Regulation of TGF-β receptor heterooligomerization and signaling by endoglin

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INTRODUCTION

Ligands from the transforming growth factor-β (TGF-β) superfamily regulate endothelial cell migration and angiogenesis (Goumans et al., 2009; Piniidiyaarachchi et al., 2009; Lee et al., 2012). They signal via a network of receptors, which in endothelial cells includes the endothelial cell coreceptor endoglin, the type II TGF-β receptor (TβRII), and the type I receptors ALK5 (TβRI) and ALK1 (expressed predominantly in endothelial cells; Massague, 1998; Goumans et al., 2003a; Bernabeu et al., 2009). Both ALK1 and endoglin, as well as ALK5 and TβRII, have essential roles in endothelial cell biology and angiogenesis, as demonstrated by germline mutations in endoglin and ALK1 resulting in the human vascular disease hereditary hemorrhagic telangiectasia (McAllister et al., 1994; Johnson et al., 1996; Bourdeau et al., 1999). In addition, mice lacking ALK1 (Oh et al., 2000; Umess et al., 2000), endoglin (Bourdeau et al., 1999; Li et al., 1999; Arthur et al., 2000), ALK5 (Larsson et al., 2001), or TβRII (Oshima et al., 1996) all exhibit embryonic lethal phenotypes due to vascular defects. Specifically, endoglin is critically important in developmental settings in the cardiovascular system, as endoglin-null mouse embryos show defects in vessel and heart development, resulting in a lethal phenotype (Li et al., 1999; Arthur et al., 2000). Moreover, in the adult vasculature, hereditary hemorrhagic telangiectasia was shown to occur after endoglin haploinsufficiency (McAllister et al., 1994; Pece et al., 1997).

The minimal TGF-β signaling complex, capable of signaling via the canonical Smad pathway and several non-Smad pathways, consists of a TβRII dimer, the dimeric TGF-β ligand, and a pair of type I receptors (Shi and Massague, 2003; Derynck and Miyazono, 2008; Ehrlich et al., 2011). TGF-β binding triggers phosphorylation and activation of the type I receptor, which induces Smad signaling by phosphorylating R-Smads, followed by their hetero-oligomerization with Smad4 and translocation to the nucleus, where they regulate transcription (Shi and Massague, 2003; Peng and Derynck, 2005;
Schmierer and Hill, 2007; Heldin et al., 2009). TGF-β stimulus in endothelial cells activates Smad2/3 and Smad1/5/8 pathways via ALK5 and ALK1, respectively (Chen and Massague, 1999; Goumans et al., 2003a; Shi and Massague, 2003; Moustakas and Heldin, 2009). In endothelial cells, ALK1 and ALK5 form a mutual complex with TβRII (Goumans et al., 2003a), and it was proposed that ALK1/ALK5 cross-talk leads to a role for ALK5 in TGF-β–induced ALK1 signaling and for ALK1 in inhibiting ALK5 signaling (Goumans et al., 2003b). ALK1 can also be activated by BMP-9 or BMP-10 to signal via Smad1/5/8 (Lebrin et al., 2004).

TGF-β signaling can be modulated by structurally diverse coreceptors. The most abundant TGF-β superfamily coreceptor in endothelial cells is endoglin (Goumans et al., 2003a; Gatza et al., 2010). Endoglin is a 95-kDa glycoprotein that binds TGF-β1 and -β3 in conjunction with TβRII (Barbara et al., 1999) and can also bind directly BMP-9 or -10 (David et al., 2007, 2009). Endoglin has been reported to increase TGF-β1 binding to TβRII (Letamendia et al., 1998; Barbara et al., 1999), with controversial effects on TGF-β signaling (Lastres et al., 1996; Letamendia et al., 1998; Barbara et al., 1999; Guerrero-Esteo et al., 2002). Endoglin-mediated regulation of ALK1 signaling to Smad1/5/8 is contentious; loss-of-function studies with either small interfering RNA (siRNA)–mediated knockdown or in endoglin-null endothelial cells suggest either positive or negative regulation (Lebrin et al., 2004; Pece-Barbara et al., 2005). BMP-9 was also reported to promote Akt activation and endothelial cell tube stability via endoglin/GAIP-interacting protein C-terminal (GIPC)–mediated scaffolding to PI3K/Akt (Lee et al., 2012). Whereas endoglin is regulated by phosphorylation of its short cytoplasmic domain by TβRII, ALK5, and ALK1 (Guerrero-Esteo et al., 2002; Koleva et al., 2006; Ray et al., 2010), the role of the interactions between endoglin and the signaling TGF-β receptors in the regulation of TGF-β responses remains to be defined.

Multiple experimental approaches have been used to study the mode, extent, and function of TGF-β receptor oligomerization (reviewed in Ehrlich et al., 2011). Crystallographic studies have shown that the extracellular domains of TβRII, ALK5, and dimeric ligand (TGF-β1 or -β3) form ternary complexes in which each receptor appears as a dimer (Groppe et al., 2008; Radaev et al., 2010). However, such studies yield only a static view of the endpoint of complex formation and do not necessarily reflect interaction between the full-length receptors situated in their native milieu, the plasma membrane. To address these issues, we developed the method of patch/FRAP (fluorescence recovery after photobleaching; Henis et al., 1990) and applied it to study homomeric and heteromeric interactions among type II and type I TGF-β superfamily receptors (Rechtman et al., 2009; Marom et al., 2011). The emerging picture is that the cell surface population of these receptors comprises a heterogeneous and dynamic mixture of monomeric, homodimeric, and heteromeric complexes modulated by ligand binding (Ehrlich et al., 2011). However, the interactions of ALK1 with itself, with TβRII, and/or with endoglin have not been investigated quantitatively in the cell membrane. Moreover, such data are lacking for the interactions of endoglin with itself, with TβRII, and with ALK5. Here we study these questions in live cells using epitope-tagged TGF-β receptor and endoglin constructs.

RESULTS
Endoglin forms stable homomeric complexes at the cell surface
The TGF-β coreceptor endoglin regulates TGF-β signaling and interacts with scaffolding proteins such as GIPC and β-arrestin2 (Lee and Blobe, 2007; Lee et al., 2008). However, the molecular composition and dynamics of endoglin complexes with the full-length signaling TGF-β receptors situated at the cell plasma membrane have not been characterized. As the first step, we explored the homologeric interactions of endoglin and their potential dependence on GIPC and β-arrestin2 binding. The extent and dynamics of homomeric or heteromeric receptor complexes can be studied by the patch/FRAP method (Henis et al., 1990), which we recently used to investigate TGF-β and bone morphogenetic protein receptor complexes (Rechtman et al., 2009; Marom et al., 2011). In patch/FRAP, one receptor is patched and laterally immobilized by cross-linking at the surface of live cells with a double layer of bivalent immunoglobulin Gs (IgGs); the effect on the lateral diffusion of a coexpressed receptor bearing a different extracellular epitope tag, labeled by monovalent fluorescent Fab′ fragments, is then measured by FRAP (see Materials and Methods). Depending on the FRAP timescale relative to the association–dissociation kinetics between the immobilized and the Fab′-labeled (non–cross-linked) receptors, one may observe a reduction in the mobile fraction (Rf) or in the lateral diffusion coefficient (D) of the latter. Reduction in Rf is obtained when the complex lifetimes are long relative to the characteristic FRAP recovery time, since bleached Fab′-labeled receptor molecules would not undergo measurable dissociation from the cross-linked patches during the FRAP measurement. Conversely, a short complex lifetime would lead to multiple association–dissociation cycles during the FRAP recovery phase, resulting in a slower diffusion rate (Henis et al., 1990; Rechtman et al., 2009).

To study endoglin homo-oligomerization, we coexpressed hemagglutinin (HA)–or myc-tagged endoglin at the surface of COS7 cells and conducted patch/FRAP studies on the tagged receptors. IgG-mediated cross-linking and immobilization of HA-endoglin significantly reduced (41%) the Rf of myc-endoglin, with no effect on D (Figure 1). Such an effect characterizes stable interactions between the differently tagged endoglin pairs (Henis et al., 1990; Rechtman et al., 2009), suggesting the formation of homomeric endoglin complexes that are stable on the time scale of the FRAP measurements (minutes). These complexes are detected already in the absence of exogenous ligand and are not affected by incubation with TGF-β1 or BMP-9. Of note, a statistical correction has to be applied to obtain the percentage of endoglin in homodimers (Ehrlich et al., 2011; Marom et al., 2011), since the probabilities of homodimer formation are 1:2:1 for dimers containing myc/myc, myc/HA plus HA/myc, and HA/HA. Because only myc-endoglin in mixed complexes with HA-endoglin would undergo immobilization and myc/myc endoglin complexes are labeled at twice the intensity of myc/HA endoglin complexes, the percentage reduction in Rf(ΔRf) in patch/FRAP studies on homomorphic complexes should be multiplied by 2 to derive the percentage of homodimerization (82%). Because endoglin is endogenously expressed in endothelial cells, we repeated the foregoing patch/FRAP studies in bEnd.3 endothelial cells transfected with myc-endoglin and HA-endoglin. These cells were chosen because they respond to TGF-β in both the Smad2/3 and Smad1/5/8 pathways (Figure 1G). The results of the patch/FRAP studies in these cells (Figure 1, E and F) were very similar to those obtained in COS7 cells, except that the initial Rf and D values of endoglin were somewhat lower in the bEnd.3 cells, reflecting the different cellular context. As in the COS7 cells, the reduction in the Rf value of myc-endoglin upon cross-linking HA-endoglin was high (47%), suggesting a high level in homodimers (47 × 2 = 94%), with no change in D, as expected for stable interactions. In line with the studies on COS7 cells, there were no significant effects of either TGF-β1 or BMP-9 on the patch/FRAP results.

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To explore whether interactions involving the cytoplasmic domain of endoglin, such as with GIPC or β-arrestin2, are involved in the observed endoglin homooligomerization, we coexpressed wild-type (WT) myc-endoglin-WT with HA-endoglin-WT or with HA-endoglin mutants lacking interaction motifs with either GIPC (endoglin-Del) or β-arrestin2 (endoglin-T650A), cross-linked myc-endoglin-WT, and measured the effects on the lateral diffusion of the HA-endoglin mutants (Figure 2). The $R_f$ and $D$ values measured for the two HA-endoglin mutants without cross-linking were indistinguishable from that of HA-endoglin-WT (or myc-endoglin-WT; Figure 1), indicating that interactions of endoglin with GIPC or β-arrestin2 have a negligible effect on its lateral mobility. Of importance, the Δ$R_f$ values of each HA-endoglin mutant upon cross-linking myc-endoglin were similar to the Δ$R_f$ measured for HA-endoglin-WT, demonstrating that the homomeric interactions of endoglin do not depend on either GIPC or β-arrestin2 binding. The results in Figures 1 and 2 are in line with the reported disulfide-bond homo-dimerization of endoglin via its extracellular domain (Gougos and Letarte, 1988). However, it may well be that the endoglin subunits in the dimer interact with each other also without such an S–S bond, since reduction of the cells with 2 mM...
endoglin—a role that is independent of ligand (Figure 3, A–D). Similar results were obtained in the presence or absence of ligand (TGF-β1 addition (Figure 5C; no endoglin). These results are in full accord with our earlier observations, which show that TGF-β1 enhances complex formation between TβRII and TβRII (Rechtman et al., 2009). Of note, cross-linking HA-TβRII in the presence of endoglin already yields maximal reduction in $R_I$ of myc-ALK5 (~50%), which does not increase further upon incubation with TGF-β1 (Figure 5C; + endoglin). We conclude that endoglin forms mutual complexes with both TβRII and ALK5, thus bringing the last two into close proximity.

**ALK1 forms stable complexes with itself, with TβRII, and with endoglin**

In spite of its involvement in TGF-β signaling in endothelial cells, neither the homomeric interactions of ALK1 nor its hetero-oligomerization with TβRII and/or endoglin have been investigated for the full-length receptors at the plasma membrane. To study the homomeric interactions of ALK1, we conducted patch/FRAP studies on cells coexpressing HA-TβRII and myc-ALK1. Cross-linking/immobilization of HA-ALK1 induced a reduction by ~30% in $R_I$ of non-cross-linked, coexpressed myc-ALK1, with no effect on the $D$ value (Figure 6). This is indicative of stable homo-oligomerization, which in the case of dimers would reflect ~60% of the receptors existing in homo-dimeric complexes. Because ALK1 can bind BMP-9, we also tested the effects of BMP-9 on ALK1 homo-oligomerization; we found no significant effects (Figure 6).

Next we investigated complex formation between ALK1 and TβRII. To this end, we conducted patch/FRAP studies on cells coexpressing HA-TβRII and myc-ALK1. In the absence of endoglin,
Endoglin complexes with TGF-β

Because endoglin appears to form complexes with TβRII, ALK5, and ALK1, we examined its ability to modulate TGF-β signaling via ALK5 (phospho-Smad2 [pSmad2] formation) and/or ALK1 (pSmad1/5/8 formation). To this end, we used endoglin-null murine embryonic endothelial cells derived from endoglin−/− mice (MEEC−/−) and their

from those obtained in the presence of TGF-β1, GIPC, or β-arrestin2 binding motifs (**p < 10−6; ***p < 10−7; Student’s t test). No significant differences were found in the D values as a result of IgG-mediated cross-linking of HA-endoglin or after addition of TGF-β1 (or BMP-9) under similar experimental conditions.

Cross-linking of HA-TβRII reduced the Rf of myc-ALK1 by ~30% (Figure 7, −endoglin). Because D was not affected, this suggests that 30% of ALK1 forms stable complexes with TβRII. Similar results were obtained in the presence of TGF-β1 or BMP-9. Subsequently, we probed for potential effects of untagged endoglin expression on HA-TβRII/myc-ALK1 interactions. Here, too, cross-linking of HA-TβRII resulted in ~30% reduction in Rf of myc-ALK1 (Figure 7A; +endoglin). Taken together, these findings suggest stable interactions between TβRII and ALK1, which are not significantly affected by either ligand or endoglin. However, they do not exclude the possibility that ALK1 may interact with endoglin independent of TβRII. To answer this question, we coexpressed myc-endoglin and HA-ALK1 and conducted patch/FRAP studies to measure the effects of cross-linking myc-endoglin on the lateral diffusion of HA-ALK1. Expression of endoglin (without cross-linking) had no effect on Rf or D of HA-ALK1; however, Rf of HA-ALK1 (but not its D value) was markedly reduced (~30%) upon myc-endoglin cross-linking (Figure 8, A and B), suggesting a significant level of stable heterocomplex formation. Similar results were obtained after addition of TGF-β1 (Figure 8, A and B) or BMP-9.

Endoglin differentially augments TGF-β–mediated activation of Smad1/5/8

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wild-type control (MEEC+/+). The cells were stimulated by increasing concentrations of TGF-β1, and the resulting phosphorylation of Smad2 and Smad1/5/8 was measured by immunoblotting (Figure 9). A low but measurable level of pSmad1/5/8 was observed in both cell lines already before addition of TGF-β1. Stimulation by TGF-β1 increased the pSmad1/5/8 levels in both cell types but was significantly higher in MEEC+/+ cells at TGF-β1 concentrations ≥10 pM. A markedly different scenario was observed for Smad2 phosphorylation, for which no significant differences between MEEC+/+ and MEEC−/− cells were detected without or with TGF-β1 stimulation (Figure 9, A and C). In light of earlier findings that demonstrated that TGF-β1-mediated phosphorylation of Smad1/5/8 proceeds via ALK1 in human umbilical vein endothelial cells and mouse embryonic endothelial cells (Oh et al., 2000; Goumans et al., 2002), we conclude that endoglin expression alters the balance between the activation of ALK1-dependent (Smad1/5/8) and ALK5-dependent (Smad2/3) TGF-β signaling.

DISCUSSION

TGF-β signaling is regulated by complex formation among numerous receptors and coreceptors. Although oligomerization of TβRII and ALK5 and its role in Smad2/3 signaling have been extensively studied (reviewed in Ehrlich et al., 2011), such information is lacking for ALK1–TβRII interactions, as well as for the coreceptor endoglin and its interactions with ALK1, ALK5, and TβRII in the intact cell membrane. Here we present biophysical studies on the extent and dynamics of complex formation among these receptors. Our main findings are as follows: 1) endoglin forms stable homomeric complexes, as well as heteromeric complexes with ALK1 and TβRII; 2) ALK5 displays limited binding to endoglin, which is significantly enhanced by TβRII; and 3) endoglin tilts the balance of activation between pSmad1/5/8 and pSmad2/3 toward the former. These findings, together with our previous results on TβRII–ALK5 interaction (Wells et al., 1999; Rechtman et al., 2009; Ehrlich et al., 2011), support a model (Figure 10) in which endoglin, assisted by recruitment of ALK5 via binding to TβRII, functions as a scaffold that is sequentially phosphorylated by ALK5 and ALK1 after their activation by TβRII and ligand. This enables optimal activation of the Smad1/5/8 pathway.

Our patch/FRAP results demonstrated that homomeric endoglin complexes do not appreciably dissociate on the time scale of FRAP measurements (minutes), indicating stable interactions. The percentage reduction in the $R_1(ΔR_1)$ value was 41%, which for homomeric interactions predicts a homodimerization level of 41 × 2 = 82%. This high dimerization level and its stable nature are in accord with the reported extracellular disulfide bond linkage between two endoglin monomers (Gougou and Letarte, 1988). Consistent with this model, endoglin homomeric interactions were independent of GIPC or β-arrestin2 binding (Figure 2). These results suggest that the interactions of endoglin with GIPC or β-arrestin2 do not link it to large immobile structures (e.g., the cytoskeleton), since endoglin mutants that lack binding to these scaffold proteins display the same $D$ and $R_1$ values as endoglin-WT.

Because endoglin does not bind TGF-β ligands on its own (Cheifetz et al., 1992; Barbara et al., 1999), its ability to modulate TGF-β signaling most likely reflects interactions with type II and/or type I TGF-β receptors. Activation of Smad2/3 by TGF-β depends on TβRII and ALK5; we therefore measured their ability to form heteromeric complexes with endoglin. Endoglin–TβRII interactions were ligand independent, stable on the FRAP time scale (patch/FRAP-mediated reduction in $R_1$ of myc-TβRII upon cross-linking of coexpressed HA-endoglin; Figure 3), and involved approximately one-third of the cell-surface TβRII population (ΔR1 of 35%). Endoglin–ALK5 interactions were different: the ΔR1 value of myc-ALK5 was 15–17%, indicating that a much lower fraction (approximately one-sixth) of ALK5 is immobilized along with cross-linked endoglin (Figure 4). Here, too, the interactions were stable over the FRAP time scale and ligand independent. Of note, the interactions of endoglin with either TβRII or ALK5 did not require GIPC or β-arrestin2 binding, indicating that they do not depend on recruitment to immobile scaffolds (e.g., the cytoskeleton). In view of the well-documented interactions between TβRII and ALK5 (Gilboa et al., 1998; Rechtman et al., 2009; reviewed in Ehrlich et al., 2011), we assessed whether coexpression with TβRII contributes to endoglin–ALK5 interactions (compare Figures 4 and 5). Expression of TβRII dramatically enhanced ALK5-endoglin association (increasing ΔR1 of myc-ALK5 after HA-endoglin cross-linking from 17 to >40%; Figures 4A and 5A). This enhancement occurred already in the absence of ligand. Accordingly, coexpression of endoglin enhanced TβRII–ALK5 interactions (approximately twofold increase in ΔR1 of myc-ALK5 upon cross-linking of HA-TβRII) without ligand (Figure 5C). The
endoglin-mediated enhancement of TβRII–ALK5 interactions was indistinguishable from that mediated by TGF-β1 without endoglin (Figure 5C), indicating that triple expression of endoglin/TβRII/ALK5 leads to a significant fraction of mutual and stable complexes containing all three receptors. These findings support our model (Figure 10) in which endoglin binding to TβRII and TβRII–ALK5 interactions enhance the proximity of endoglin and ALK5, a prerequisite for efficient phosphorylation of endoglin by ligand-activated ALK5 and ALK1 (Ray et al., 2010). Moreover, TβRII–ALK5 interactions appear to be different without endoglin and in the triple complex with endoglin, since although association with endoglin enhances TβRII–ALK5 interactions similarly to TGF-β1, it does not suffice to activate the Smad2/3 pathway (Figure 9).

Endothelial cells express both endoglin and ALK1, which mediate Smad1/5/8 signaling after TGF-β stimulation (Goumans et al., 2002; Lebrin et al., 2004). It is therefore possible that endoglin modulates TGF-β signaling via ALK1 by altering TβRII–ALK1 association. The present studies represent the first measurements of ALK1 homomeric (Figure 6) and heteromeric (Figures 7 and 8) complex formation at the surface of intact cells. ALK1 homomeric interactions resembled ALK5, showing a high level of homodimerization (∼60%) already without ligand (Figure 6). This dimerization was insensitive to TGF-β1 (unpublished data), in line with the inability of ALK1 alone to bind this ligand. Of interest, in contrast to the TGF-β1-mediated increase in TβRII–ALK5 interaction (Figure 5C; Rechtman et al., 2009), TGF-β1 did not increase the already high (∼30%) TβRII–ALK1 association (Figure 7A). However, even in endoglin-null cells (MEEC−/−), TGF-β1 stimulated signaling to Smad1/5/8 via ALK1 (Figure 9), indicating that ligand binding likely modifies the TβRII–ALK1 complex to an active state. Similar to ligand, coexpression with endoglin did not affect the stability or percentage of interacting TβRII and ALK1 molecules. Here, too, endoglin expression enhanced signaling to Smad1/5/8, both without and with ligand (Figure 9), supporting the notion that endoglin can modulate TGF-β signaling via Smad1/5/8 (Ray et al., 2010). Of note, endoglin exhibited strong and stable association with TβRII and ALK5, whereas the D value is unaffected. This indicates the formation of stable ALK1 homomeric complexes. As shown, addition of BMP-9 had no significant effects.

**Figure 5:** Patch/FRAP studies show ternary complex formation between endoglin, TβRII, and ALK5. (A, B) Effects of TβRII expression on endoglin/ALK5 interactions. COS7 cells were cotransfected with myc-ALK5 together with HA-endoglin and excess untagged TβRII (both replaced by pcDNA3 vector as control). Myc-ALK5 diffusion was measured by FRAP with or without cross-linking of HA-endoglin, as described in Figure 3. TGF-β1 was added where indicated; BMP-9 addition yielded similar results and is not shown. (A) Rf values and (B) D values of 50–75 measurements in each condition. Significant differences (**p < 10−22) between the indicated pairs were observed only for Rf values of myc-ALK5 upon cross-linking of HA-endoglin either in the presence or absence of TGF-β1 (or BMP-9). (C, D) effects of endoglin expression on TβRII/ALK5 interactions. COS7 cells were cotransfected with myc-ALK5 together with HA-TβRII or a combination of HA-TβRII and excess untagged endoglin. Empty pcDNA3 vector served as cotransfection control. Myc-ALK5 diffusion was measured by FRAP with or without cross-linking of HA-TβRII and ± TGF-β1, as described in Figure 3. (C) Rf values and (D) D values of 50–75 measurements in each case. Endoglin expression was sufficient to induce highly significant differences (**p < 10−4; ***p < 10−15; Student’s t test) in the Rf values of myc-ALK5 when HA-TβRII was cross-linked. This effect was as high as the TGF-β1–induced association between ALK5 and TβRII in the absence of endoglin.

**Figure 6:** Patch/FRAP studies demonstrate stable ALK1 homomeric complexes. COS7 cells were cotransfected with pairs of expression vectors encoding myc-ALK5 and HA-ALK1, replacing HA-ALK1 by empty vector in control experiments. Labeling, IgG cross-linking (XL) of HA-ALK1, and patch/FRAP studies (measuring the lateral diffusion of myc-ALK5) were as in Figure 1. (A) Average Rf and (B) average D values derived from multiple patch/FRAP measurements. Bars are mean ± SEM of 50–100 measurements. Asterisks indicate significant differences between the Rf values of the pair indicated by brackets (**p < 10−10; Student’s t test). The Rf value of myc-ALK1 is markedly reduced after cross-linking of HA-ALK1, whereas the D value is unaffected. This indicates the formation of stable ALK1 homomeric complexes. As shown, addition of BMP-9 had no significant effects.
ALK1 forms stable heterocomplexes with TβRII. Experimental conditions were as in Figure 3, except that myc-ALK1 replaced myc-TβRII, and HA-TβRII was used instead of HA-endoglin. In some experiments, untagged endoglin was coexpressed (marked as “+endoglin”). The lateral diffusion of myc-ALK1 was measured under the indicated experimental conditions. (A) Average $R_t$ and (B) average $D$ values. Bars are mean ± SEM of 30–70 measurements. Asterisks indicate significant differences between the $R_t$ values of the pair indicated by brackets (**p < 10^{-5}, ***p < 10^{-10}; Student’s t test). The studies show a clear reduction in $R_t$ of myc-ALK1 after cross-linking of HA-TβRII, and this reduction is unaffected by endoglin. Similar results were obtained in the presence of TGF-β1 or BMP-9 (since both had no effect, the latter is not shown).

ALK1 strongly interacts with endoglin to form stable heteromeric complexes. COS7 cells were cotransfected with pairs of expression vectors encoding HA-ALK1 and myc-endoglin, myc-ALK1 and HA-endoglin-Del, or myc-ALK1 and HA-endoglin-T650A. In control experiments, the vector encoding endoglin or its mutants was replaced by empty vector. IgG-mediated patching/cross-linking of myc-endoglin (A, B) was as in Figure 2; patching/cross-linking of HA-endoglin was as in Figures 1 and 3. The lateral diffusion of HA-ALK1 (A, B) or myc-ALK1 (C, D) was measured without or with myc-endoglin or HA-endoglin cross-linking. (A, C) Average $R_t$ and (B, D) average $D$ values. Bars are mean ± SEM of 45–65 measurements. Asterisks indicate significant differences between $R_t$ values of the pair indicated by brackets (**p < 10^{-4}; Student’s t test). Cross-linking of coexpressed myc-endoglin-WT, HA-endoglin-Del, or HA-endoglin-T650A elicited similar reduction in $R_t$ of HA-ALK1 or myc-ALK1, suggesting that the strong ALK1/endoglin association does not rely on endoglin binding to GIPC or β-arrestin2. TGF-β1 or BMP-9 addition did not alter ALK1-endoglin association; only the first is shown, as both ligands had no effect.

With ALK1 already without TβRII transfection; this association was ligand independent and persisted with endoglin mutants lacking binding to GIPC or β-arrestin2 (Figure 8). Thus, unlike ALK5, ALK1-endoglin association is independent of TβRII. In contrast to the lack of dependence of TGF-β1–mediated Smad 2/3 activation on endoglin, Smad1/5/8 activation was markedly augmented by endoglin (Figure 9). These results cannot be explained by ligand-mediated modulation of the interactions between endoglin, TβRII, and ALK1, as all of these interactions were insensitive to TGF-β1. Therefore a molecular mechanism capable of supporting the potentiation of Smad1/5/8 signaling (both basal and ligand induced) by endoglin expression (Figure 9) is required. We propose that TβRII and ALK1 bind to endoglin stably and directly. On the other hand, ALK5 binding to endoglin is augmented by TβRII residing in the same complex (Figure 10). Thus endoglin and TβRII act together to recruit ALK5 to endoglin (Figure 10). Ligand binding allows 1) activation of the endoglin-bound ALK5 by TβRII and the ensuing phosphorylation of endoglin by ALK5 (Ray et al., 2010) and 2) phosphorylation of endoglin by ligand-activated ALK1; 3) these lead to enhanced Smad1/5/8 signaling, without affecting signaling via Smad2/3, tilting the balance of TGF-β–mediated signaling toward Smad1/5/8 (Figure 10).

Although the effects of the Smad2/3 and Smad1/5/8 pathways on endothelial cell function and angiogenesis are controversial, multiple studies suggest that the balance of signaling between these pathways regulates the functional response to TGF-β ligands. The present studies, which support a central role for endoglin in differentially regulating signaling through the Smad2/3 and Smad1/5/8 pathways, including preferentially promoting ALK1 signaling to Smad1/5/8 (Figure 9), are consistent with the central role of endoglin and ALK1 in both physiological and pathophysiological angiogenesis. Indeed, both endoglin and ALK1 are being targeted in antiangiogenic cancer therapy. Whether these agents function by disrupting the complexes characterized here remains to determined.

MATERIALS AND METHODS

Reagents

Recombinant TGF-β1 was obtained from PeproTech (Rocky Hill, NJ), and BMP-9 from R&D Systems (Minneapolis, MN). Fatty acid–free bovine serum albumin (BSA; fraction V) was from Sigma-Aldrich (St. Louis, MO). Media and Hank’s balanced salts solution...
Endoglin complexes with TGF-β

Plasmids
Expression vectors encoding human TβRII (in pcDNA3) or TβRII (in pcDNA1) with extracellular myc or HA epitope tags, as well as untagged TβRII, were described earlier (Henis et al., 1994; Gilboa et al., 1998; Ehrlach et al., 2001; Shapira et al., 2012). HA-tagged WT endoglin (endoglin-L), HA-endoglin-Del (lacking the last three C-terminal amino acids, resulting in loss of binding to GIPC), and HA-endoglin-T650A (a point mutation that abrogates endoglin binding to β-arrestin2) in pDisplay were described (Lee and Blobe, 2007; Lee et al., 2008). HA- or myc-tagged ALK1 in pcDNA3.1 were delineated previously (Lee et al., 2009; Tian et al., 2012). Myc-tagged endoglin was generated by PCR incorporation of myc sequence into untagged pcDNA3.1 endoglin.

Cell culture and transfections
COS7 cells (American Type Culture Collection [ATCC], Manassas, VA) were grown in DMEM with 10% fetal calf serum (FCS; Biological Industries) as described (Gilboa et al., 2000; Shapira et al., 2012). Murine endothelial polya bEnd.3 cells (ATCC) were grown in the same medium under similar conditions. Murine embryonic endothelial cells (MEEC) were from Cell Signaling Technologies (Danvers, MA). Mouse anti-β-actin was from Millipore (Billerica, MA), and antibodies to pSmad1/5/8, pSmad2, tSmad1, tSmad2, and β-actin were from Cell Signaling Technologies (Danvers, MA).

For patch/FRAP experiments, COS7 or bEnd.3 cells were grown on glass coverslips in six-well plates and transfected by TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI) with different combinations of expression vectors encoding myc- and HA-tagged (or untagged) receptors. For endoglin, which expresses at higher levels than the TGF-β receptors, 300 ng of plasmid DNA was used per transfection, and the DNA amounts of TβRI, ALK5, or ALK1 vectors were adjusted to yield similar cell-surface expression levels, determined by quantitative immunofluorescence as described earlier (Marom et al., 2011). The total DNA level was complemented by empty vector to 2 μg.

IgG-mediated patching/cross-linking
At 24 h posttransfection, COS7 or bEnd.3 cells transfected with various combinations of expression vectors for endoglin and TGF-β receptors were serum starved (30 min, 37°C), washed with cold HBSS supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4) and 2% BSA (HBSS/HEPES/BSA), and blocked with normal goat γ-globulin (200 μg/ml, 30 min, 4°C). They were then labeled successively at 4°C (to avoid internalization and enable exclusive cell surface labeling) in HBSS/HEPES/BSA (45-min

H.B.S. were from Biological Industries (Beit Haemek, Israel) or Invitrogen (Carlsbad, CA). Rabbit anti-Smad2, anti–phospho-Smad2 (pSmad2), anti-Smad1, and anti-pSmad1/5/8 (antibody 9511, recognizing the C-terminal S*XS* motif, where S* stands for phospho-Ser) antibodies were from Cell Signaling Technologies (Danvers, MA). Mouse anti-β-actin was from MP Biomedicals (Solon, OH). Anti–myc tag 9E10 mouse ascites (Evans et al., 1985), anti-influenza hemagglutinin epitope tag (anti-HA) 12CA5 mouse ascites, and HA.11 rabbit antiserum to the HA tag were purchased from Covance Research Products (Denver, PA). IgG and monovalent Fab’ fragments were prepared as described (Henis et al., 1994). Chicken anti-myc affinity-purified IgY was from EMD Millipore-Chemicon (Temecula, CA). Peroxidase-conjugated donkey anti-rabbit (DxR) and sheep anti-rabbit (SxR) antibodies used for immunoblotting were from Amersham/GE Life Sciences (Pittsburgh, PA). Goat γ-globulin, Cy3-conjugated AffiniPure Goat F(ab’2), and fluorescein isothiocyanate (FITC)-conjugated AffiniPure donkey IgG against chicken IgG (DxC) were from Jackson Immunoresearch Laboratories (West Grove, PA). Alexa Fluor 488–GtR IgG and Alexa Fluor 546–GtR F(ab’2)2 were from Invitrogen-Molecular Probes (Eugene, OR); fluorescent F(ab’2)2 was converted to Fab’ as described (Gilboa et al., 1998).

HBSS were from Biological Industries (Beit Haemek, Israel) or Invitrogen (Carlsbad, CA). Rabbit anti-Smad2, anti–phospho-Smad2 (pSmad2), anti-Smad1, and anti-pSmad1/5/8 (antibody 9511, recognizing the C-terminal S*XS* motif, where S* stands for phospho-Ser) antibodies were from Cell Signaling Technologies (Danvers, MA). Mouse anti-β-actin was from MP Biomedicals (Solon, OH). Anti–myc tag 9E10 mouse ascites (Evans et al., 1985), anti-influenza hemagglutinin epitope tag (anti-HA) 12CA5 mouse ascites, and HA.11 rabbit antiserum to the HA tag were purchased from Covance Research Products (Denver, PA). IgG and monovalent Fab’ fragments were prepared as described (Henis et al., 1994). Chicken anti-myc affinity-purified IgY was from EMD Millipore-Chemicon (Temecula, CA). Peroxidase-conjugated donkey anti-rabbit (DxR) and sheep anti-rabbit (SxR) antibodies used for immunoblotting were from Amersham/GE Life Sciences (Pittsburgh, PA). Goat γ-globulin, Cy3-conjugated AffiniPure Goat F(ab’2), and fluorescein isothiocyanate (FITC)-conjugated AffiniPure donkey IgG against chicken IgG (DxC) were from Jackson Immunoresearch Laboratories (West Grove, PA). Alexa Fluor 488–GtR IgG and Alexa Fluor 546–GtR F(ab’2)2 were from Invitrogen-Molecular Probes (Eugene, OR); fluorescent F(ab’2)2 was converted to Fab’ as described (Gilboa et al., 1998).
incubations) with 1) monovalent mouse Fab’ anti-myc (40 μg/ml) together with HA.11 rabbit anti-HA (20 μg/ml) and 2) Alexa Fluor 546–Fab’ Goat (40 μg/ml) together with Alexa Fluor 488–IgG Goat (20 μg/ml). This protocol results in the HA-tagged receptor cross-linked and immobilized by IgGs, whereas the myc-tagged receptor, whose lateral diffusion is then measured by FRAP (see later description), is labeled exclusively by monovalent Fab’.

Alternatively, for immobilizing the myc-tagged receptor and measuring the lateral diffusion of Fab’-labeled HA-tagged receptor, the following labeling protocol was used: 1) monovalent mouse Fab’ anti-HA (40 μg/ml) together with chicken IgY anti-myc (20 μg/ml) and 2) Cy3-Fab’ Goat (40 μg/ml) together with FITC-IgG DonC (20 μg/ml). This protocol results in the myc-tagged receptor being immobilized and the HA-tagged receptor labeled by monovalent Fab’. In experiments with TGF-β1 or BMP-9, the ligand (250 pM TGF-β1 or 5 ng/ml BMP-9) was added after starvation along with the normal goat γ-globulin and maintained at this concentration during the following labeling steps and FRAP measurements.

**FRAP and patch/FRAP**

Cells coexpressing epitope-tagged receptors labeled fluorescently by anti-tag Fab’ fragments as described were subjected to FRAP and patch/FRAP studies as described previously (Rechtman et al., 2009; Marom et al., 2011). The FRAP measurements were conducted at 15°C, replacing samples within 20 min to minimize internalization during the measurement. An argon-ion laser beam (Innova 70C; Coherent, Santa Clara, CA) was focused through a fluorescence microscope (Axioimager.D1; Carl Zeiss Microlmaging, Jena, Germany) to a Gaussian spot of 0.77 ± 0.03 μm (Plan Apochromat 63×/1.4 numerical aperture oil-immersion objective). After a brief measurement at monitoring intensity (528.7 nm, 1 μW), a 5-mW pulse (20 ms) bleached 60–75% of the fluorescence in the illuminated region, and fluorescence recovery was followed by the monitoring beam. Values of D and R were extracted from the FRAP curves by nonlinear regression analysis, fitting to a lateral diffusion process (Petersen et al., 1986). Patch/FRAP studies were performed similarly, except that IgG-mediated cross-linking/patching of an epitope-tagged TGF-β receptor or endoglin (described in the foregoing) preceded the measurement (Henis et al., 1990; Rechtman et al., 2009).

This enabled us to determine the effects of immobilizing one receptor type on the lateral diffusion of the coexpressed receptor (labeled exclusively with non–cross-linking Fab’), allowing identification of complex formation between them and distinction between transient and stable interactions (Henis et al., 1990; Rechtman et al., 2009).

**Smad phosphorylation assay and Western blotting**

MEEC+/− (WT) or MEEC−/− (endoglin-null) cells were serum starved for 6 h, followed by a 30-min incubation with increasing doses of TGF-β1 as indicated in the legend to Figure 9. The cells were then lysed and subjected to electrophoresis on 10% SDS–PAGE, followed by immunoblotting as described (Ray et al., 2010). The blots were probed by rabbit anti-pSmad1/5/8 (1:500), rabbit anti-total Smad1 (1:1000), rabbit anti-pSmad2 (1:1000), rabbit anti-total Smad2 (1:1000), or mouse anti-β-actin (1:10000), followed by peroxidase DonC or Goat IgG (1:5000). The bands were visualized by ECL (Amersham) and quantified by densitometry (ImageJ; National Institutes of Health, Bethesda, MD).

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**FIGURE 10:** Model of endoglin regulation of Smad signaling via TβRII, ALK5, and ALK1 interactions. All receptors are drawn as monomers for simplicity. TβRII and ALK1 can bind to endoglin stably and directly, whereas ALK5 binding to endoglin is augmented by TβRII residing in the same complex. TGF-β1 binding to the complex (not drawn, for simplicity) enables activation of the endoglin-bound ALK5 by TβRII and the phosphorylation of endoglin first by ALK5 (designated 1-P) and subsequently by ligand-activated ALK1 (designated 2-P since this is a second phosphorylation event, which was shown to occur only after the first one; Ray et al., 2010). In this complex, the signaling through ALK5/Smad2/3 is not affected by endoglin, whereas signaling via ALK1/Smad1/5/8 is enhanced.

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