

CHARACTERIZATION OF CHICKEN ENDOGLIN, A MEMBER OF THE ZONA PELLUCIDA FAMILY OF PROTEINS, AND ITS TISSUE EXPRESSION

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ABSTRACT

Endoglin is a TGF- β co-receptor expressed in endothelial cells, where it plays a crucial role in angiogenesis, cardiovascular development and vascular remodelling. In humans, mutations in the endoglin gene give rise to Hereditary Hemorrhagic Telangiectasia type 1 (HHT1), an autosomal dominant disorder associated with vascular lesions in skin, mucosa and internal organs. So far, endoglin cDNA has been sequenced in several species from mammals, amphibians and birds. While in mammals the characterization of endoglin protein expression and function is well documented, little is known about the protein homologue in birds. *In silico* analysis by multiple sequences alignment showed a low homology score of 30-33 between the full length chicken endoglin protein and several mammalian homologues. However, a high homology score (80-85) was observed with the cytoplasmic and transmembrane regions and the overall structure of the zona pellucida (ZP) and orphan domains of the extracellular region appear to be conserved. Transient expression of chicken endoglin allowed the identification of a 180-kDa disulfide linked homodimer similar to the mammalian homologues. To further characterize its tissue expression, the novel specific monoclonal antibody (mAb) 7H5A8 was generated against chicken endoglin transfectant cells. The mAb 7H5A8 specifically recognized chicken endoglin by western blot, immunoprecipitation, immunofluorescence flow cytometry as well as immunofluorescence microscopy assays and displayed a positive staining of the endothelium in veins and arteries from frozen tissue sections of lung and bursa of Fabricius. These results may help to further understand the endoglin expression in vertebrates.

Keywords: endoglin, zona pellucida, endothelium, antibody, TGF-beta

1. Introduction

Endoglin is an auxiliary TGF- β receptor expressed in endothelial cells that plays a major role in cardiovascular development, as well as in angiogenesis and vascular remodeling and homeostasis (Lopez-Novoa & Bernabeu, 2010; Bernabeu et al., 2009; Lebrin & Mummery, 2008). Mutations in the human *ENDOGLIN* gene (*ENG*) are responsible for the Hereditary Hemorrhagic Telangiectasia (HHT) type 1, a disease characterized by vascular malformations (McAllister et al., 1994). This pathological condition leads to frequent nose bleeds, telangiectases on skin and mucosa and may cause arteriovenous malformations in different organs including brain, lung, and liver (Shovlin et al., 2010). In addition to endothelial cells, endoglin can be expressed at lower levels in smooth muscle cells, activated macrophages and fibroblasts (Bernabeu et al., 2007). Endoglin expression is markedly upregulated in proliferating endothelial cells, where it plays a crucial role in angiogenesis during development and in adult animals (Li et al., 2001; Bernabeu et al., 2009; ten Dijke et al., 2008; Lopez Novoa & Bernabeu, 2010). In fact, endoglin knockout mice die *in utero* because of defects in the vascular system (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999). While endoglin expression and function in mammals is widely documented, similar studies in birds are scarce. Chicken endoglin is expressed during heart development (Vincent et al. 1998) and during pre-circulation vascular development, where it seems to play an important role in the transition from endothelial progenitors to functional endothelial cells (Alev et al., 2010), and in post-circulation chick development, where endoglin is expressed intraembryonically in cardiac endothelium and in developing vessels (Mercado-Pimentel et al., 2007). Moreover, chicken endoglin is involved in angiogenesis (Raab et al. 1999) and in the process of epithelial-to-mesenchymal transformation during cardiac valve formation (Mercado-Pimentel et al., 2007).

Endoglin cDNA has been sequenced in several species from mammals, amphibians and birds, including humans (Gougos and Letarte, 1990; Bellón et al. 1993), pig (Yamashita H et al. 1994), rat (Meurer et al., 2005) and mouse (St-Jacques et al., 1994; Ge and Butcher, 1994). Human endoglin is expressed as a 180-kDa disulfide-linked homodimer (Gougos and Letarte 1990) that contains a highly glycosylated extracellular domain of 561 amino acids. Structurally, endoglin belongs to the Zona Pellucida (ZP) family of proteins that share a ZP domain of ~260 amino acid residues at their extracellular region (Jovine et al. 2005; Llorca et al. 2007; Plaza et al., 2010). The three-dimensional structure of the extracellular domain of endoglin at 25Å resolution, using single-particle electron microscopy has been elucidated (Llorca et al. 2007). Endoglin is arranged as a dome made of antiparallel orientated monomers enclosing a cavity at one end. Each subunit comprises three well-defined regions, two of them corresponding to the ZP domain. The third region does not show any significant homology to other protein family/domain and thereby has been named the “orphan” domain. While in mammals the characterization of endoglin protein expression and function is well documented, similar studies in chicken remain to be performed. The knowledge about chicken endoglin sequence is of significant phylogenetic interest because chicken is the most distant species where an endoglin protein, with significant homology to the mammalian homologues, has been reported. Moreover, a widely used method to study angiogenesis is the chick embryo chorioallantoic membrane (CAM), which is very useful model in different fields of medicine, providing information on the timing of tissue changes before the establishment of adult vascularization (Ribatti, 2008). Because endoglin is an endothelial marker of neoangiogenesis (Lopez-Novoa & Bernabeu, 2010), the generation of chicken endoglin related reagents and tools may be very useful to further analyze the angiogenesis process. *In silico* analysis of the chicken endoglin cDNA sequence reported by us (AY702002) reveals an open reading frame coding for 644 amino acids with predicted domains for leader sequence, extracellular, transmembrane and cytoplasmic regions. In this paper we have made a comparative sequence

analysis between chicken endoglin and nine mammalian homologues and we have characterized the expression of recombinant endoglin protein *in vitro*. Moreover, we have generated a novel mAb against chicken endoglin that served to demonstrate the expression of endoglin in the endothelium from chicken tissue sections.

2. Materials and Methods

2.1. *In silico* analysis of chicken endoglin

Multiple sequence alignment was performed using ClustalW2 (Larkin *et al.*, 2007) at European Bioinformatic Institute web site (www.ebi.ac.uk). To predict the secondary structure, Jpred3 server (<http://www.compbio.dundee.ac.uk/www-jpred/>) at University of Dundee was used (Cole *et al.*, 2008). MotifScan resource (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was used to predict conserved structural motifs (Pagni *et al.*, 2007). N- and O-glycosylation predictions were performed using the NetNGlyc and NetOGlyc servers, respectively. Putative phosphorylation sites were predicted using NetPhos server (Blom *et al.*, 1999). All these tools are available at the Center for Biological Sequence Analysis (Technical University of Denmark) webpage (www.cbs.dtu.dk).

2.2. Plasmids

A 2,484-bp fragment of full length chicken endoglin cDNA (AY702002) was inserted into an *EcoRI* site of plasmid pcDNA3, resulting in the expression vector pcDNA3-ch.Eng. The wild type sequence in vector pcDNA3-ch.Eng was used to derive a hemagglutinin (HA)-tagged chicken endoglin expression vector. Specific oligonucleotides were used to prime the PCR synthesis of endoglin (amino acids 26–644) and the amplified 1,880-bp fragment was cloned into *SfiI/SmaI* sites of pDisplay (Invitrogen) vector. The resulting vector (pDisplay-HA-ch.Eng) allows expression of the protein on the cell surface, using the leader sequence of the vector, and the mature protein contains an HA epitope at the NH₂ terminus. Recombinant Newcastle disease virus from the lentogenic strain B1 (rNDV) expressing chicken (rNDV-ch.Eng) or human (rNDV-h.Eng) endoglin have been described (Ayllon, 2009), and were rescued by reverse genetic techniques as reported (Nakaya *et al.*, 2001)

2.3. Cell culture and generation of cell transfectants

The monkey epithelial kidney cells COS-7, the mouse fibroblast cell line L929, the African-green monkey kidney cell line Vero and the white leghorn chicken B cell line DT40 were cultured in DMEM (COS-7, L929 and Vero) or RPMI-1640 (DT40) media (Gibco, Scotland, UK). All culture media were supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 100 U/ml penicillin/streptomycin (complete media). Additionally, DT40 cells were supplemented with 1% of chicken serum and 50 μ M 2-mercaptoethanol. COS-7 cells were transiently transfected with expression vectors encoding chicken endoglin using SuperFect (Qiagen), as indicated by the manufacturer. The adherent mouse fibroblasts cell line L929, was used to generate stable transfectants expressing HA-tagged chicken endoglin, as described for human endoglin (Bellon *et al.*, 1993). Briefly, cells were co-transfected in serum-free medium with pDisplay-HA-ch.Eng vector in the presence of DC-Cholesterol (3 β -[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol) and DOPE (dioleoyl-phosphatidylethanolamine) (Sigma) at a 1:1 ratio, as transfection agent. Positive clones (L929-HA-ch.Eng) were selected in the presence of 400 mg/ml of the antibiotic Geneticin (G-418 sulphate, Gibco). Pooled clones were used in biochemical characterizations and immunizations. Cell suspensions of the chicken B cell line DT40 were used to generate stable transfectants expressing HA-tagged chicken endoglin, as described for human endoglin (Lastres *et al.*, 1996). DT40 cells were transfected with pDisplay-HA-ch.Eng by electroporation of 3×10^7 cells in RPMI 1640 medium using a BTX 600 electroporator (350V, 950 μ F, 200 Ω) with plasmid DNA in 2-mm cuvettes. After selection in RPMI medium containing 10% FCS and 2 mg/ml of Geneticin, endoglin-positive cells (DT40-HA-ch.Eng),

were sorted by immunofluorescence flow cytometry under sterile conditions. Pooled clones were used in biochemical studies. Cells were maintained in a NAPCO incubator at 37°C in a humidified atmosphere with 5% CO₂.

2.4. Generation of monoclonal antibodies to chicken endoglin

Balb/c mice were immunized with murine L929-HA-ch.Eng cell transfectants expressing full-length chicken endoglin tagged with an HA epitope at the N-terminus. Mice were intraperitoneally injected with 1×10^6 L929-HA-ch.Eng cells in phosphate buffered saline (PBS), on days -45, -30 and -15. On day -3, mice were intravenously injected with 0.5×10^6 L929-HA-ch.Eng cells in PBS as a boost for the immunological response. On day 0, spleen cells were fused with SP2 mouse myeloma cells at a ratio of 4:1 (spleen cells:myeloma cells), using polyethylene glycol 1500 (Roche) and seeded in 96-well culture plates. After one week of growth in HAT (hypoxanthine, aminopterin and thymidine) (Sigma) selection medium, the reactivity of hybridoma supernatants was assayed by immunofluorescence flow cytometry using L929-HA-ch.Eng cells and using with anti-HA monoclonal antibodies (12CA5, Roche), as a positive control, and the hybridoma supernatant X63, as a negative control. Selected positive hybridomas were cloned by the limiting dilution method in 96-well plates. Among the positive hybridomas, the clone 7H5A8 producing an IgG1 antibody, was chosen for further characterization.

2.5. Western blot, immunoprecipitation and biotinylation analyses

For western blot and immunoprecipitation analyses, cells were lysed at 4°C with Lysis Buffer: 50 mM Hepes (Gibco), 150 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na₃VO₄ and protease inhibitors (Roche). Aliquots of total cell lysates, containing equivalent amounts of total protein, were precleared for 4 hours at 4°C with protein-G Sepharose (GE Healthcare). Immunoprecipitations were carried out using specific antibodies coupled to protein-G Sepharose. After overnight incubation at 4°C, immunoprecipitates were isolated by centrifugation and washed three times with lysis buffer. Total lysates and immunoprecipitates were separated by standard SDS-PAGE (6% polyacrylamide gel), under reducing or non-reducing conditions. When required, protein samples were dissolved in sample buffer containing 3M urea to fully denature the protein, prior to separation by SDS-PAGE, following the technical bulletin of the manufacturer (MWS-877X, Sigma). After SDS-PAGE, proteins were electrotransferred to a PVDF membrane (BioRad) for immunodetection with the indicated antibodies. Proteins were revealed by incubation with a rabbit polyclonal anti-mouse IgG/HRP (Dako), followed by incubation with SuperSignal chemiluminescent substrate (Pierce, Rockford, IL), according to the manufacturer's instructions. Labelling of cell surface endoglin was carried out with EZ-Link Sulfo NHS Biotin reagent (Pierce), as indicated by the manufacturer. Briefly, after washing the cells three times with cold PBS, they were incubated with the biotin reagent (diluted in PBS to 0.5 mg/ml) for 2 hours at 4°C and with mild agitation. The reaction was stopped by washing 3 times with 100 mM glycine PBS. Finally, cells were lysed with Lysis Buffer as indicated above. Total cell lysates were immunoprecipitated with anti-HA mAb, 7H5A8 mAb or a negative control antibody (X63). Immunoprecipitates were separated by SDS-PAGE and the presence of the recombinant chicken endoglin was revealed with streptavidin-peroxidase (PO) using a chemiluminescent substrate.

2.6. Flow Cytometry

Immunofluorescence flow cytometry was carried out as described (Guerrero-Esteo et al., 1999). Briefly, cells were incubated with the primary antibodies 7H5A8 (anti-chicken endoglin), 12CA5 (anti-HA) or X63 (negative control) for 30 min at 4°C. After two washes with PBS, cells were incubated with Alexa Fluor^R 488 anti-mouse IgG (Invitrogen) at 4°C for 30 minutes. Finally, cells were washed twice with PBS and their fluorescence was estimated

with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), using of logarithmic amplifiers.

2.7. Immunohistochemistry (IHC)

To assess the specificity of the mAb 7H5A8 for endoglin in endothelia, chicken frozen tissue sections with a high degree of vascularization (lung and bursa of Fabricius) were purchased (Gentaur, Belgium). Samples were processed for immunohistochemistry using the Novolink™ Min Polymer Detection System (Novocastra Laboratory, UK), according to the manufacturer's instructions. Endogenous peroxidase and nonspecific staining were blocked by incubation with ready to use peroxidase-blocking and protein block solutions, respectively, followed by rinsing in Tris Buffered Saline solution 1X (TBS).

The immunohistochemical method involved a sequential incubation with either the primary antibody to detect endoglin (mAb 7H5A8) or with the mAb X63 (negative control) for 45 minutes. Then, samples were treated with a ready to use post primary block solution for 30 minutes, followed by incubation with the NovoLink™ Polymer (rabbit anti-mouse IgG–Poly-HRP) solution for 30 minutes. After a couple of rinses with TBS, immunoprecipitates were visualized by treatment with 3,3'-diaminobenzidine for 5 minutes and samples were counterstained with hematoxylin. Then, samples were dehydrated by quick steps in running water, ethanol 50%, ethanol 70%, ethanol 95%, ethanol 100% and xylene and slides were mounted with HI-MO mounting medium (Bio Optica, Milan, Italy). Samples were observed by light-microscopy (Zeiss, Axiovert 25) and the photos were taken using an Olympus E-330 camera.

2.8. Immunofluorescence and confocal microscopy

Immunofluorescence was performed on both frozen tissue sections and adherent cultured cells, using the same procedure. Samples were fixed with formaldehyde (3.5%) at 4°C for 5 minutes, rinsed with PBS and incubated with the primary antibodies 7H5A8 hybridoma supernatants, X63 hybridoma supernatant, mAb anti-HA and mAb QH1 for 1 hour at 4°C. The mAb QH1 recognizes an endothelial marker in quail and was purchased from DSHB (University of Iowa, USA). Samples were rinsed twice with PBS and then incubated with the secondary antibody Alexa Fluor® 488 anti-mouse IgG (Invitrogen, Molecular Probes, Eugene, Oregon, USA) diluted 1:100 in BSA 1% for 1 hour at 4°C. After two washes with PBS, samples were incubated with DAPI 1µg/ml and Alexa Fluor 546 Phalloidin 5U/ml (Molecular Probes, Eugene, Oregon, USA) and subsequently mounted using ProLong Gold antifade reagent (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Samples were observed using a fluorescence microscope (Zeiss Axioplan Universal Microscope) equipped with CCD camera (Digital Camera Leica DFC 350 FX and computer Power Mac G4 OS X) and by spectral confocal microscopy (TCS SP2, Leica Microsystems, Nussloch, Germany).

3. Results

3.1. *In silico* analysis of chicken endoglin protein

A comparative analysis of the chicken endoglin protein and 9 different mammalian species was carried out using the alignment program ClustalW2 (Fig. S1). Overall, a low homology score (<33) between the full length chicken protein and the mammalian homologues was found (Table 1). This is compatible with the distant position of chicken endoglin in a comparative phylogenetic tree (Fig. S2). By contrast, a high homology score (80-85) was observed with the cytoplasmic and transmembrane regions (Table 2). This high degree of conservation suggests that the cytoplasmic region should play an important common role in cellular functions among distinct species. In addition, the overall structure of the zona pellucida (ZP) domain of the extracellular region appears to be conserved (Fig. S1). Indeed, a conserved ZP structural domain from residues K354 to G548 (raw score: -12.7; N-score: 13.619; E-value: 5.1×10^{-7}) was predicted using the MotifScan server, in agreement with the

ZP domain previously described in human endoglin (Llorca et al., 2007). Moreover, the secondary structure of chicken endoglin was predicted as a beta-strand enriched protein using the Jpred3 server, which fits well with the overall structure of endoglin and the putative ZP domain in particular (Llorca et al., 2007). Nevertheless, this structural domain in chicken endoglin contains only 6, instead of the 8 cysteine residues that characterize the mammalian ZP domains (Llorca et al., 2007; Jovine et al., 2005; Plaza et al., 2010). Furthermore, only 4 out of those 6 cysteine residues are conserved in other species (Fig. S1). On the other hand, the N-terminal region of chicken endoglin does not show any structural homology domain, so it was designated as orphan domain (OD), as reported for the human endoglin (Llorca et al., 2007). It is worth noting the low homology score obtained for the alignment when comparing the OD and ZP domains in chicken versus the nine mammalian species analyzed (best score for OD \leq 30; best score for ZP \leq 25). Because mammalian endoglin has been shown to be O- and N-glycosylated (Gougos and Letarte, 1990), the potential glycosylation sites of chicken endoglin were analyzed *in silico*. Thus, NetNGlyc server showed a 7 matching pattern for N-glycosylation at Asn residues, all of them in the extracellular region (positions 79, 249, 258, 299, 377, 390 and 551). In addition, the NetOGlyc server showed a unique Thr residue at position 333, as susceptible of being O-glycosylated. However, we should take into account that these bioinformatic tools are designed to predict glycosylation sites using matrices or consensus motifs based on mammalian proteins, so the results with chicken endoglin should be taken only as a hint. Moreover, the NetPhos server predicted 8 residues in the cytoplasmic tail that are prone to be phosphorylated, including 6 Ser residues (positions 609, 615, 621, 624, 625 and 629) and 2 Thr residues (616 and 636). This is in agreement with the Ser/Thr phosphorylation observed in human and porcine endoglin (Lastres et al., 1994; Yamashita et al., 1994; Koleva et al., 2006).

3.2. Expression of chicken endoglin in mammalian and chicken cell lines

Because of the lack of a reliable source of antibodies to chicken endoglin (Raab et al., 1999), its detection remains a challenge. In order to bypass this restriction, an expression vector encoding HA-tagged chicken endoglin (pDisplay-HA-ch.Eng) was generated, as described in Materials and Methods. Thus, expression of this recombinant protein would allow its detection by using commercially available anti-HA antibodies. Indeed, Western blot analysis of monkey COS-7 cells transiently transfected with pDisplay-HA-ch.Eng demonstrated the presence of ~90-kDa protein under reducing conditions that corresponds to the expected monomeric primary structure (Fig. 1A). The analysis under non-reducing conditions reveals the presence of an oligomeric structure as well as the monomer. Similar results were obtained when analyzing stable murine cell transfectants expressing HA-tagged chicken endoglin (Fig. 1B). Interestingly, in these two mammalian cell types, the size of the oligomer was much higher than the one expected for the interchain disulfide dimer of endoglin (~180kDa). This oligomerization effect was also detected in DT40 chicken cells stably expressing HA-tagged chicken endoglin, excluding a mammalian specific effect (Fig. 1C). Because endoglin contains a ZP domain that is well known to enhance the oligomerization capacity of proteins (Jovine et al., 2002; Plaza et al., 2010), we hypothesized that these oligomers are formed by non-covalent interactions of the proteins. In order to eliminate these non-covalent interactions, we carried out a denaturing SDS-PAGE analysis in the presence of urea. Under these conditions, the western blot analysis revealed the expected size for the monomer and the disulfide-linked dimer of chicken endoglin in two different mammalian cell types (Fig. 1D). As a control, the size of human endoglin was shown to be similar to that of the chicken homologue.

To assess whether chicken endoglin was expressed on the cell surface, two types of experiments were carried out. First, immunofluorescence flow cytometry analyses demonstrated that ectopically expressed recombinant chicken endoglin was recognized by

anti-HA antibodies on the surface of stable transfectants in chicken and murine cells (Fig. 2A). Second, stable transfectants expressing HA-tagged chicken endoglin were subjected to cell surface biotinylation, followed by immunoprecipitation with anti-HA antibodies. As shown in Fig. 2B, recombinant chicken endoglin was clearly detected in the specific immunoprecipitates in monomeric and oligomeric forms. These data suggest that chicken endoglin is expressed at the cell surface as previously described for human endoglin.

3.3. Generation and characterization of the mAb 7H5A8 specific for chicken endoglin.

To generate antibodies to chicken endoglin, mice were immunized with the murine L929-HA-ch.Eng cell transfectants expressing full-length chicken endoglin tagged with an HA epitope at the N-terminus. The characterization of these transfectants has been documented in Figs. 1B and 2A. By using a murine background in the cells injected as antigen, the immune response was expected to be focused against the xenogeneic chicken endoglin. Hybridoma supernatants were screened for the presence of specific antibodies against chicken DT40 stable transfectants expressing recombinant chicken endoglin (see Figs. 1C and 2B) by immunofluorescence flow cytometry. This screening yielded mAb 7H5A8 as specific for chicken endoglin. This is illustrated in Fig. 3A by the strong reactivity of the mAb 7H5A8 for the DT40 stable transfectants expressing recombinant chicken endoglin and by the lack of reactivity against parental cells. This result suggests that the mAb 7H5A8 recognizes an antigenic epitope on the extracellular domain of chicken endoglin. As a positive control, cell surface endoglin was also stained with anti-HA mAb. Western blot analysis also demonstrated the specific reactivity of the mAb 7H5A8 for chicken endoglin when the electrophoretic separation was carried out under non-reducing conditions (Fig. 3B). By contrast, the mAb 7H5A8 showed a weak reactivity against the monomeric endoglin when the electrophoresis was carried out under reducing conditions. A parallel control experiment with anti-HA antibodies evidenced the strong reactivity of these antibodies against monomeric and oligomeric endoglin. In addition, the mAb 7H5A8 was able to recognize both wild type and HA-tagged chicken endoglin by immunoprecipitation experiments (Fig. 3C), ruling out the possibility that the mAb 7H5A8 might be specific for the HA tag.

Next, the binding of the mAb 7H5A8 to recombinant chicken endoglin expressed on cultured cell lines was assessed by immunofluorescence microscopy. As shown in Fig. 4, monkey COS-7 cells transiently transfected with chicken endoglin (Fig. 4A), monkey Vero cells infected with recombinant Newcastle disease virus expressing chicken endoglin (Fig. 4B) and murine L929 fibroblasts stably transfected with chicken endoglin (Fig. 4C) showed a positive fluorescent staining with the mAb 7H5A8. The staining of mAb 7H5A8 was found predominantly on the cell surface, in agreement with the membrane localization of endoglin.

Finally, the reactivity of the mAb 7H5A8 was tested in chicken frozen tissue sections of lung and Bursa of Fabricius, two organs that are highly vascularized. Immunohistochemistry of lung and bursa sections demonstrated the positive staining of mAb 7H5A8 in vessels (Fig. 5A). Immunofluorescence microscopy experiments also showed a clear staining of veins and arteries in bursa samples counterstained with phalloidin and DAPI (Fig. 5B). Analysis at a higher magnification showed a positive staining with the mAb 7H5A8 in the tunica intima of bursa arteries (Fig. 5C). This localization of chicken endoglin is in agreement with the endothelial expression reported for mammalian endoglin. In the same experiment, staining with the mAb QH1, specific for avian vessels, was included as a positive control. Moreover, staining experiments with mAb 7H5A8 in formaldehyde-fixed paraffin embedded chicken tissues did not yield positive results (data not shown), suggesting that the mAb 7H5A8 recognizes an epitope on the endoglin protein in a native state, but is unreactive against the denatured protein.

4. Discussion

Endoglin is expressed in endothelial cells, where it plays a crucial role in angiogenesis, cardiovascular development and vascular remodeling (Lopez-Novoa & Bernabeu, 2010; ten Dijke et al., 2008; Lebrin & Mummery, 2008; Raab et al., 1999). While in mammals the characterization of endoglin protein expression and function has been widely studied, little is known about the avian homologue. Here, we have analyzed for the first time the protein sequence and the ectopic expression of chicken endoglin. A high degree of identity between chick and mammalian endoglins was found at the transmembrane and short cytoplasmic tail domains. Remarkably, 100% conservation was detected in the C-terminal 24 amino acids, containing a PDZ-binding motif as well as target residues that are known to be phosphorylated in mammalian endoglin by T β RII, ALK1 and ALK5 (Guerrero-Esteo et al., 2002; Yamashita et al., 1994; Lastres et al., 1994; Koleva et al. 2006; Ray et al., 2010). This high conservation degree suggests that the cytoplasmic region plays a common functional role among different species. In this regard, the endoglin cytoplasmic domain has been postulated to modulate the TGF- β cellular responses through its interaction with, and phosphorylation by, the TGF- β signaling receptors (Bernabeu et al., 2007). Of note, endoglin phosphorylation influences its subcellular localization and deletion of the putative C-terminal PDZ-binding motif results in endoglin hyperphosphorylation of distal threonine residues (Koleva et al., 2006). These data reveal that kinase-mediated phosphorylation of endoglin is a complex process involving a negative regulation by the PDZ-binding motif. Another level of regulation may be provided by the interaction of the endoglin cytoplasmic domain with other cytosolic proteins as described in humans. This is the case of zyxin and zyxin-related protein 1 (ZRP-1) involved in actin organization and assembly around focal adhesions (Conley et al., 2004; Sanz-Rodriguez et al., 2004), Tctex2b, a member of the Tctex1/2 family of cytosolic dynein light chains, linking endoglin to the microtubule based transport machinery (Meng et al., 2006) or beta-arrestin2 involved in endoglin internalization in endocytic vesicles (Lee and Blobel, 2007). It will be interesting to investigate whether these mammalian endoglin binding proteins also have homologues that interact with chicken endoglin.

In silico analysis of chicken endoglin also revealed the existence of a conserved ZP structural domain (residues K354 to G548). This consensus ZP domain is present in a large family of proteins and is potentially involved in endoglin receptor oligomerization (Jovine et al., 2005; Llorca et al., 2007). Supporting this view, analysis by standard SDS-PAGE under non-reducing conditions revealed the presence of endoglin oligomers with a molecular weight higher than the expected size of the disulfide linked homodimer (Fig. 1C). This oligomerization effect is likely due to the contribution of non-covalent interactions mediated by the ZP domains. Indeed, these oligomeric interactions were inhibited under strong denaturing conditions in the presence of urea (Fig. 1D).

The N-terminal region of chicken endoglin (H26-S351) showed a relatively low homology with the other vertebrate homologues. This region corresponds to the orphan domain, which has been structurally characterized in human endoglin (Llorca et al., 2007). It has been postulated that this domain is responsible for binding to the TGF- β superfamily members in humans, including TGF- β 1, TGF- β 3, activin, BMP7 or BMP9 (Lopez-Novoa & Bernabeu, 2010). However, preliminary experiments in our laboratory failed to demonstrate functional binding of human TGF- β 1 to chicken endoglin. The homologous avian ligands that bind to chicken endoglin remain to be identified.

Expression studies of recombinant chicken endoglin demonstrated that it is a 180-kDa disulfide-linked homodimer that is expressed at the cell surface (Figs. 1 and 2). Generation of stable cell transfectants allowed the production of the mAb 7H5A8 against chicken endoglin. Previous studies used polyclonal or monoclonal antibodies with mammalian specificities that crossreacted with chicken endoglin, yielding a specific, although weak reactivity (Luque et

al., 1997; Raab et al., 1999). This is the first time that a mAb is described against chicken endoglin using chicken endoglin as an antigen and it will be a useful tool to further advance in the knowledge of this protein and its role in vascular biology. The mAb 7H5A8 specifically recognized chicken endoglin in immunoprecipitation, immunofluorescence flow cytometry, immunohistochemistry and immunofluorescence microscopy studies. The optimal binding of the mAb 7H5A8 was obtained when the chicken endoglin protein was in a native structure. By contrast, weak or null recognition was obtained in our hands when the protein was denatured as it was the case of Western blot upon SDS-PAGE under reducing conditions or staining of formaldehyde paraffin embedded tissues. One of the interesting results from the immunohistochemistry and immunofluorescence microscopy experiments is that the mAb 7H5A8 recognizes chicken endoglin in the endothelia from veins and arteries, a finding that is in line with the localization of endoglin in human vascular endothelium (Lopez-Novoa & Bernabeu, 2010; Mahmoud et al. 2009). Furthermore, whole-mount in situ hybridization studies on chicken embryos demonstrated expression of endoglin during embryonic pre-circulation stages mainly confined to areas of vasculogenesis, being strongest in endothelial cells undergoing active vascularization and lower or undetectable in differentiated vessels (Alev et al., 2010). Moreover, using the chick chorioallantoic membrane as an angiogenic model, the involvement of chicken endoglin in angiogenesis was demonstrated (Raab et al., 1999).

The important role that endoglin plays in endothelial biology and its predominant expression in this cell lineage suggests a close association with the phylogenetic origin of endothelial cells. Supporting this hypothesis, the endoglin family is expressed in vertebrates, but not in invertebrates and the main difference between invertebrate and vertebrate vascular system is the presence of a true endothelium. So far, there is no consensus phylogenetic theory to explain the origin of the endothelial cells in vertebrates or a comprehensive approach to the transition between the vertebrate and the invertebrate types of circulatory system (Muñoz-Chapuli et al. 2005). The study of endothelial genes and their encoded proteins such as endoglin may help to understand the complex phylogenetic transition to the vascular endothelial system in vertebrates.

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FIGURE LEGENDS

Figure 1. Expression of chicken endoglin in mammalian and chicken cell lines. **A.** COS-7 cells were transiently transfected with the expression vector encoding HA-tagged chicken endoglin, pDisplay-HA-ch.Eng (HA-ch.Eng) or the pDisplay empty vector (Control). Proteins from total cell lysates were separated by SDS-PAGE followed by western blot analysis. Chicken endoglin was detected with an anti-HA mAb. **B.** Stable murine L929 fibroblast transfectants expressing HA-tagged chicken endoglin (HA-ch.Eng) and parental L929 cells (Control) were lysed and immunoprecipitated with anti-HA antibodies. Total cell lysates (left panel) or immunoprecipitates (right panel) were separated by SDS-PAGE, followed by western blot analysis using anti-HA mAb. **C.** Stable chicken DT40 cell transfectants expressing HA-tagged chicken endoglin (HA-ch.Eng) and parental DT40 cells (Control) were lysed. As a mammalian control, COS-7 cells transiently transfected, as described in panel A, were included. Total cell lysates were separated by SDS-PAGE followed by western blot analysis using anti-HA mAb. **D.** Stable L929 fibroblast transfectants expressing HA-tagged chicken endoglin (HA-ch.Eng) and COS-7 cells transiently transfected with HA-tagged chicken endoglin (HA-ch.Eng) or HA-tagged human endoglin (HA-h.Eng) were lysed with sample buffer containing 3M urea to fully denature the proteins. Total cell lysates were separated by SDS-PAGE under denaturing conditions as described in Materials and Methods. Western blot analysis was performed using anti-HA mAb. Asterisks indicate the presence of unspecific bands. Proteins were separated by SDS-PAGE under either reducing (R) or non reducing (NR) conditions, as indicated.

Figure 2. Detection of chicken endoglin on the cell surface. **A.** Flow cytometry analysis. Stable chicken DT40 cell transfectants and stable murine L929 fibroblast transfectants expressing HA-tagged chicken endoglin (HA-ch.Eng) as well as the corresponding parental cells were analyzed by immunofluorescence flow cytometry with anti-HA antibodies. The percentage of positive cells is indicated by the lower horizontal bar. **B.** Stable chicken DT40 cell transfectants expressing HA-tagged chicken endoglin (HA-ch.Eng) and parental DT40 cells were biotinylated on the cell surface and lysed. Total cell lysates were immunoprecipitated with an anti-HA antibody (α -HA) or a negative control antibody (cAb). Immunoprecipitates were separated by SDS-PAGE and the presence of the recombinant chicken endoglin was revealed with streptavidin-peroxidase (PO). Asterisks indicate the presence of unspecific bands. Proteins were separated by SDS-PAGE under either reducing (R) or non reducing (NR) conditions, as indicated.

Figure 3. Reactivity of the mAb 7H5A8 for recombinant chicken endoglin. **A.** Flow cytometry analysis. Stable chicken DT40 cell transfectants expressing HA-tagged chicken endoglin (HA-ch.Eng-DT40) and the corresponding parental DT40 cells were analyzed by immunofluorescence flow cytometry with 7H5A8 and anti-HA mAb; a negative control antibody (cAb) was also included. The percentage of positive cells is indicated by the lower horizontal bar. **B.** Western blot analysis. Stable chicken DT40 cell transfectants expressing HA-tagged chicken endoglin (HA-ch.Eng) were lysed. Total cell lysates were separated by SDS-PAGE followed by western blot analysis using 7H5A8 and anti-HA mAbs. **C.** Biotinylation studies on cells expressing HA-tagged and untagged chicken endoglin. COS-7 cells were transiently transfected with expression vectors encoding either HA-tagged chicken endoglin, (HA-ch.Eng) or wild type untagged chicken endoglin (ch.Eng). Cells were biotinylated on the cell surface and lysed. Total cell lysates were immunoprecipitated with 7H5A8 mAb or a negative control antibody (cAb), as indicated. Immunoprecipitates were

separated by SDS-PAGE and the presence of the recombinant chicken endoglin was revealed with streptavidin-peroxidase (PO).

Figure 4. Immunofluorescence of cells transduced with chicken endoglin using the mAb 7H5A8. **A.** COS-7 cells were transiently transfected (ch.Eng) or not (control) with untagged chicken endoglin for 24 hrs. Cells were stained with DAPI to visualize the nuclei (blue stain), with phalloidin to visualize the actin filaments (red stain) and with the mAb 7H5A8 coupled to an Alexa 488-labelled secondary antibody (green fluorescence), as indicated. Overlapping of the three colours is shown in the last row (Merge). Images were captured by confocal microscopy at X400 magnification. **B.** Vero cells were infected with either a recombinant Newcastle disease virus expressing chicken endoglin (rNDV-ch.Eng), a recombinant Newcastle virus expressing human endoglin (rNDV-h.Eng) or a negative control Newcastle virus (Mock). Cells were stained with the mAb 7H5A8 coupled to FITC-labelled secondary antibody (green fluorescence). Images were captured at X100 (upper panels) or X600 (lower panels) magnification. **C.** Stable L929 fibroblast transfectants expressing HA-ch.Eng or parental cells were stained with DAPI to visualize the nuclei (blue stain) and with the mAb 7H5A8 coupled to an Alexa 488-labelled secondary antibody (green fluorescence), as indicated. Overlapping of the two colours is shown in the last row (Merge). Images were captured at X200 magnification.

Figure 5. Staining of chicken tissues with the mAb 7H5A8. **A.** Immunohistochemistry on frozen chicken sections of Bursa of Fabricius and lung. Samples were stained with the mAb 7H5A8 or a negative control mAb (X63) followed by incubation with peroxidase-coupled rabbit anti-mouse IgG. The primary antibody was visualized by incubation with 3,3'-diaminobenzidine. Samples were counterstained with hematoxylin and photographs were taken by light microscopy at the indicated magnification. **B.** Immunofluorescence on frozen chicken sections of Bursa of Fabricius. Samples were stained with DAPI to visualize the nuclei (blue stain), the mAb 7H5A8 coupled to an Alexa 488-labelled secondary antibody (green fluorescence) and phalloidin to visualize actin filaments (red stain), as indicated. As a negative control, samples were stained with the mAb X63 (bottom row). Overlapping of the three colours is shown in the last column (Merge). The pictures were taken at an original magnification of X400. **C.** Immunofluorescence on frozen chicken sections of Bursa of Fabricius and lung. Samples were stained with DAPI to visualize the nuclei (blue stain), phalloidin to visualize actin filaments (red stain) and the mAb 7H5A8 coupled to an Alexa 488-labelled secondary antibody (green fluorescence), as indicated. As a negative control, Bursa sections were stained with mAb X63 (Cont-Ab). As positive controls, lung and Bursa sections were stained with the vascular endothelium specific mAb QH1. Photographs were taken at an original magnification of X400 or X600, as indicated.

Table 1. Sequence homology analysis of full length chicken endoglin*

Organism 1	Length (aa)	Organism 2	Length (aa)	Score
Chicken	644	Cow	647	33
Chicken	644	Dog	721	33
Chicken	644	Human	658	33
Chicken	644	Orangutan	672	32
Chicken	644	Opossum	673	31
Chicken	644	Marmoset	692	30
Chicken	644	Mouse	653	30
Chicken	644	Pig	653	30
Chicken	644	Rat	650	30

*The alignment score is indicated in the right column

Table 2. Sequence homology analysis of transmembrane and cytoplasmic domains of chicken endoglin*

Organism 1	Length (aa)	Organism 2	Length (aa)	Score
Chicken	70	Opossum	70	85
Chicken	70	Orangutan	72	82
Chicken	70	Human	72	82
Chicken	70	Dog	72	82
Chicken	70	Mouse	72	81
Chicken	70	Rat	72	81
Chicken	70	Cow	72	81
Chicken	70	Marmoset	72	81
Chicken	70	Pig	72	80

*The alignment score is indicated in the right column

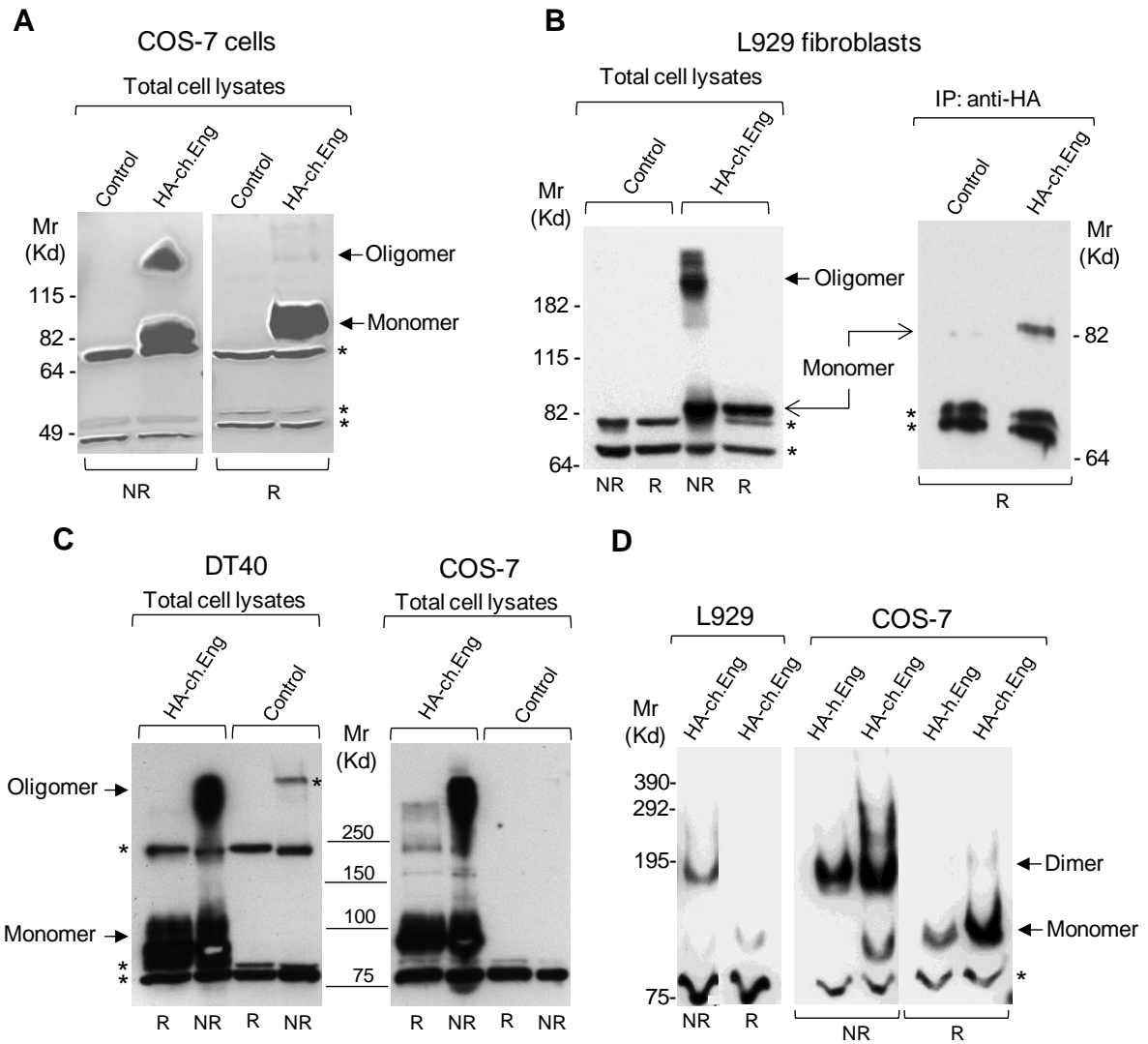


Figure 1

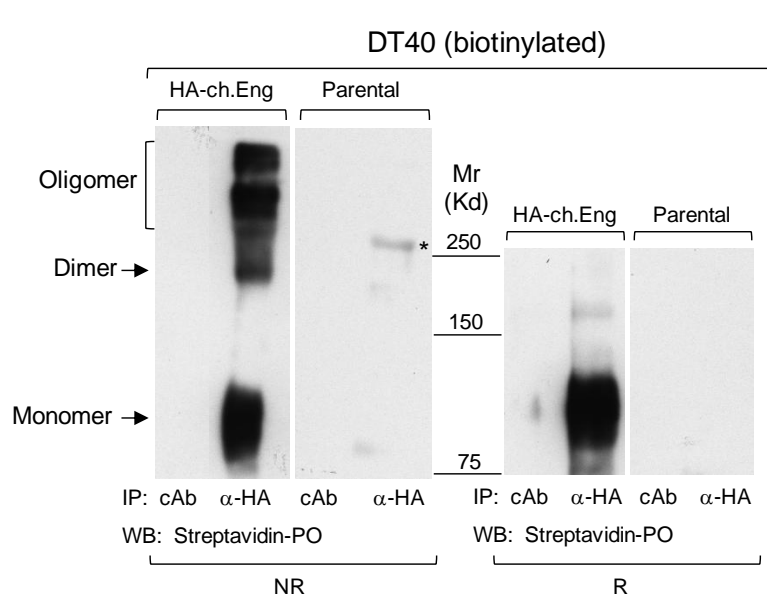
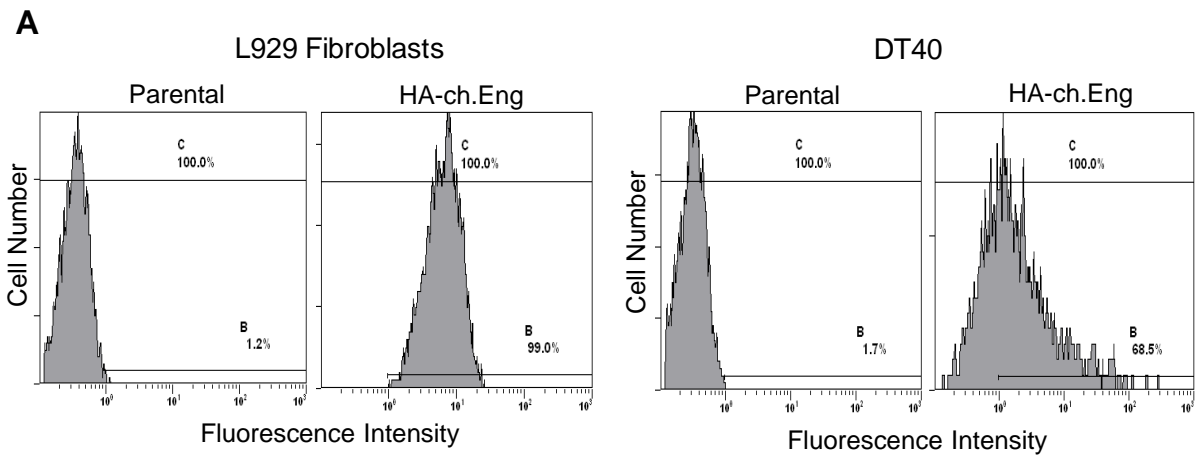


Figure 2

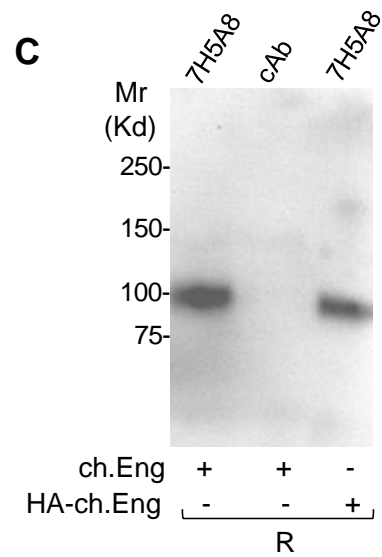
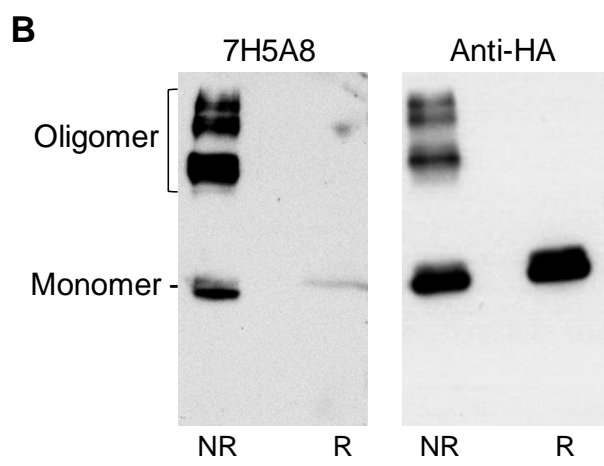
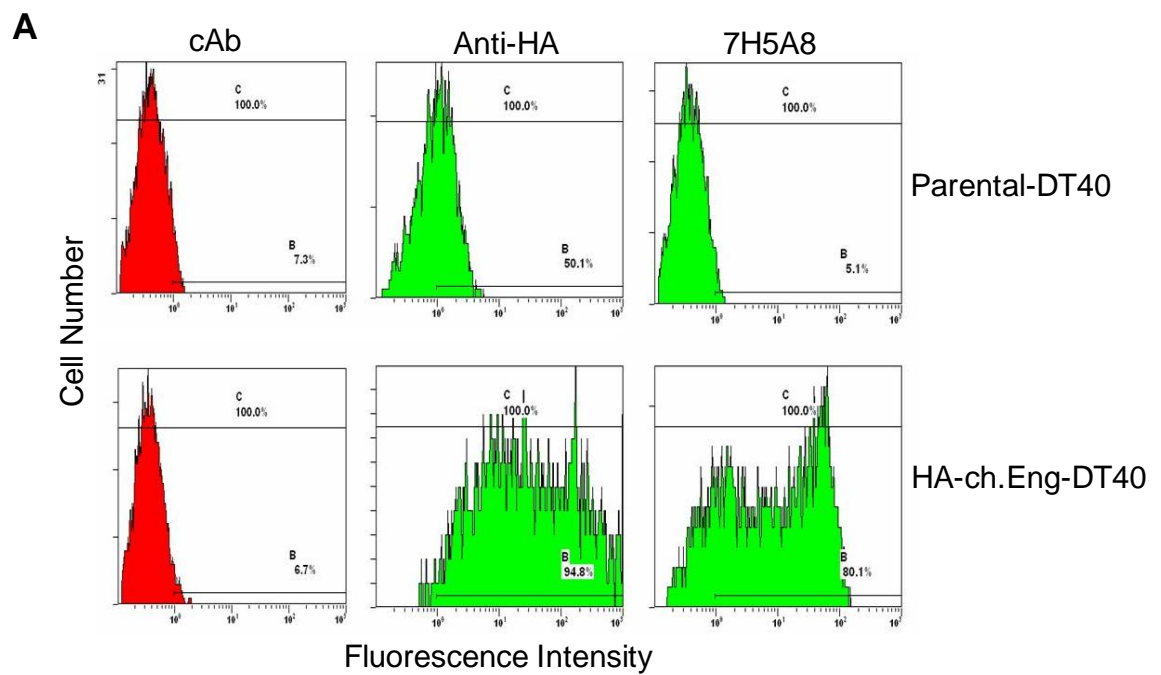


Figure 3

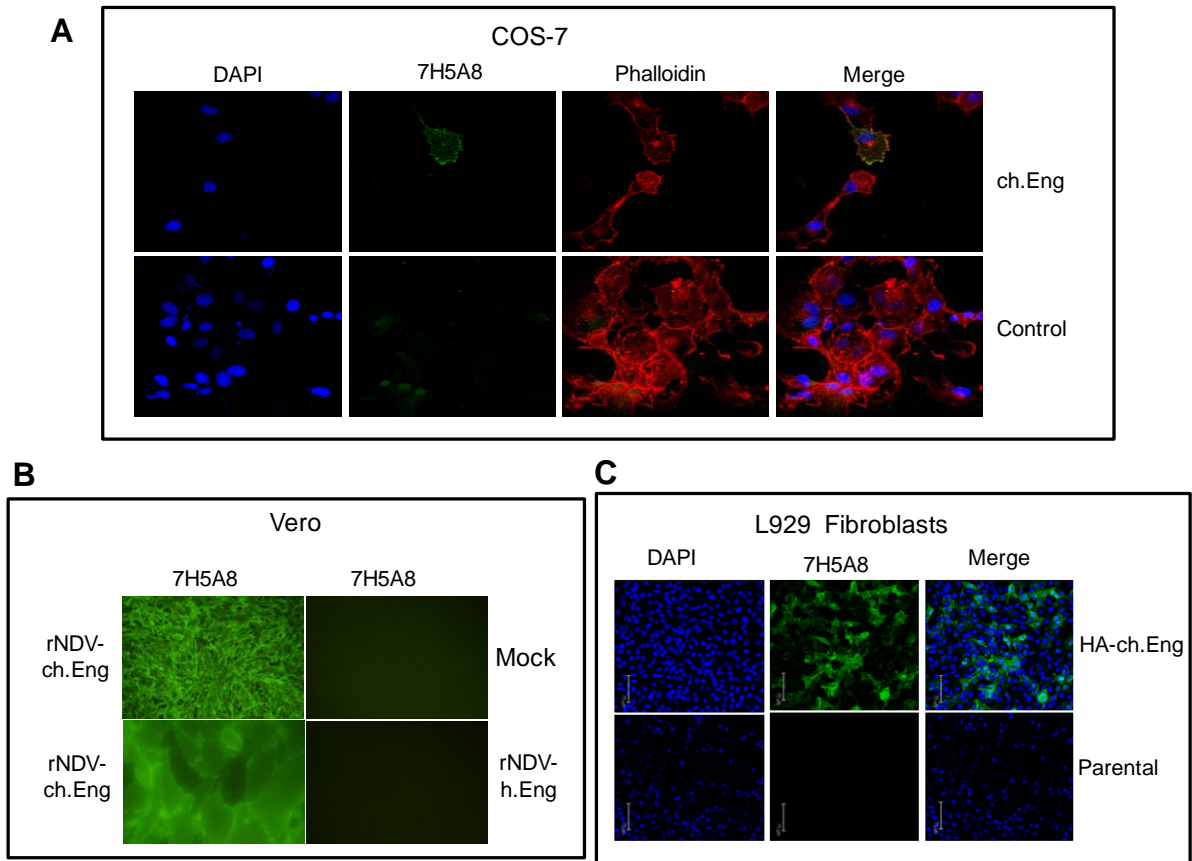


Figure 4

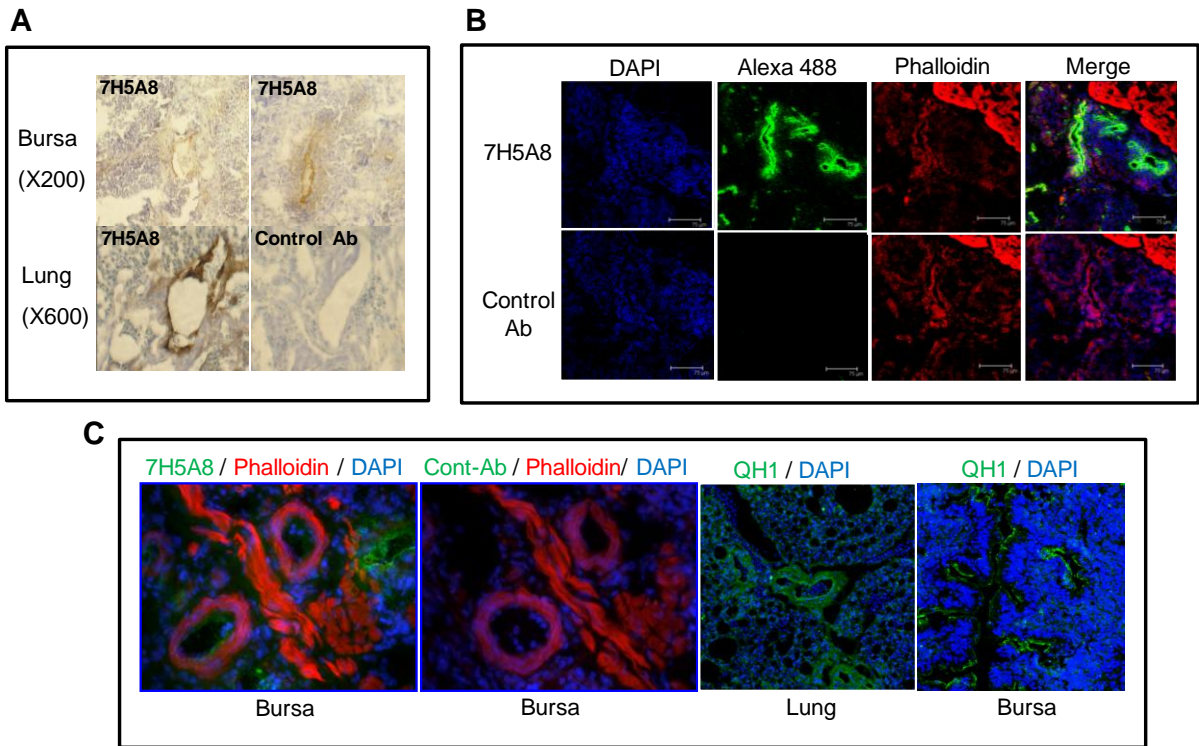


Figure 5