ligand ICAM-1/Fc was observed (Fig. 4C).

The αMβ2 heterodimer is also known as the macrophage antigen Mac-1 and is considered a maturation marker, among other features. The decreased protein levels of αM (CD11b) in both L-Eng and S-Eng U937 transfectants (Fig. 4A) suggests an earlier differentiation stage versus mock cells. Supporting this view, treatment of U937 transfectants with 1α,25-Dihydroxyvitamin D3 ($1,25(OH)_2$-D3), a well known differentiating agent of myeloid cells, leads to a marked increased cell adhesion to plastic, a hallmark of macrophage differentiation, in mock transfectants, whereas this enhanced adhesion was much more

reduced in both endoglin transfectants (Fig. 5A). It is worth noting that based on our microarray data and selection, the expression level of the vitamin D receptor, VDR, is not affected by endoglin isoform overexpression (log_2FCL-Eng/M: 0.28, FDR: 0.02; log_2FCS-Eng/M: 0.25, FDR: 0.07). The vitamin D-induced differentiation of U937 cells was confirmed by the upregulated expression levels of the classical differentiation marker integrin αM (CD11b) as shown in Figure 5B. Interestingly, we observed that after 72 hours of vitamin D treatment, the levels of the differentiation markers CD14 and CD11b in both endoglin transfectants were lower than those in mock cells (Fig. 5C), suggesting again an earlier differentiation stage of endoglin cells versus control cells.

Endoglin isoforms inhibit cell adhesion to endothelial cells

The cell adhesion properties of endoglin transfectants to an endothelial monolayer were analysed. In agreement with the lower adhesion ability of these endoglin transfectants to immobilised ligands (Fig. 4), we found that the percentage of U937 cells bound to a resting monolayer of HUVECs was reduced by endoglin isoform overexpression in comparison with control Mock cells (Fig. 6A). Moreover, activation of the HUVEC monolayer with TNFα resulted in a marked increased adhesion of Mock U937 transfectants, whereas this effect was much more reduced in both L-Eng as S-Eng cells (Fig. 6A). In parallel, similar results were obtained after activation by scratching of the endothelial monolayer, thus reproducing a wound healing assay. Indeed, a clear increase in the number of Mock transfectant cells bound to the wound boundary were found, this effect being much more reduced in endoglin transfectants (Fig. 6A).

To further corroborate the involvement of integrins in the adhesion to the HUVEC monolayer, U937 transfectants were pre-treated with $MnCl_2$ to allow integrin activation by Mn^{2+} ions. Interestingly, we found that Mn^{2+} ions added to the normal medium do not affect significantly *per se* cell adhesion. However, a clear Mn^{2+}-dependent increase in cell adhesion,

after activation of the HUVEC monolayer with TNFα, was found. This enhancing effect was reduced significantly in endoglin U937 transfectants (Fig. 6B).

Differential regulation of Activin A in L-endoglin *versus* S-endoglin U937 cells

The *INHBA* gene, which encodes the inhibin βA chain, is known to play a critical role in monocyte-to-macrophage differentiation and polarization, two key processes in inflammation resolution. As shown in Figure 2, an opposite regulation of the *INHBA* gene between S-Eng versus L-Eng cells was evidenced at the mRNA level by qPCR. Moreover, the activin A concentration in conditioned culture supernatants from all three U937 transfectants was determined with a commercial ELISA assay. Activin-A levels were more than 2-fold higher in L-Eng transfectants versus Mock cells, whereas in S-Eng cells activin-A levels showed a 9.4- or 4.1-fold reduction versus L-Eng or Mock cells, respectively (Fig. 7A). Of note, this activin A was biologically active because culture supernatants from L-Eng transfectants were able to induce significantly the transcriptional activity of the specific Smad3 reporter $p(CAGA)_{12}$-Luc, which was abolished by the ALK4, 5 and 7 inhibitor SB431542 and significantly inhibited by an anti-activin A blocking antibody (Fig. 7B).

Next, we wanted to assess the role of activin A on integrin expression, since downregulation of integrins by activin-A was previously reported in other cellular types including trophoblasts and fibroblasts, among others (Carracedo et al., 2010; de Kretser et al., 2012; Stoikos et al., 2010). Thus, we found that treatment of parental U937 cells with activin A resulted in a statistically significant repression of integrins α1, αL and αM, but not β2. These results were confirmed not only by qPCR but also by flow cytometry at the protein level (Fig. 7C-D). Interestingly, activin-A induced the expression of endoglin, in agreement with the TGF-β1-dependent upregulation of endoglin observed in human U937 cells and primary macrophages (Lastres et al., 1996). To assess a likely transcriptional regulation, we assayed two artificial reporter vectors bearing sequences from the integrin αL proximal promoter.

These vectors were also transiently co-transfected with L-Eng, S-Eng or an empty vector and then treated or not with activin A. In all cases, activin-A repressed the activity of both integrin reporter vectors (Fig. 7E), suggesting a transcriptional regulation of integrins by activin-A. Because activin-A levels are increased in L-endoglin transfectants, these results suggest that activin-A might contribute to the downregulation of integrins in these L-endoglin cells.

DISCUSSION

Most studies of endoglin have been focussed on the endothelial context, where its major isoform (L-Eng) plays a key role as a pro-angiogenic molecule, whilst the minor one (S-Eng) is associated with endothelial senescence (Blanco et al., 2008). Nonetheless, endoglin is also expressed in early haematopoietic precursors in bone marrow (Baik et al., 2012) and is progressively repressed during the myeloid lineage differentiation process, so mature monocytes express very low levels of endoglin (Lastres et al., 1992). However, endoglin expression can be induced in response to a proinflammatory focus in activated monocytes and can be easily detected in tissue macrophages, as well (Bellon et al., 1993; Lastres et al., 1992; O'Connell et al., 1992).

Thus, analysis of endoglin isoforms in the myeloid lineage contributes to a better understanding of the gene expression changes implicated in this process. We have set up stable transfectants in pro-monocytic cells U937, termed control Mock, L-Eng and S-Eng, and then carried out a DNA microarray analysis in order to obtain the gene fingerprint influenced by endoglin isoforms. The results showed that most of the genes modulated by endoglin isoforms affect the cellular motility function and particularly processes such as chemotaxis, adhesion and transmigration, which are three basic steps in the recruitment of leucocytes to an inflammatory focus. These processes have been characterised in the present

work by the functional analysis of the chemokine receptor CCR2 (*CCR2*), a pool of integrins (α1, *ITGA1*; αL, *ITGAL*; αM, *ITGAM*; and β2, *ITGB2*) and a member of the TGF-β superfamily activin A (*INHBA*).

Our data demonstrate that endoglin expression strongly represses CCR2, clearly decreasing the chemotaxis response to MCP-1 in the U937 transfectants. By contrast, the response to other chemokines is not affected because the gene expression of their corresponding receptors does not change in the presence of ectopic endoglin (see Supplementary material Table S3). Nonetheless, endoglin isoform overexpression leads to downregulation of other chemokines such as CCL3 and CXCL10, among others, suggesting a failure in the recruitment of other leucocytes to the inflammatory focus. On the other hand, MCP-1/CCR2 signalling is also reduced in endoglin transfectants. Indeed, the expression of the recently discovered MCP-1-induced protein (MCPIP, encoded by the *ZC3H12A* gene) by MCP-1 treatment was partially inhibited in endoglin transfectants. Thus, low MCPIP levels ameliorate NFκB activation during the control of the inflammatory response due to a low MCPIP deubiquitinase activity (Kolattukudy and Niu, 2012; Liang et al., 2010). These results are compatible with those previously reported describing CCR2 downregulation in monocyte-derived macrophages cultured *in vitro* (Kaufmann et al., 2001), in which endoglin increases as well (Lastres et al., 1992).

Integrins constitute a superfamily of heterodimeric glycoproteins involved in cell-cell and cell-matrix adhesion. Consistent with this, we have demonstrated that endoglin overexpression in U937 cells clearly represses integrin levels at the cell membrane, which results in a reduced binding of endoglin transfectants to ligands, such as collagen type 1 and ICAM-1. Moreover, adhesion to endothelial monolayers is also compromised. It is well known that the loss of adhesion molecules associated with the dedifferentiation process is a general feature of most leukaemias (Herter and Zarbock, 2013). Indeed, many therapies are focussed on avoiding this phenomenon by inducing a new differentiation process. Thus,

vitamin D derivates and analogs are promising molecules currently under study for the treatment of myeloid leukaemia (Kim et al., 2012). According to our results, endoglin isoform overexpression in U937 cells produces a certain backwards step in the differentiation stage denoted by integrin downregulation, especially for αM integrin (CD11b). In this work, we demonstrated that treatment with 1,25$(OH)_2$-D3 for 3 days markedly differentiated U937 control cells as evidenced by a high rate of adherent cells to the culture plastic and the induction of cell surface αM integrin. In parallel, 1,25$(OH)_2$-D3 treatment was also associated with increased αM integrin expression in endoglin transfectants but to a lesser extent than that for control cells. In addition, endoglin isoform expression partially decreases CD14 levels after 1,25$(OH)_2$-D3 treatment, confirming that ectopic endoglin overexpression not only reversed monocyte development but also precluded the further differentiation to a macrophage. Indeed, it is well known that endoglin is upregulated in many types of leukaemia (Catchpoole et al., 2007; Chakhachiro et al., 2013).

The downregulation of several integrin family members in L-Eng and S-Eng U937 transfectants suggests a regulatory mechanism dependent on the extracellular domain, which is shared by both endoglin isoforms. However, an additional regulation of the integrin expression mediated by cytokines secreted by U937 cells is not excluded. One of the key regulatory molecules for the myeloid lineage is activin A, which particularly regulates macrophage polarization to either a pro-inflammatory M1 phenotype (classical activation) or an anti-inflammatory M2 state (alternative activation). Our results show that L-Eng transfectants upregulate the *INHBA* gene ~30-fold with respect to the control Mock, thus, we assume that the βA chain associates preferentially with itself in homodimers to form activin A molecules. We do not disregard the formation of other members such as activin AB (βA/βB chains heterodimer) or inhibin A (α/βA chains heterodimer), which eventually might represent a minor proportion of the total activin molecules. Interestingly, we found that activin A represses most of the integrins, as revealed by the microarray data. This is

consistent with previous results showing that activin A modulates the expression of some integrins in other cell types. For example, activin A represses via Smad2 several integrins including α1, α2, α3, α5, β1, β2, β4 and αvβ5 as well as cell binding to their respective ligands in trophoblast HTR8 cells (Stoikos et al., 2010). On the other hand, activin A modulates α11 integrin via Smad3 in mouse embryonic fibroblasts and regulates myofibroblast differentiation (Carracedo et al., 2010). Hence, these results and our data are consistent because endoglin (L-Eng) promotes Smad2 signalling pathway (Santibanez et al., 2007), but inhibits Smad3 signalling (Blanco et al., 2005). Moreover, since activin-A levels are increased in L-Eng transfectants, these results suggest that activin-A might contribute to the downregulation of integrins in these L-endoglin cells. By contrast, the activin A repression observed in S-Eng transfectants points to a different regulatory mechanism for integrin expression and activity. In this regard, activin A has been shown to induce the expression of IL-1β (Wang et al., 2009), a pro-inflammatory cytokine that increases the expression of integrins and their ligands, suggesting a potential IL-1β-mediated regulation in S-Eng cells.

Based on all these results, we propose that an increase in endoglin expression compromises the initial monocyte recruitment mediated by MCP-1/CCR2, the cellular adhesion to the endothelial monolayer and the subsequent cell positioning in the tissue. Thus, the inside-out signalling triggered by MCP-1 chemokines would be unable to increase the avidity of LFA-1 and other integrins, resulting in a weak attachment to their ligands. Indeed, this hypothesis is consistent with previous reports where MCP-1 is a key chemokine for tissue repair and reendothelization (Niu and Kolattukudy, 2009; Willenborg et al., 2012). In addition, these events would be modulated, in turn, by the release of activin A by the endoglin-expressing monocytes, contributing to a further loss of integrin expression. Because endoglin haploinsufficiency is the basis for the pathogenesis of HHT (Lopez-Novoa and Bernabeu, 2010; Shovlin, 2010) and endoglin is involved in blood cell-mediated vascular repair (van

Laake et al., 2006), it will be of interest to investigate whether these endoglin-modulated functions of monocytic cells may contribute to the generation of the vascular lesions in HHT patients.

In conclusion, we have investigated for the first time the gene expression fingerprinting specifically associated with two endoglin isoforms (L-endoglin and S-endoglin) in human monocytic cells. We have demonstrated that endoglin expression in promonocytic U937 cells affects drastically to essential biological functions, especially those related with cellular movement, including cell adhesion and transmigration. Accordingly, i) endoglin reduces the chemoattractant properties of U937 cells in response to MCP-1 by downregulating its receptor CCR2; and ii) diminishes cell adhesion by repressing an important set of integrins in a process mediated, at least, by the induction and release of activin A. Interestingly, some of these functions were differentially modulated by L-endoglin or S-endoglin. Taken together, these results provide new insights into endoglin function in monocytes.

ACKNOWLEDGEMENTS

We thank Drs. Miguel A. Vega and Angel Corbí (Centro de Investigaciones Biológicas, CSIC, Madrid) for valuable comments and contributions at early stages of this work, Dr. Carlos Cabañas (Centro de Biología Molecular Severo Ochoa CSIC-UAM, Madrid) for providing ICAM-1 recombinant protein, and Dr. Pedro Lastres for assessment with flow cytometry. This study has been supported by grants from *Ministerio de Economia y Competitividad* of Spain (SAF2010-19222 to CB), Genoma España (MEICA to CB), and *Centro de Investigación Biomédica en Red de Enfermedades Raras* (CIBERER, CB). FJB is a scientific researcher from the CIBERER, an initiative of the *Instituto de Salud Carlos III* (ISCIII) of Spain supported by FEDER funds.

Competing interests

The authors declare that they have no competing interests

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FIGURE AND TABLE LEGENDS

Figure 1. Differentially expressed genes.

A: Venn diagram showing the up- and down-regulated genes due to endoglin isoform expression in U937 cells. A total of 66 upregulated and 137 downregulated genes are common to both L-endoglin and S-endoglin transfectants. **B:** Representative group of differentially expressed genes. Particular genes with an opposite regulation in L-endoglin *versus* S-endoglin cells are indicated by arrows.

Figure 2. Validation of microarray results by qPCR.

Genes were selected from the candidates involved in "Cellular movement" function according to IPA analysis. *ENG*, endoglin; *INHBA*, inhibin βA chain; *IL1B*, interleukin 1β; *CCR2*, chemokine (C-C motif) receptor 2, *CXCL10*, chemokine (C-X-C motif) ligand 10, *ITGA1*, integrin α1; *ITGAL*, integrin αL; *ITGAM*, integrin αM; *ITGB2*, integrin β2; *COL1A1*, type I collagen α1 chain; *PPARG*, peroxisome proliferator-activated receptor γ; *AZU1*, azurocidin 1; *CAMP*, cathelicidin antimicrobial peptide; *HP*, haptoglobin; *CTSL2*, cathepsin L2.

Figure 3. Analysis of CCR2 downregulation.

A: Chemotaxis assay to MCP-1 (CCL2) in nude transwells. SDF-1α (CXCL12) was assayed as a chemoattractant positive control. **B:** Transmigration ability to MCP-1 through an endothelial monolayer of HUVECs. **C:** Quantitative PCR to monitor the upregulation of MCP-1-induced protein (MCPIP, *ZC3H12A* gene) in response to MCP-1 treatment. **D:** Luciferase reporter assay to analyse the crosstalk between the MCP-1/CCR2 and TNFα/NFκB pathways in U937 tranfectants.

Figure 4. Effect of endoglin isoforms on the integrin-mediated adhesion properties of U937 cells.

A: Differential expression of integrins in U937 transfectants was validated by flow cytometry (Fully grey, control Mock; continuous line, L-Eng; dotted line, S-Eng). **B**: Suppression of

endoglin by nucleofection with siRNA in L-Eng and S-Eng transfectants counteracts the downregulation of integrins. After nucleofection, the cell surface expression of integrins and endoglin was measured using immunofluorescence flow cytometry. The fold induction respect to cells nucleofected with scrambled siRNA (siSC) is indicated. **C**: Effect of endoglin haploinsufficiency on the expression of integrins. Transcript levels of integrins from HHT-1 blood derived macrophages (n=4) were compared to those of macrophages from control subjects (n=4), as revealed by quantitative RT-PCR. **D:** Effect of endoglin silencing on the expression of integrins in endothelial cells. HUVECs were nucleofected with endoglin siRNA and transcript levels were measured by quantitative RT-PCR. The fold induction of each integrin is represented relative to the corresponding values of HUVECs nucleofected with scrambled siRNA (siSC). **E:** Adhesion of endoglin transfectants to surface-coated collagen type 1. **F:** Adhesion of endoglin transfectants to immobilised ICAM1-Fc. Continuous line, control Mock; dashed line, L-Eng; dotted line, S-Eng.

Figure 5. Differentiation induction with 1,25(OH)2-D3.

A: Adhesion to the substrate of U937 transfectants upon differentiation with $1,25(OH)_2$-D3 treatment. **B:** The expression of αM as a differentiation marker was analysed by flow cytometry in untreated (Control) and Vitamin D treated cells. **C:** The comparative expression of αM and CD14 as differentiation markers in the three types of transfectants was analysed by flow cytometry in cells treated $1,25(OH)_2$-D3. As an internal control, endoglin expression was also analysed. Fully grey, control Mock; continuous line, L-Eng; dotted line, S-Eng.

F**igure 6. Binding of U937 transfectants to an endothelial monolayer.**

A: Confluent HUVECs were assayed in resting conditions and after activation by either TNFα treatment or scratching the cell monolayer (wound healing-WH, from the dashed to the dotted line). The graph on the left represents the quantification of total bound cells. **B:** The integrin contribution to the adhesion ability of U937 transfectants to HUVEC was corroborated in the presence of $MnCl_2$.

Figure 7. Role of activin A in U937 cells.

A: Activin A was quantified in culture supernatants of U937 transfectants by ELISA. **B:** The biological activity of activin A released into the culture medium was assayed by analysing the transcriptional activity of the Smad3-responsive p(CAGA)12-Luc reporter in the presence or in the absence of the ALK4/ALK5/ALK7 inhibitor SB431542 or a blocking monoclonal antibody against activin A. **C, D:** The expression level of integrin α1 (*ITGA1*), αL (*ITGAL*) and αM (*ITGAM*) decreased by the activin A treatment not only at the mRNA level (**C**, quantitative PCR) but also at the membrane protein level (**D**, flow cytometry). By contrast, no significant differences were observed for integrin β2 (*ITGB2*). **E:** Activin A partially inhibits the transcriptional activity of the integrin αL (CD11a) proximal promoter in parental U937 cells transiently transfected with endoglin isoforms.

SUPPLEMENTARY MATERIAL

Figure S1. Endoglin expression analysis of U937 cell transfectants.

Table S1. Genetic description of HHT-1 patients.

Table S2. Sequences and features of the primers used for qPCR.

Table S3. List of all differentially expressed genes affected by L-endoglin or S-endoglin isoforms (FC>1, FDR<0.05).

Table 1. Biological functions mainly affected by endoglin isoforms expression in U937 cells.

The top 5 Biological Functions are shown based on the number of affected genes and their p-value respect to Mock control transfectants.

Biological Function (top 5)	Mock vs. L-Eng			Mock vs. S-Eng		
	# Genes	p-value		# Genes	p-value	
		min.	max.		min.	max.
Cellular Movement	93	$2.4x10^{-7}$	$2.4 x10^{-2}$	82	$1.5 x10^{-8}$	$8.7 x10^{-3}$
Cell-To-Cell Signaling and Interaction	92	10^{-5}	$2.4 x10^{-2}$	78	$9.2 x10^{-8}$	$8x10^{-3}$
Hematological System Development and Function	84	$1.9x10^{-5}$	$2.4 x10^{-2}$	96	$9.2 x10^{-8}$	$8.7 x10^{-3}$
Inflammatory Response	73	$7.2x10^{-6}$	$2.4 x10^{-2}$	86	$1.2 x10^{-11}$	$8.7 x10^{-3}$
Immune Cell Trafficking	61	$1.9x10^{-5}$	$2.4 x10^{-2}$	61	$9.2 x10^{-8}$	$8.7 x10^{-3}$

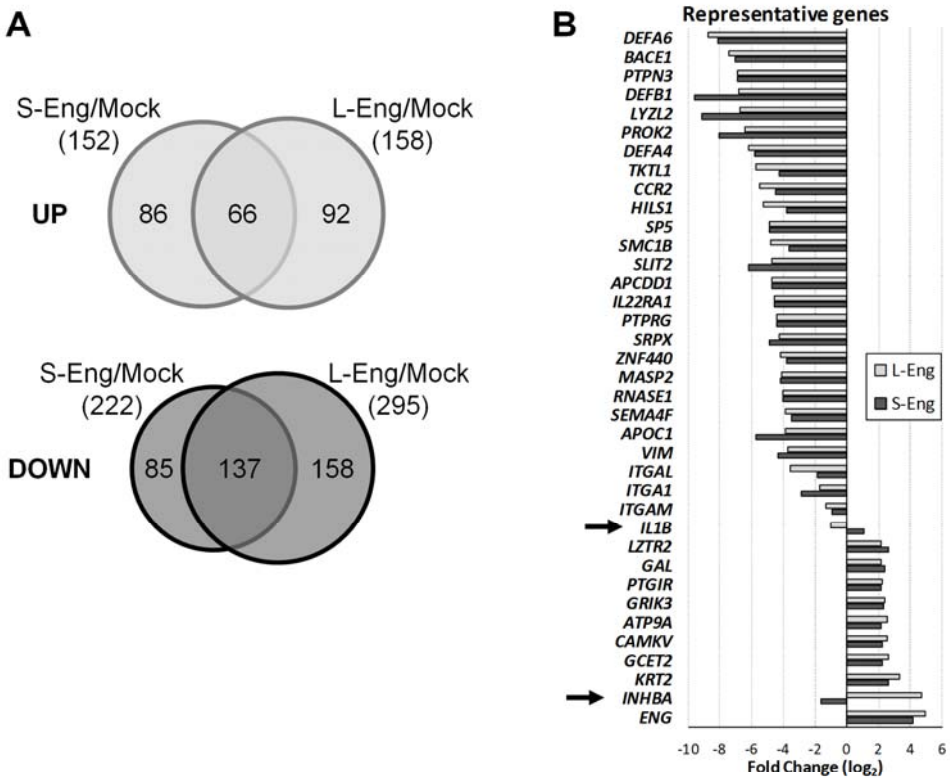


Figure 1.

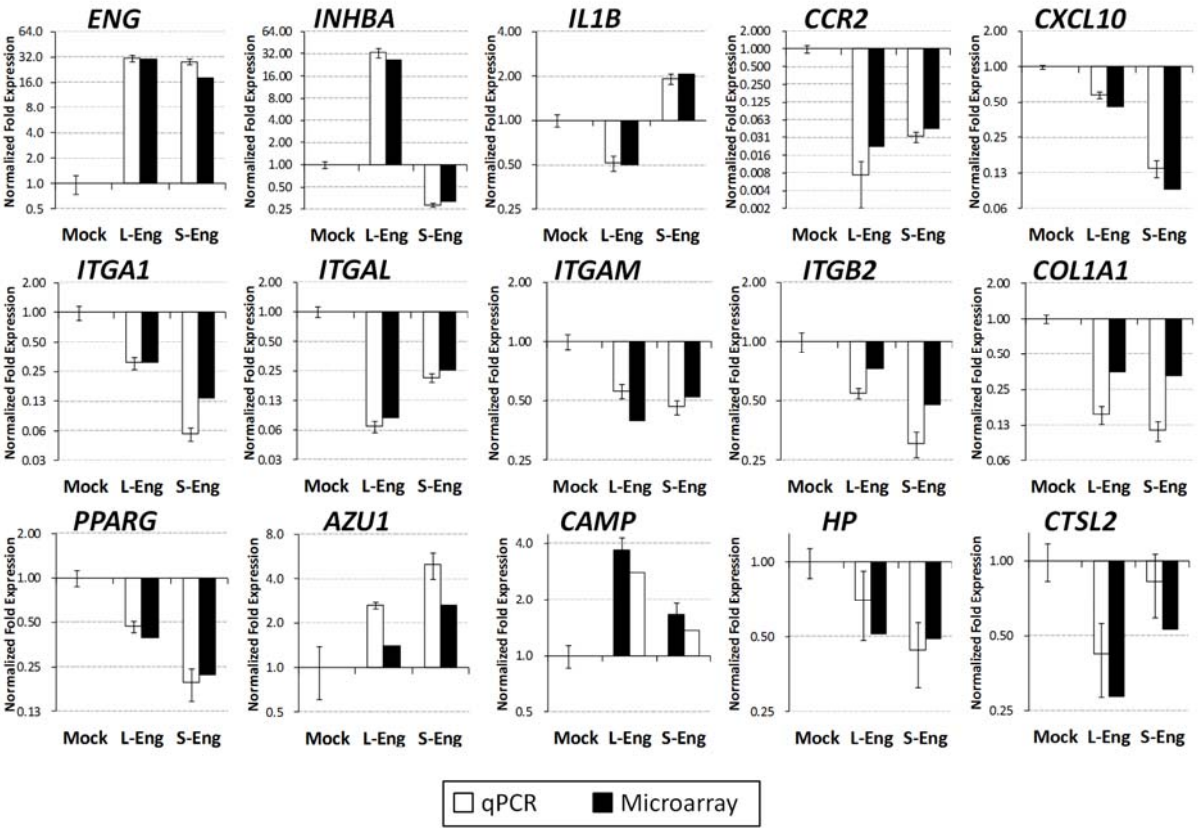


Figure 2.

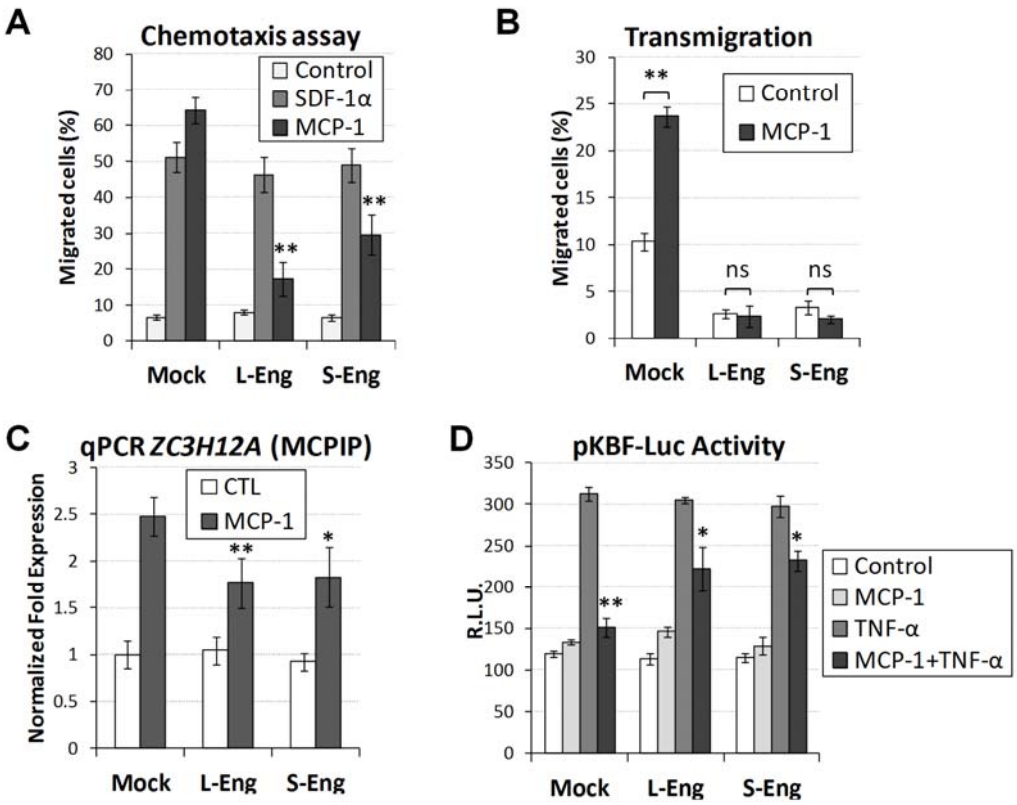


Figure 3.

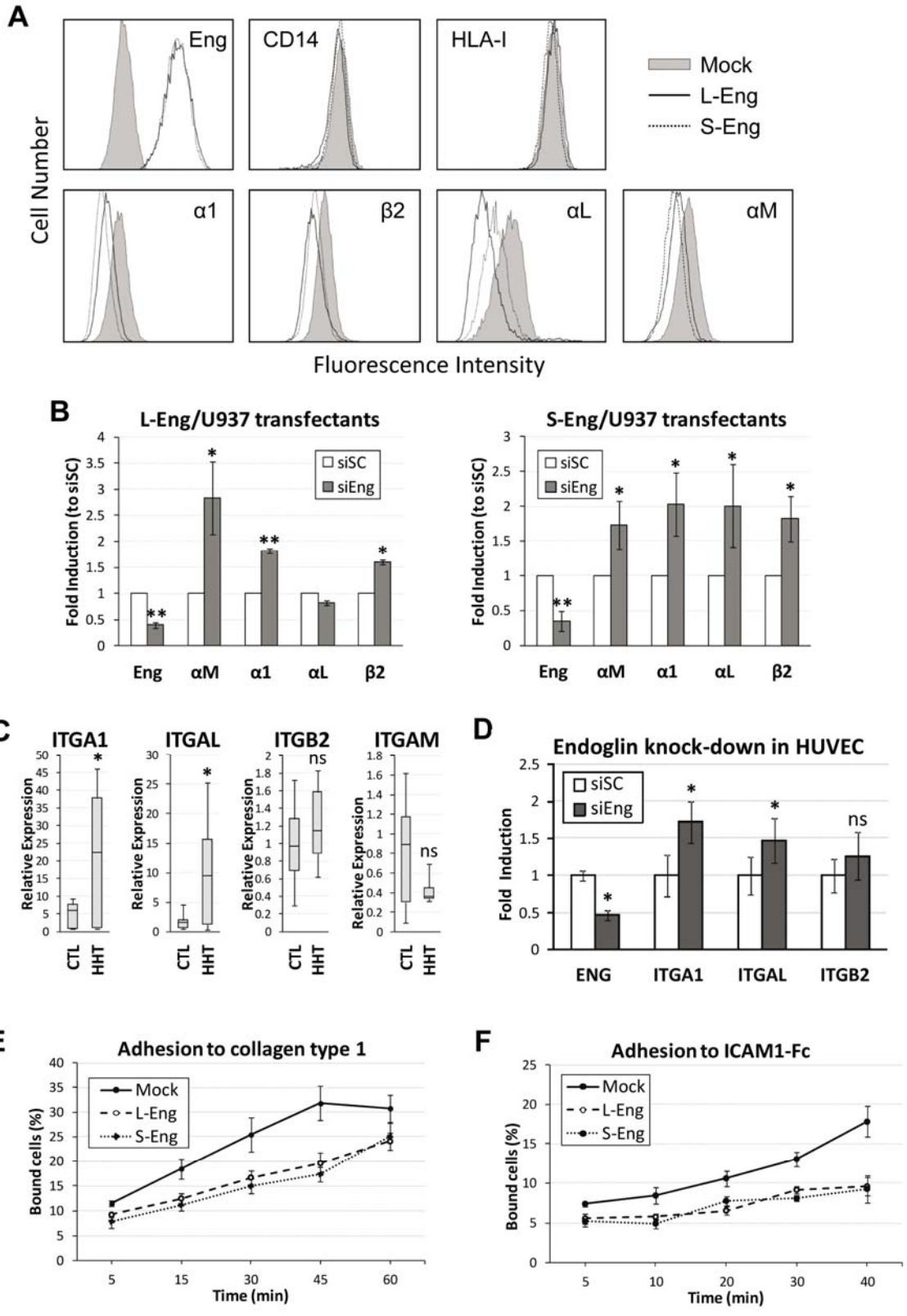


Figure 4

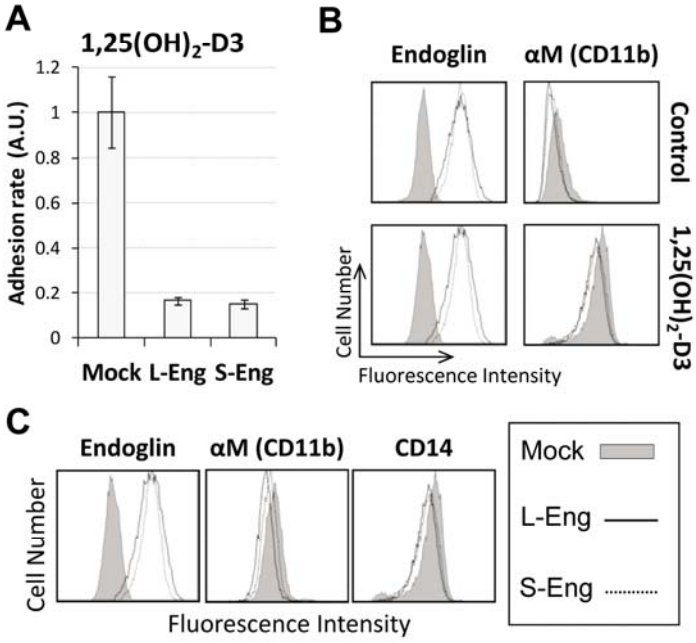
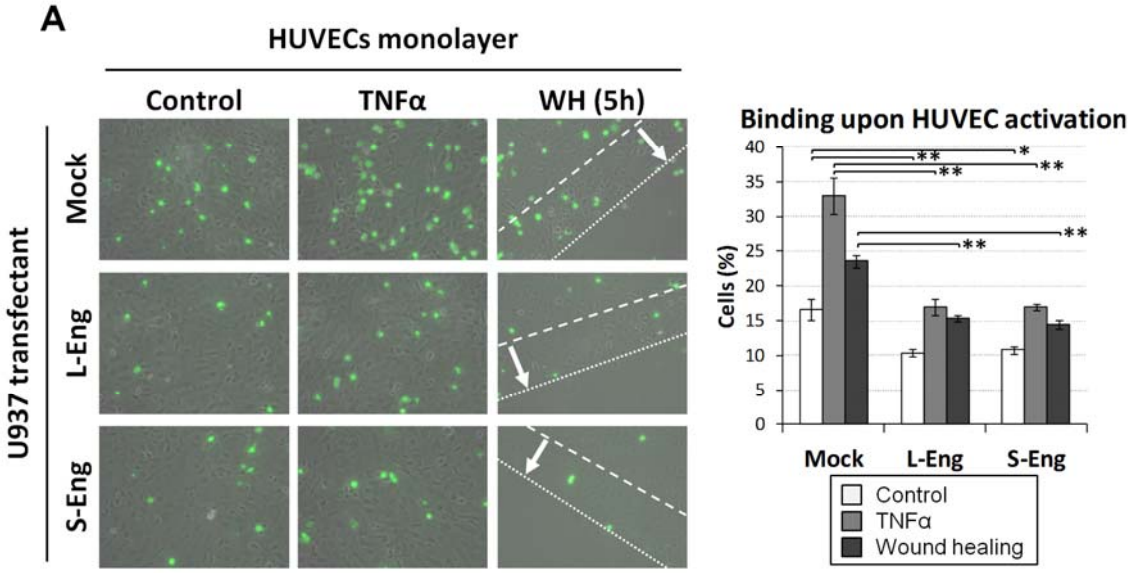


Figure 5



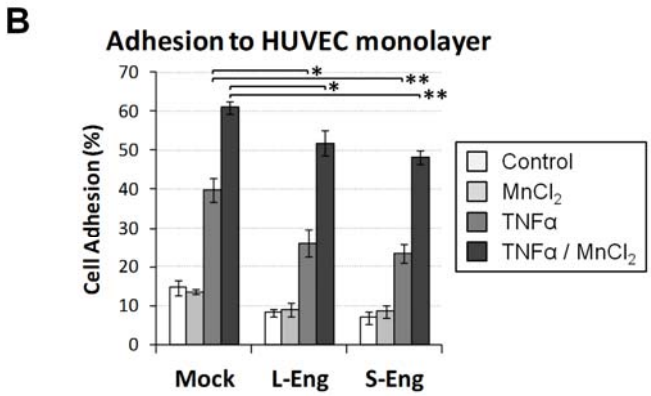
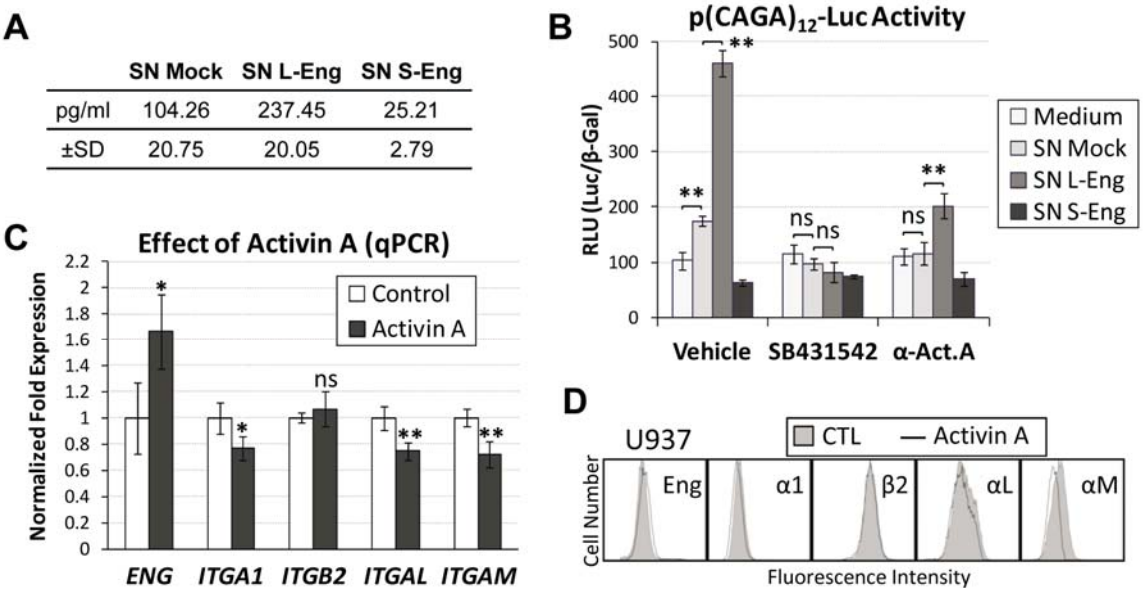


Figure 6.

A

	SN Mock	SN L-Eng	SN S-Eng
pg/ml	104.26	237.45	25.21
±SD	20.75	20.05	2.79



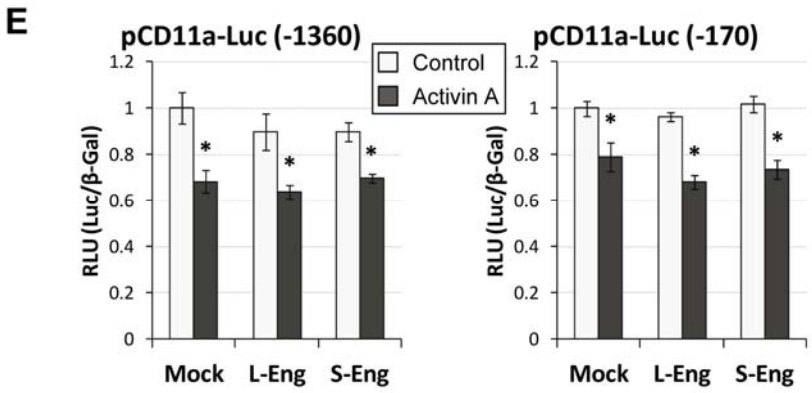


Figure 7