The Interaction of Endoglin with β-Arrestin2 Regulates Transforming Growth Factor-β-mediated ERK Activation and Migration in Endothelial Cells*

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In endothelial cells, transforming growth factor β (TGF-β) signals through two distinct pathways to regulate endothelial cell proliferation and migration, the ALK-1/Smads 1/5/8 pathway and the ALK-5/Smads 2/3 pathway. TGF-β signaling through these pathways is further regulated in endothelial cells by the endothelial specific TGF-β superfamily co-receptor, endoglin. The importance of endoglin, ALK-1, and ALK-5 in endothelial biology is underscored by the embryonic lethal phenotypes of knock-outs in mice due to defects in angiogenesis, and by the presence of disease-causing mutations in these genes in human vascular diseases. However, the mechanism of action of endoglin is not well defined. Here we define a novel interaction between endoglin and the scaffolding protein β-arrestin2. Both co-immunoprecipitation and fluorescence confocal studies demonstrate the specific interaction between endoglin and β-arrestin2 in endothelial cells, enhanced by ALK-1 and to a lesser extent by the type II TGF-β receptor. The endoglin/β-arrestin2 interaction results in endoglin internalization and co-accumulation of endoglin and β-arrestin2 in endocytic vesicles. Whereas endoglin did not have a direct impact on either Smad 2/3 or Smad 1/5/8 activation, endoglin antagonized TGF-β-mediated ERK signaling, altered the subcellular distribution of activated ERK, and inhibited endothelial cell migration in a manner dependent on the ability of endoglin to interact with β-arrestin2. Reciprocally, small interfering RNA-mediated silencing of endogenous β-arrestin2 expression restored TGF-β-mediated ERK activation and increased endothelial cell migration in an endoglin-dependent manner. These studies define a novel function for endoglin, and further expand the roles mediated by the ubiquitous scaffolding protein β-arrestin2.

Transforming growth factor β (TGF-β) regulates diverse cellular processes including proliferation, differentiation, and apoptosis through a heteromeric complex of the type I (TβRI or ALK-5), type II (TβRII), and type III (TβRIII) TGF-β receptors (1). TGF-β signaling in normal epithelial cells begins with ligand binding to TβRII, which enables TβRII to recruit and phosphorylate TβRI. TβRI is then activated to recruit and phosphorylate the downstream transcription factors, Smads 2 and 3. Phosphorylated Smads 2 and 3 then form a complex with the co-Smad, Smad4, and the complex translocates to the nucleus to activate or repress genes in a context specific manner (2–5). TβRIII has primarily been considered to function as co-receptor, presenting ligand to its signaling partner, TβRII. However, recent studies have identified an essential role for TβRII during development, as demonstrated by the embryonic lethal phenotype of TβRIII knock-out mice and the essential, non-redundant role of TβRII during chick heart development (6, 7).

Endoglin is another TGF-β superfamily co-receptor expressed predominantly in vascular endothelial cells. Endoglin shares significant sequence homology with TβRII in the transmembrane and cytoplasmic domains, but participates in two distinct TGF-β signaling pathways in endothelial cells (8, 9). One pathway antagonizes endothelial cell proliferation through ALK-5 (TβRI), TβRII, and Smads 2/3. The other pathway promotes endothelial cell growth through an endothelial cell-specific type I receptor, ALK-1, and TβRII to activate Smad1/5/8 (10, 11). The balance of signaling between these opposing pathways regulates endothelial cell biology, including the activation and maturation phases of angiogenesis (12). Previous studies have suggested that endoglin inhibits the ALK-5/Smad 2/3 pathway while promoting ALK-1/Smad 1/5/8 signaling (11–13). However, the mechanism by which endoglin mediates these effects is unclear.

Like TβRII, endoglin demonstrates an essential role in mouse embryonic development, with a lethal phenotype in endoglin-null (endoglin−/−) mouse embryos due to defects in vascular development (14). In addition, mutations in endoglin or ALK-1 in humans result in the autosomal dominant disease, hereditary hemorrhagic telangiectasia, characterized by dilated vessels and arteriovenous malformations that lead to recurrent hemorrhage and shunting in the lung, brain, and the gastrointestinal tract (15–17). Conversely, the overexpression of endoglin in endothelial cells during tumor-induced angiogenesis suggests a role for endoglin in angiogenesis associated with many common cancers, including cancers of the breast.

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2 The abbreviations used are: TGF-β, transforming growth factor β; ERK, extracellular signal-regulated kinase; HMEC, human microvascular endothelial cell; MEEC, mouse embryonic endothelial cell line; MAPK, mitogen-activated protein kinase; HEK, human embryonic kidney; HA, hemagglutinin; GFP, green fluorescent protein; siRNA, small interfering RNA; PBS, phosphate-buffered saline.
and colon (12, 16, 18–20). How endoglin contributes to hereditary hemorrhagic telangiectasia and tumor-induced angiogenesis remains to be defined.

To define mechanisms by which the TGF-β superfamily coreceptors, TβRII and endoglin, exert their influence on signaling in a cell type-specific manner, we have been investigating how these receptors function and signal. We have demonstrated that the cytoplasmic domain of TβRII is essential for enhancing TGF-β signaling (21, 22), and functions in part by associating with autophosphorylated TβRII, and the scaffolding proteins GIPC and β-arrestin2 (22, 23). As the cytoplasmic domains of endoglin and TβRII are highly conserved, including precise conservation of the binding site for β-arrestin2, here we investigated the interaction of endoglin with β-arrestin2 and the impact on downstream TGF-β signaling and endothelial cell biology.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Antibodies**—HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10%
Roche Applied Science and Sigma, respectively. Endoglin antibody P3D1 was obtained from Chemicon. Phospho- and total ERK-specific antibodies were purchased from Cell Signal- ing. β-Arrestin2 polyclonal antibody was raised from rabbit immunized with β-arrestin2-specific peptide sequence (KPHDHIPLPRPQSAAP).

Transfection and Protein Expression—Lipofectamine 2000 was used to transiently transfect HEK 293 cells as described according to manufacturer’s protocol (Invitrogen). MEECs were nucleofected with Amaza nucleofection system. Briefly, ~1 × 10⁶ cells were electroporated with 4–6 μg of DNA using solution R and allowed expression for 40–48 h before harvest. Adenoviral infection of HMECs was performed at 100 multiplicity of infection. All cDNAs were PCR amplified with HA or FLAG epitope tag incorporated into the primers. Adenovirus of endoglin and GFP were generated according to the manufacturer’s instructions (Stratagene). The siRNA sequence targeting mouse β-arrestin2 5'-AAGGACCGGA- AAGUGUUCGUG-3 is reported elsewhere (43). Briefly, knockdown of β-arrestin2 expression was achieved by nucleofection of 15 μg of RNA in 10⁶ cells and incubated for 72 h.

Immunofluorescence—HEK 293 or endoglin-TβRII-MEECs expressing HA-tagged endoglin alone (B), HA-tagged endoglin alone (B), β-arrestin2-GFP with HA-tagged endoglin (C–E), β-arrestin2-GFP, HA-tagged endoglin and TβRII (F–H), β-arrestin2-GFP with HA-tagged endoglin-T650A (I–K), or β-arrestin2-GFP with endoglin-S (L–N) were fixed, permeabilized, and stained to visualize epitope-tagged endoglin localization. Confocal images (× 100) of β-arrestin2-GFP (A, C, F, I, and L) and HA-endoglin (B, D, G, J, and M) were obtained. β-Arrestin2-GFP co-localizes with HA-endoglin in the merged images (E and H), but not with endoglin-T650A (K). Data are representative of three independent experiments.

fetal bovine serum. Human microvascular endothelial cells (HMEC) were grown in MCDB-131 medium (Invitrogen), supplemented with 10% fetal bovine serum, 1 μg/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (Sigma), and 2 mM L-glutamine. Endoglin-null (endoglin−/−) and control mouse embryonic endothelial cell lines (MEEC) (endoglin+/+) were grown in MCDB-131 supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate (Invitrogen), 100 μg of heparin (Sigma), and 50 μg/ml endothelial cell growth supplement (Sigma). HA and FLAG epitope antibodies were purchased from transient endoglin expression in HEK 293 and MEECs for 1 h at 25 °C. Cells were washed with PBS, incubated with Cy3-conjugated rabbit secondary antibody for 1 h at room temperature, washed, then mounted with Vectashield. In phospho-ERK staining, MEECs were prepared as previously mentioned, then probed with phospho-ERK antibody (Cell Signaling). Cy3-conjugated rabbit secondary was again used before mounting on slides. Immunofluorescence images were obtained using Zeiss laser scanning confocal microscope (LSM-510).
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Co-immunoprecipitation—HEK 293 or endoglin−/− MEECs expressing FLAG epitope-tagged β-arrestin2 and/or HA epitope-tagged endoglin (expressed at ∼3–5-fold of endogenous levels) were washed with PBS, then lysed on ice with lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 10 mM NaF, 10% (w/v) glycerol) supplemented with protease inhibitors (Sigma protease inhibitor mixture) and phosphatase inhibitors (Sigma phosphatase inhibitor mixture). The lysates were preclarified by centrifugation, and incubated with FLAG antibody and protein G-agarose beads for 4 h at 4 °C. The immunoprecipitates were collected by centrifugation, pellets were washed with lysis buffer, and stored in 2× sample buffer prior to Western blot analyses.

Wound Healing Migration Assay—Cell monolayers of endoglin+/+ and endoglin−/− MEECs in 12-well plates were wounded and then monitored for up to 14 h. Wound-induced cell migration was measured by monitoring the distance between cells lining the wound edge and then normalized to time 0 h.

RESULTS

Endoglin Associates with β-Arrestin2 in Epithelial and Endothelial Cells—We previously reported a novel association between TβRIII and the scaffolding protein β-arrestin2 in HEK 293 cells (23). The interaction between TβRIII and β-arrestin2 was stimulated by TβRIII-mediated phosphorylation of the cytoplasmic domain of TβRIII, and resulted in their co-localization in internalized endocytic vesicles and subsequent down-regulation of TGF-β-mediated inhibition of cellular proliferation (23). As endoglin and TβRIII have precise conservation of the β-arrestin2 binding site (Fig. 1A), we examined whether endoglin also interacts with β-arrestin2. Initially, HA-tagged endoglin and FLAG-tagged β-arrestin2 were transiently co-expressed in HEK 293 cells followed by co-immunoprecipitation. Immunoprecipitation of FLAG-tagged β-arrestin2 with FLAG antibody resulted in the co-precipitation of HA-tagged endoglin (Fig. 1B, lane 2).

As endoglin forms complexes with several TGF-β receptors, including TβRIII, ALK-1, and ALK-5, we examined the effect of expression of these signaling receptor partners for endoglin on the endoglin/β-arrestin2 interaction by co-expression in HEK 293 cells. The endoglin/β-arrestin2 interaction was consistently increased in the presence of TβRIII (Fig. 1B, lane 3) and more so by ALK-1 (Fig. 1B, lane 4), but only by a modest extent in the presence of ALK-5 (Fig. 1B, lane 5).

The interaction of TβRIII with β-arrestin2 depends upon threonine 841 in the cytoplasmic domain of TβRIII (23). Based upon the sequence homology of the cytoplasmic domains of TβRIII and endoglin (Fig. 1A), we reasoned that the interaction of endoglin and β-arrestin2 might depend upon the corresponding threonine 650 in the cytoplasmic domain of endoglin. As predicted, site-directed mutagenesis of threonine residue 650 into alanine (endoglin-T650A) abrogated the endoglin/β-arrestin2 interaction, as determined by co-immunoprecipitation studies (Fig. 1B, lane 6 versus lane 2).

The enhanced co-immunoprecipitation of endoglin and β-arrestin2 in the presence of ALK-1 suggested that the endoglin/β-arrestin2 interaction is mediated, at least in part, by phosphorylation of endoglin by ALK-1. Indeed we and others (24, 25) have observed phosphorylation of endoglin by ALK-1, and this appears to occur primarily on threonine residues (24, 25). To further investigate the role of ALK-1, wild-type ALK-1, and ALK-1 (Q200D), a point mutant that renders the kinase constitutively active, were used in a co-immunoprecipitation study. In the absence of TGF-β stimulation or serum, the endoglin/β-arrestin2 interaction was significantly reduced when compared with cells in the presence of serum (Fig. 1, C, lane 2 versus B, lane 2). As before, wild-type ALK-1 increased the endoglin/β-arrestin2 interaction (Fig. 1C, lane 3 versus lane 2), and constitutively active ALK-1 (Q200D) further increased the endoglin/β-arrestin2 interaction (Fig. 1C, lane 4 versus lane 5), whereas there were no detectable levels of endoglin-T650A interacting with β-arrestin2, even with ALK-1 present (Fig. 1C, lanes 5 and 6). Taken together, these results suggest that threonine 650 provides a structural queue for the interaction of endoglin with β-arrestin2, and ALK-1, presumably through phosphorylation of endoglin on or near this region, is able to increase this interaction.

As endoglin is primarily expressed in endothelial cells, we also examined whether endoglin interacts with β-arrestin2 in endothelial cells. For these studies we used an endoglin-null (endoglin−/−) MEEC to allow selective expression of either the wild-type or mutant endoglin (endoglin-T650A) and comparison of their specificity toward β-arrestin2 in an endothelial cell environment. Again, in MEECs, wild-type endoglin transiently co-expressed with β-arrestin2 co-immunoprecipitated (Fig. 1D, lane 3), whereas endoglin-T650A did not (Fig. 1D, lane 2). As with other β-arrestin2 interacting receptors, we were not able to demonstrate interaction of endogenous endoglin with endogenous β-arrestin2. However, we were able to demonstrate the interaction of endogenous β-arrestin2 with overexpressed endoglin using β-arrestin2 antibody (Fig. 1E, lane 3), with pre-immune β-arrestin2 serum and endoglin antibody serving as negative and positive controls, respectively (Fig. 1E, lanes 1 and 3). Taken together these studies establish that endoglin associates with β-arrestin2 in both HEK 293 cells and MEECs, and this association is promoted primarily by ALK-1 and TβRIII, the two receptors that are known to phosphorylate endoglin.

Endoglin and β-Arrestin2 Co-internalize in Endocytic Vesicles—As β-arrestin2 functions, in part, to mediate the internalization of interacting receptors via clathrin-coated pits (26, 27), we investigated whether the endoglin/β-arrestin2

### Table 1

| Transfection with β-arrestin2-GFP | β-Arrestin2-GFP vesicle formation (%) |
|----------------------------------|-------------------------------------|
| Endo               | 36 ± 10 |
| Endo + TβRII       | 52 ± 12 |
| Endo + ALK-1       | 59 ± 2 |
| Endo + ALK-5       | 14 ± 3 |
| Endo-T650A         | <5     |
| Endo-S             | <5     |
**β-Arrestin2 Associates with Endoglin**

resulted in the internalization of endoglin into endocytic vesicles, where endoglin co-localized with β-arrestin2-GFP (Fig. 2, C–E). In contrast, expression of the alternatively spliced form of endoglin lacking the β-arrestin2 interaction motif, endoglin-S (Fig. 1A), or endoglin-T650A, failed to redistribute β-arrestin2-GFP (Fig. 2, I and L), and both endoglin-S and endoglin-T650A remained on the cell surface even in the presence of β-arrestin2 (Fig. 2, J and M), confirming the site of interaction between endoglin and β-arrestin2, and the specificity of this interaction.

As the association between endoglin and β-arrestin2 was enhanced in the presence of ALK-1 and TβRII in the co-immunoprecipitation studies, we next examined how the presence of the signaling co-receptors influenced the co-localization and internalization process of endoglin/β-arrestin2. Whereas expression of ALK-1, TβRII, or ALK-5 did not change the internalization profile of the endoglin/β-arrestin2 complex (Fig. 2, F–H, data not shown), there was a significant increase in the frequency of cells displaying endocytic vesicles. We made a quantitative assessment of the contribution of expression of TβRII, ALK-1, and ALK-5 by determining the percentage of cells expressing both endoglin and β-arrestin2 that exhibited an endocytic pattern of β-arrestin2-GFP as opposed to a diffuse cytoplasmic pattern. As indicated in Table 1, ~35% of the cells expressing β-arrestin2-GFP and endoglin displayed internalization in HEK 293 cells. In the presence of TβRII and ALK-1, the percentage of cells expressing β-arrestin2-GFP and endoglin displaying an internalization pattern increased to ~50 and ~60% of cells, respectively. These results correlated closely with the effects of TβRII and ALK-1 on co-immunoprecipitation of β-arrestin2-GFP and endoglin (Fig. 1B).

To establish whether the endoglin/β-arrestin2 interaction resulted in their co-internalization using the β-arrestin2-GFP interaction assay (28). These studies were performed both in HEK 293 cells, to explore the contribution of other endoglin interacting receptors, and in endoglin−/− MEECs. As in other cell systems, diffuse cytoplasmic distribution of β-arrestin2-GFP was observed in HEK 293 cells (Fig. 2A). However, co-expression with endoglin resulted in the redistribution of β-arrestin2-GFP into endocytic vesicles (Fig. 2C). In the absence of β-arrestin2, endoglin was localized at the plasma membrane (Fig. 2B). Co-expression with β-arrestin2 association resulted in their co-internalization using the β-arrestin2-GFP interaction assay (28). These studies were performed both in HEK 293 cells, to explore the contribution of other endoglin interacting receptors, and in endoglin−/− MEECs. As in other cell systems, diffuse cytoplasmic distribution of β-arrestin2-GFP was observed in HEK 293 cells (Fig. 2A). However, co-expression with endoglin resulted in the redistribution of β-arrestin2-GFP into endocytic vesicles (Fig. 2C). In the absence of β-arrestin2, endoglin was localized at the plasma membrane (Fig. 2B). Co-expression with β-arrestin2

![Image](image-url)

**FIGURE 3. Recruitment and co-localization of β-arrestin2 with endoglin in endothelial cells.** β-Arrestin2-GFP alone (A), endoglin alone (B), β-arrestin2-GFP with HA-tagged endoglin (C–E), or with HA-tagged endoglin-T650A (F–H) were transiently expressed in endoglin−/− MEECs by electroporation. Cells were fixed, permeabilized, and stained to visualize epitope-tagged endoglin localization, and confocal images (×100) of β-arrestin2-GFP (A, C, and E), and HA-tagged endoglin constructs (D and G) obtained. β-Arrestin2-GFP exclusively co-localized with vivo-endoglin wild-type in the merged image (E). Endoglin and β-arrestin2 siRNA were electroporated into endoglin−/− MEECs (I–K). Cells were stained with β-arrestin2 antibody to visualize endogenous expression of β-arrestin2 before and after treatment with β-arrestin2 siRNA (L versus I, respectively). Data are representative of two independent experiments.
Endoglin Regulates TGF-β-mediated Mitogen-activated Protein Kinase (MAPK) Signaling—Having established the interaction of endoglin and β-arrestin2, we sought to identify a role for the endoglin/β-arrestin2 interaction. As the interaction of β-arrestin2 with endoglin/β-arrestin2 resulted in the down-regulation of TGF-β signaling, we first examined the Smad pathways immediately downstream of endoglin, including Smad 2 and Smad 1/5/8 in endoglin−/− and endoglin+/+ MEECs using phospho-specific Smad antibodies. TGF-β treatment of endoglin−/− and endoglin+/+ MEECs resulted in comparable levels of both Smad 1/5/8 and Smad 2 phosphorylation (Fig. 4A) and Smad 1/5 and Smad 2 nuclear translocation (data not shown). To test whether β-arrestin2 directly impacts the Smad pathways, siRNA silencing was again employed. Whereas β-arrestin2 expression was significantly reduced (Fig. 4B), there was no effect on levels of Smad 1/5/8 and Smad 2 phosphorylation in either endoglin−/− or endoglin+/+ MEECs (Fig. 4C). Taken together, these results suggest that in endothelial cells, endoglin or the interaction of endoglin with β-arrestin2 do not have prominent effects on TGF-β1 signaling through Smads.

Although Smad-mediated signaling is well established as a prominent mechanism for TGF-β signaling, TGF-β is also known to signal through Smad-independent pathways, including the MAPK pathways (29–32). Our findings that endoglin did not have a significant impact on Smad activation, and β-arrestin2 has been demonstrated to link interacting receptors to the MAPK pathways (33–35), we explored how expression was significantly reduced (Fig. 4D). To determine whether endoglin participates in MAPK activation in endothelial cells, we monitored the levels of phosphorylated ERK1/2, p38, and c-Jun NH2-terminal kinase (JNK) activation in endoglin−/− and endoglin+/+ MEECs in response to TGF-β1. Whereas TGF-β1 activated all three MAPK pathways in both endoglin−/− and endoglin+/+ MEECs (data not shown), the ERK pathway in particular clearly displayed an endoglin-dependent TGF-β1 response (Fig. 5). When assessing ERK1/2 phosphorylation, endoglin−/− MEECs consistently demonstrated enhanced ERK1/2 phosphorylation in response to TGF-β1 treatment compared with endoglin+/+ MEECs (Fig. 5), suggesting a negative role for endoglin on ERK1/2 signaling. Specifically, in dose–response experiments, TGF-β1 resulted in increased ERK1/2 phosphorylation from 2 to 100 pm endoglin−/− MEECs, with little to no activation in endoglin+/+ MEECs (Fig. 5A). To characterize the kinetic properties of ERK activation in these cells, time course experiments were performed using 10 pm TGF-β1. Consistently, ERK1/2 activation in endoglin+/+ MEECs remained minimal, whereas time-dependent activation was observed in endoglin−/− MEECs, with maximal activation at 60 min (Fig. 5B). Moreover, varying serum deprivation conditions prior to TGF-β1 treatment had an impact on the degree to which endoglin down-regulated ERK activation, with a shorter time interval of serum deprivation resulting in more active repression of ERK activation in response to TGF-β1 in endoglin+/+ cells, and stronger ERK induction in endoglin−/− cells (Fig. 5C).

To establish the relevance of endoglin-mediated regulation of ERK1/2 activation in endothelial cells, we used another endothelial cell system, HMECs. As HMECs endogenously express endoglin, it was hypothesized that increasing the expression of endoglin would further attenuate ERK activation. To this end, we employed adenoviral infection to overexpress either endoglin or GFP as negative control. Consistent with the results in MEECs, increasing the expression of endoglin sup-
pressed ERK1/2 activation (Fig. 5D). These results confirmed an inhibitory role of endoglin on ERK1/2 activation, and supported the observation that the difference in ERK1/2 activation seen in wild type and endoglin null MEECs was due to a difference in endoglin expression.

The Endoglin/β-Arrestin2 Interaction Mediates the Down-regulation of ERK Activation—The TGF-β dose-response and time course experiments in different endothelial cell lines strongly suggested an endoglin-dependent down-regulation of TGF-β-mediated ERK activation. Given that β-arrestin2 also serves as a scaffolding protein to link interacting receptors to the MAPK pathways, we explored whether the endoglin/β-arrestin2 complex had a role in regulating ERK pathway activation in endothelial cells. Endoglin+/+ MEECs, endoglin−/− MEECs, and endoglin−/− cells ectopically expressing wild-type endoglin, endoglin-T650A, or endoglin-S were treated with TGF-β and ERK1/2 activation assessed (Fig. 6A). As before, the endoglin+/+ MEECs demonstrated an attenuated response to TGF-β1 compared with endoglin−/− MEECs (Fig. 6A, lane 2 versus lane 4) and phospho-ERK levels were significantly reduced upon ectopic expression of endoglin in endoglin−/− MEECs (Fig. 6A, lane 6 versus lane 4). In contrast, expression of either endoglin-T650A or endoglin-S, neither of which is able to interact with β-arrestin2 (Figs. 1–3), failed to suppress ERK activation as efficiently as wild-type endoglin (Fig. 6A, lanes 8 and 10 versus lane 6). These results strongly suggested that the endoglin/β-arrestin2 complex modulates the down-regulation of ERK activation in endothelial cells.

To further investigate a role for β-arrestin2 in ERK activation we utilized siRNA-mediated silencing of endogenous β-arrestin2 expression. Whereas β-arrestin2 silencing in endoglin+/+ MEECs restored TGF-β-mediated ERK activation (Fig. 6B, lanes 1 and 2 versus 5 and 6), endoglin−/− MEECs were less affected (Fig. 6B, lanes 3 and 4 versus 7 and 8), supporting a role for β-arrestin2 in regulating ERK activation in an endoglin-dependent fashion.

Subcellular localization of MAPK pathway components has significant implications on signaling, with ERK activation restricted to the cytoplasmic or nuclear compartments having distinct signaling specificities and properties (36–39). Given the importance of the spatial organization of activated ERKs in MAPK signaling, and the role of β-arrestin2 in altering the sub-
β-Arrestin2 Associates with Endoglin

![Image](374x26 to 401x38)

**FIGURE 6. Down-regulation of ERK activity is mediated by endoglin/β-arrestin2 interaction.** Endoglin+/+ and endoglin−/− MEECs were assessed for ERK activation in response to TGF-β1 (10 pm for 10–15 min) (A, lanes 1–4, respectively). The effect of wild-type endoglin, endoglin-T650A, and endoglin-S expression on ERK activity was assessed in endoglin−/− cells (A, lanes 5–10, respectively). Whole cell extracts were prepared by direct lysis with sample buffer and the levels of ERK1/2 phosphorylation assessed by use of total ERK antibody (first panel). Equal protein levels were verified by use of total ERK antibody (second panel). Endoglin and β-arrestin2 expression levels were also verified (lower two panels). B, TGF-β1-mediated ERK activation (10 pm for 10–15 min) was assessed in endoglin+/+ and endoglin−/− MEECs upon no treatment (lanes 1–4, upper panel) or treatment with β-arrestin2 siRNA (lanes 5–8, upper panel). Total ERK and β-arrestin2 levels were detected using ERK and β-arrestin2-specific antibodies (middle and lower panels, respectively). Data are representative of three independent experiments.

cellular localization of endoglin, we investigated the effect of endoglin on the subcellular localization of phosphorylated ERK in MEECs (Fig. 7, Table 2). In endoglin+/+ MEECs, phospho-ERK was localized in a diffuse cytoplasmic and nuclear distribution (Fig. 7A) and TGF-β treatment yielded no discernable change in the distribution while decreasing the intensity of the phospho-ERK signal (Fig. 7B), consistent with results seen by Western blot analysis (Fig. 6). In contrast, we consistently observed a strong perinuclear accumulation of phospho-ERK in 70–80% of the endoglin−/− cells (Fig. 7C). The strong perinuclear accumulation of phospho-ERK was increased upon 10 min of TGF-β1 stimulation (Fig. 7D), and persisted up to 60 min in endoglin−/− MEECs (data not shown). Whereas TGF-β1 treatment increased the intensity of the phospho-ERK signal (Fig. 7D), it did not significantly alter the perinuclear localization profile.

Because ectopic endoglin expression restored the endoglin+/+ phenotype to endoglin−/− MEECs in terms of localization of ERK1/2 phosphorylation. Wild-type endoglin expression altered the distribution of phospho-ERK in endoglin−/− MEECs, from a strong perinuclear pattern to a diffuse cytoplasmic and nuclear pattern irrespective of TGF-β treatment, such that only about 10% cells retained a perinuclear localization (Fig. 7, E and F). This pattern was similar to the endoglin+/+ MEECs, and confirmed that the difference in phospho-ERK1/2 localization between endoglin-null and wild-type MEECs was due to endoglin. In contrast, endoglin-T650A was only partially successful in restoring the endoglin+/+ MEEC phenotype, with endoglin−/− MEECs expressing endoglin-T650A displaying both diffuse cytoplasmic and perinuclear localization phenotypes under non-stimulated and TGF-β-stimulated conditions (Fig. 7, G and H), and increased phospho-ERK intensity with TGF-β stimulation (Fig. 7H), with ~55 and 40% perinuclear localization in the unstimulated and stimulated conditions, respectively. In all cases, the duration of TGF-β stimulation from 10–15 to 60 min did not alter their diffuse cytoplasmic or perinuclear localization (data not shown).

To determine whether endoglin was specifically responsible for the cytoplasmic/perinuclear retention of ERK, we subjected these cells to normal serum conditions. Serum has been shown to induce ERK activation and nuclear translocation in cultured cells (40), and as Fig. 7 (I and J) demonstrates, the presence of serum caused a significant nuclear accumulation of phospho-ERK in both endoglin−/− and endoglin+/+ MEECs. Taken together, our data strongly implicate a role for the endoglin/β-arrestin2 complex in regulating the ERK pathway, both in terms of ERK induction and in the context of their spatial organization.

The Endoglin/β-Arrestin2 Interaction Regulates Endothelial Cell Migration—To establish whether the endoglin/β-arrestin2 interaction regulated further downstream biological responses we examined endothelial cell migration using the well characterized wound-healing assay. We initially investigated the migration of endoglin+/+ and endoglin−/− MEECs, and observed that the endoglin−/− cells migrated faster than the endoglin+/+ MEECs (Fig. 8, A and B). To determine whether this difference in migration rates was due to endoglin, we expressed endoglin or endoglin-T650A in endoglin−/− MEECs. Expression of endoglin, but not endoglin-T650A, effectively reduced the migration rate of endoglin−/− MEECs (Fig. 8C), suggesting that endothelial cell migration was regulated, in part, by the endoglin/β-arrestin2 interaction. Consistent with these findings, the migration rate of endoglin+/+ MEECs was markedly enhanced upon siRNA-mediated β-arrestin2 knockout, whereas endoglin−/− MEECs were not significantly affected (Fig. 8D).

**DISCUSSION**

Defining mechanisms for TGF-β signaling is essential for understanding many aspects of cell biology, cancer biology, and angiogenesis, as well as for effectively targeting the TGF-β signaling pathway for the treatment of human diseases. In the present studies we investigated the interaction between endoglin and the scaffolding protein, β-arrestin2, their impact on downstream signaling to both Smad-dependent and Smad-
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Endoglin/β-arrestin2 interaction alters the subcellular distribution of activated ERK. Endoglin+/+ and endoglin−/− MEECs were serum starved for 3–4 h prior to 10 μM TGF-β treatment for 15 min. Cells were fixed, permeabilized, and probed with phospho-ERK antibody. The distribution of phospho-ERK in endoglin+/+ cells (A and B), endoglin−/− (C and D), endoglin+/− cells with re-expression of endoglin (E and F), or endoglin-T650A (G and H) is shown. As a control, endoglin+/+ and endoglin−/− MEECs were fixed and permeabilized without serum starvation (I and J). The density of phospho-ERK signal in the cytoplasmic and perinuclear regions of the given cells (A–H), or the nucleus (I and J), was quantified using Image J. Data are representative of three independent experiments.

TABLE 2
Perinuclear localization of phospho-ERK (% cells)

| MEECs     | −TGF-β | +TGF-β | %    |
|-----------|--------|--------|------|
| Endoglin++ | 0      | 0      | 0    |
| Endoglin−− | 80 ± 5 | 70 ± 5 |      |
| Endoglin−+/wt-endoglin | 10 ± 10 | 10 ± 5 |      |
| Endoglin−+/endo T650A  | 55 ± 8 | 40 ± 15 |      |

The effects of endoglin on the activation of the Smad pathways (Smad 1/5 versus Smad 2/3) in endoglin−− and endoglin+/− MEECs have recently been reported (42). In this study, it was noted that whereas endoglin did not significantly alter the levels of Smad 2/3 activation, the levels of Smad 1/5/8 activation in endoglin−− were higher than that of control endoglin+/+ cells, especially during the early time points. Our data are partially inconsistent with these former results, as we observed no discernable difference in Smad 1/5/8 activation in the two cell
β-Arrestin2 Associates with Endoglin

We further characterized the impact of the endoglin/β-arrestin2 interaction on the subcellular distribution of phospho-ERK. We were able to demonstrate that the two distinct subcellular distributions of phospho-ERK are at least partly the result of the endoglin/β-arrestin2 interaction, as evidenced by the partial restoration of perinuclear accumulation of phospho-ERK by endoglin (Fig. 7 and Table 2). In addition, we also observed that there was no significant nuclear accumulation of ERK in endoglin−/− or endoglin+/+ MEECs in response to TGF-β1. This was also apparent in endoglin−/− cells ectopically expressing endoglin or endoglin-T650A, and in HMECs (data not shown). As cytosolic ERK activation is thought to mediate short-term responses, we hypothesize that TGF-β1-mediated ERK activation in endothelial cells is a transient or short-term response, designed to target a subset of cytoplasmic proteins in the course of the MAPK signaling cascade. Current efforts are aimed at identifying these cytoplasmic proteins targeted by TGF-β1-responsive ERK in endothelial cells and the functional consequences of this signaling mechanism.

The endoglin/β-arrestin2 interaction also is of functional significance in endothelial cells, with endoglin, but not endoglin-T650A, exerting a negative influence on cell migration, and silencing of endogenous β-arrestin2 increasing endothelial cell migration in an endoglin-dependent manner. As endothelial cell migration is an important factor in regulating both normal and disease-associated angiogenesis, these studies suggest that the regulation of endoglin trafficking and signaling by β-arrestin2 has an important role in regulating angiogenesis. The mechanism by which β-arrestin2 regulates endothelial cell migration and the effects on angiogenesis are currently under investigation.

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