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Endoglin mediates fibronectin/ 5 1 integrin and TGF-β pathway crosstalk in endothelial cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 April 2012

Thank you for the submission of your manuscript to The EMBO Journal and please accept my apologies for the delay in getting back to you. We have only now received the full set of reports from the three referees that were asked to evaluate your study, which I copy below.

As you will see from their reports, while the referees find your paper interesting, they also point out to a number of concerns that prevent its publication as it stands now. Besides a number of technical concerns, of which the quality of the blots presented is repeatedly remarked by all three referees, several more fundamental issues are raised, particularly by referee #1. In this regard, I would like to draw your attention to points 4, 5 and 6 in his/her report, while other points are of peripheral importance in light of the comments of the other referees. I would also like to point out to the concerns of referees #2 and #3 with figure 7, which will need to be addressed as well.

Should you be able to address the criticisms of the referees in full, and particularly the points I mention above, we could consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms may require a lot of additional time and effort and be technically challenging. In this regard, do not hesitate to contact me at any point during the revision process if you have any questions or need further input.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process iniciative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Tian et al. studied functional crosstalk between the endoglin/ALK1 and fibronectin/integrin signaling pathways in endothelial cells. Some of the findings are potentially interesting in the field; however, they failed to show clear mechanisms in the crosstalk between the two pathways. In addition, I think that technical qualities are not so high.

Specific points

1. They have shown the effects of TGF-b anfd BMP-9 in Figs. 1 and 2. However, they have not shown any data using BMP-9 in other experiments. Since BMP-9 binds to endoglin and ALK1 with a high affinity and induces phosphorylation of Smad1/5/8, it is important to determine whether BMP-9 exhibits effects similar to TGF-b in other experiments (particularly Figs. 4 and 8).
2. Figs. 1 and 2. The findings that endoglin plays an important role in stimulation of Smad1/5/8 pshophorylation in endothelial cells are interesting. They also showed that fibronectin/integrin a5v1 plays a critical role in phosphorylation of Smad1/5/8 by TGF-b or BMP-9. However, the data shown in Fig. 3 are not of high qualities. In Fig. 3B, TGF-b induced Smad1/5/8 phosphorylation in endoglin-silenced cells, and the effect of fibronectin appearas to be marginal. Fig. 3E is poor; more clear data should be provided.
3. As described above, it is important to show whether BMP-9 exhibits effects similat to TGF-b in the induction and phosphorylation of integrin a5v1. Figs. 4F and G. are poor and immunoblots of t-b1 were over-exposed. Why did KD-ALK-1 induce phosphorylation of p-b1 in Fig. 4G? p-b1 and T-b1 are not explained in the figure legend.
4. Figs. 5 and 6. Most of the experiments have been carried out in over-expression systems. Endogenous interaction between endoglin and a5 is shown only in Fig. 5D, but it is unclear whether the interaction does occur in non-transfected conditions. Knockdown of endoglin should also be done. Fig. 5E, lane 2 should be GFP-a5 (+) and lane 3 should be HA-endo mutant (wild).
5. Figs. 8D and E. They have not shown whether endoglin is involved in tubule formation and apoptosis of endothelial cells.
6. Fig. 9. They used only endoglin MO and T650 mutant. This does not clearly show the functional interaction between endoglin and integrin a5v1 in vivo.
7. The manuscript has not been carefully prepared in several points and some parts are difficult to follow. Materials and Methods should be placed after Discussion. Page 17, line 9. This sentence is incomplete.

Referee #2:

In this manuscript, Tian et al. investigated the interaction between TGF-beta and integrin signaling pathways in the regulation of endothelial cell biology and angiogenesis. They show that the ECM component fibronectin, and its primary cellular receptor alpha5beta1 integrin, specifically increases TGF-beta1 and BMP9 induced phosphorylation of Smad1/5/8, while having no effects on Smad2/3

signaling downstream of ALK5. Analyzing the mechanism, the authors propose that fibronectin induces alpha5beta1 integrin clustering followed by endoglin/ALK1 cell surface complex formation and increased Smad1/5/8 signaling. On the other hand, TGF-beta1 increases alpha5beta1 integrin activation and downstream signaling to FAK in an endoglin-dependent manner. In addition, the findings suggest that endoglin mediates a fibronectin/alpha5beta1 integrin-induced functional switch of TGF-beta1 from a promotor to a suppressor of endothelial cell migration and TGF-beta1-mediated apoptosis to stabilize newly formed tubules during angiogenesis.
Generally this is an interesting paper on the function of fibronectin/alpha5beta1 integrin signaling complexes in TGF-beta1-dependent angiogenesis, highlighting important new insights on the functions of these proteins via endoglin and ALK1 pathways in vitro, and also in vivo.

Major comments:

1) Fig. 2C: The authors state that fibronectin increases both basal and TGF-beta1 induced Smad1/5/8 phosphorylation, while collagen and laminin have little to no effect. However, western blot analysis clearly shows that collagen reduces TGF-beta1 induced Smad1/5/8 phosphorylation.
2) Fig. 2C/D and Suppl. Fig. 3: Whereas the western blot analysis in Fig. 2C displays a strong increase of Smad1/5/8 phosphorylation following TGF-beta1 stimulation in the absence of exogenous fibronectin, there is no increase of the signal in Suppl. Fig. 3 and only a weak increase in Fig. 2D following TGF-beta1 stimulation as compared to unstimulated control. These assays should be repeated.
3) Fig. 2D/E: The authors should comment about the different optimal concentrations of exogenous fibronectin to increase TGF-beta1 or BMP-9 induced Smad1/5/8 phosphorylation, respectively.
4) Fig. 5D and Suppl. Fig. 12: Endogenous endoglin specifically co-precipitates endogenous integrin alpha5 (subunit of the fibronectin receptor alpha5beta1) but not integrin beta4 (subunit of the laminin receptor alpha6beta4). Does endoglin co-precipitate with beta3, beta7 or alphav which are subunits of other fibronectin receptors?
5) Fig. 6B: How many cells were counted from how many independent Duolink assays? What is the reference parameter for quantification: number of Endo/ALK-1 complexes per cell?
6) Fig. 7A: The authors state that overexpressed HA-endoglin colocalizes with overexpressed GFP alpha5 at the cell membrane and in intracellular vesicles. The colocalization is unconvincing. Which type of microscopical analysis method was used? For colocalization analysis, the authors should use confocal laser scanning microscopy. In addition, for exact allocation of intracellular vesicles co-stainings with endosomal/lysosomal markers are necessary. However, it is also possible, that endoglin and alpha5 are localized in plasma membrane clusters.
7) Fig. 8A: What is the concentration of TGFbeta1 in the transwell migration assay? Were the cells pre-incubated with TGFbeta1 or is TGFbeta1 applied to the lower well compartment?
8) Fig. 8D: The authors assign apoptotic cells by the use of phase contrast microscopy (black arrows). This is not convincing, and the authors should use fluorescence based assays to detect apoptotic cells instead of transmitted light.
What is the reference parameter for quantification of tubule degradation?
9) In some cases western blots appear to be in signal saturation which is problematic for subsequent quantification (especially Fig. 2F, 4G)
10) The paper is too long (especially the results section) and could be improved by focusing on the important findings.

Referee #3:

The authors of this manuscript describe the crosstalk of TGF- and 5 1 integrin signaling via endoglin and vice versa the impact of 5 1 integrin on endoglin/Alk1 signaling. Both pathways play pivotal roles in angiogenesis. The data give insights to the way TGF- 1 changes from a promoter of endothelial cell migration to a suppressor of cell migration via the described crosstalk. The experiments are carefully performed and complementary approaches are being used to show the results. While the basic molecular studies are comprehensive, the angiogenic assays and in vivo validation using zebrafish are short but confirm the model presented at the end.
The manuscript is well written and highlights the novel findings in the right context.

There are some concerns, which should be addressed by the authors:

1. Fig. 2C: while fibronectin indead showed enhanced TGF- 1 induced Smad1/5/8 phosphorylation, collagen shows decreased phosphorylation. Please comment on this.

2. Fig. 2F: here and in many figures to follow, some of the blots are overexposed and with that a quantification relative to e.g. total Smad1 and Smad 2 (as shown here) is impossible. Please show shorter exposure times of the same blot.

3. Fig 3D: this is a/the crucial experiment of this study. The data here are clear and well presented. How often has this been repeated? Please highlight this finding more in your text.

4. Fig. 4A and B: in A (time course of stimulation) needs p- 1 blot for completeness, while Fig. 4B needs 5 blot. Please add data or comment on this.

5. Fig. 4F and 4G: as mentioned already before here total - 1 is overexposed and therefore it is impossible to control equal loading or potential changes of total - 1 integrin levels. Please show shorter exposures.

6. Fig. 5C: the reciprocal immunoprecipitation (i.e. immunoppt of endogenous 5- or 1- integrin and detection of exogenous HA-tagged endoglin) is less convincing.

7. The authors try to map the interaction sites between endoglin and 5 integrin using a number of endoglin extracellular deletion mutants (Fig. 5H). None of those abolished binding eventhough the TMCT mutant (lacking the complete extracellular domain) failed to do so. It is very important here to check for cell surface expression of all mutants before a statement as done in the results and discussion section (5 1 interact with endoglin with the extracellular domain) can be placed.
8. Where in endoglin and Alk1 are the tags (HA and myc) localized, extracellularly or intracellularly?

9. Fig. 7B: for completeness, please include here also the interaction with -arrestin.

10. Fig. 7D: the HA blot is difficult to see; what is the transfection efficiency in this experiment? How often has this been repeated? The statements of this experiment is too strong considering, how complex clathrin-mediated endocytosis is regulated. I strongly suggest to slow down on any endocytosis arguments in this study, since the data for this are incomplete and not realy necessary. For this please also rewrite the paragraph in the discussion (Our data here demonstrate that endoglin T650A mutant, which cannot bind barrestin2 (Lee&Blobe, 2007), suppresses endoglin/integrin 5 1 complex internalization and ....).

11. The authors mention in the discussion, that the data support a model in which fibronectin induces clustering of integrin 5 1. This would be very interesting as a potential add-on study.

1st Revision - Authors' Response 17 July 2012

We thank the referees for carefully reading the manuscript and providing constructive criticisms. Our point by point response to each referee is outlined below.

Referee #1: We thank the reviewer for finding that "Some of the findings are potentially interesting in the field…"

1. They have shown the effects of TGF-beta and BMP-9 in Figures 1 and 2. However, they have not shown any data using BMP-9 in other experiments. Since BMP-9 binds to endoglin and ALK1 with a high affinity and induces phosphorylation of Smad1/5/8, it is important to determine whether BMP-9 exhibits effects similar to TGF-beta in other experiments (particularly Figures 4 and 8).

To address this issue, we investigated the effects of BMP-9 on integrin alpha5beta1 expression and beta1 subunit phosphorylation. BMP-9 did not increase integrin alpha5beta1 expression, but only modestly increased integrin beta1 phosphorylation. These data are included as new Supplementary Figure S9.

Unlike TGF-beta1, BMP-9 reduced MEEC migration through non-ECM coated transwell. This is consistent with other reports that BMP-9 is a vascular quiescence factor which inhibits endothelial migration (David et al, 2008) (Park et al, 2012). Although fibronectin slightly enhanced BMP-9-mediated decreases in migration, this effect is not as significant as the effect of fibronectin on TGF-beta-mediated migration. In addition, fibronectin had no significant effect on BMP-9's effect on tubule formation. We have added this data as new Supplementary Figures S23 and S24.

2. Figures 1 and 2. The findings that endoglin plays an important role in stimulation of Smad1/5/8 phosphorylation in endothelial cells are interesting. They also showed that fibronectin/integrin alpha5beta1 plays a critical role in phosphorylation of Smad1/5/8 by TGF-beta or BMP-9. However, the data shown in Figure 3 are not of high qualities. In Figure 3B, TGF-beta induced Smad1/5/8 phosphorylation in endoglin-silenced cells, and the effect of fibronectin appears to be marginal. Figure 3E is poor; more clear data should be provided.

In response to these concerns, we present another replicate along with lighter exposures, which demonstrates the dependence on endoglin more clearly (new Figure 3B). In terms of Figure 3E, which demonstrates a role for ALK-1 in

mediating fibronectin's effects, we believe the data presented support this conclusion quite convincingly and clearly.

3. As described above, it is important to show whether BMP-9 exhibits effects similar to TGF-beta in the induction and phosphorylation of integrin alpha5beta1. Figures 4F and G. are poor and immunoblots of T-beta1 were over-exposed. Why did KD-ALK-1 induce phosphorylation of p-beta1 in Figure 4G? p-b1 and T-b1 are not explained in the figure legend.

To address this issue, we investigated the effects of BMP-9 on integrin alpha5beta1 expression and beta1 subunit phosphorylation. BMP-9 did not increase integrin alpha5beta1 expression, but did modestly increase integrin beta1 phosphorylation. These data are included as new Supplementary Figure S9.

In terms of Figures 4F, 4G, we provide shorter exposures of these experiments, and explained "p-b1" and "T-b1" in the revised figure legend.

In terms of why KD-ALK-1 induces phosphorylation of integrin beta1 (Figure 4G), the reviewer brings up an interesting point. While we don't know the precise mechanism, in a reciprocal manner, over-expression of constitutively active ALK1 (ca ALK1) decreases basal and TGF-beta induced integrin beta1 phosphorylation (Figure 1, below). These results suggested that ALK1 may negatively regulate integrin beta1 phosphorylation. In either case, TGF-beta induces integrin beta1 phosphorylation whether in the presence of KD-ALK1 or ca-ALK1, suggesting that TGF-beta-induced beta1 integrin phosphorylation may not be mediated through ALK1.

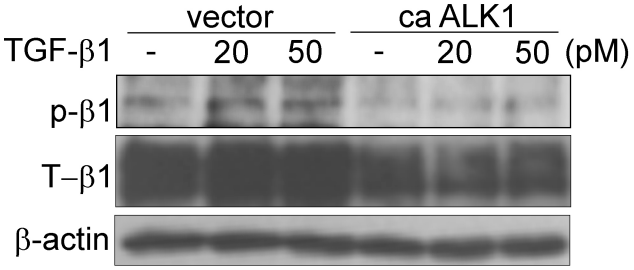

Fig.1 HMEC-1 cells were adenovirally infected with vector or constitutive active ALK1 (ca-ALK1) for 48h, serum starved for 6h prior to treatment with indicated doses of TGF-beta1 for 30min, and the cell lysates analyzed with the indicated antibodies.

4. Figures 5 and 6. Most of the experiments have been carried out in over-expression systems. Endogenous interaction between endoglin and alpha5

is shown only in Figure 5D, but it is unclear whether the interaction does occur in non-transfected conditions. Knockdown of endoglin should also be done. Figure 5E, lane 2 should be GFP-a5 (+) and lane 3 should be HA-endo mutant (wild).

To address this concern, we performed additional co-IP experiments to detect the endogenous interaction between integrin beta1 subunit and endoglin in MEEC+/+ cells, but not in MEEC-/- cells (new Figure 5D). In addition, we detected the interaction of endogenous endoglin and endogenous integrin alpha5 and endogenous integrin beta1 in wild type HMEC-1 cells, with endoglin knockdown in HMEC-1 cells as a control (new Figure 5E). The label in original Figure 5E (Figure 5F in revised version) has been fixed.

5. Figures 8D and E. They have not shown whether endoglin is involved in tubule formation and apoptosis of endothelial cells.

To address this issue, we investigated the role of endoglin in tubule formation and apoptosis in HMEC-1 cells in the presence and absence of endoglin (shRNA mediated silencing). Compared to HMEC-1 WT cells, TGF-beta decreased tubule formation more severely in HMEC-1 with shRNA-mediated silencing of endoglin (new Figure 8D), which is consistent with our recent publication in ***Mol Biol Cell(Lee et al, 2012)***. Importantly, in the absence of endoglin, fibronectin was unable to rescue tubule formation (new Figure 8D), or the effects of TGF-beta on apoptosis (new Figure 8E), further supporting the specific crosstalk between fibronectin and endoglin.

6. Figure 9. They used only endoglin MO and T650 mutant. This does not clearly show the functional interaction between endoglin and integrin a5b1 in vivo.

To address this issue, we generated morpholinos (Endo-MO) to suppress endogenous endoglin translation in Fli1-EGFP embryos, and detected whether the endoglin TMCT mutant, the only mutant we identified that cannot bind with integrin alpha5beta1, could rescue angiogenesis. We found endoglin TMCT mutant failed to rescue the MO phenotype compared to WT endoglin rescue. These results support a functional interaction between endoglin and integrin alpha5beta1in vivo. We have added this result as new Figure 9B in the revised manuscript.

7. The manuscript has not been carefully prepared in several points and

some parts are difficult to follow. Materials and Methods should be placed after Discussion. Page 17, line 9. This sentence is incomplete.

We carefully proofed the manuscript, and the mistakes have been fixed. We placed Materials and methods after Discussion and fixed the sentence in Page 17, line 9. In addition, we rewrote some paragraphs in the Results section to focus on the key findings.

Referee #2:

We thank the reviewer for finding that **"**this is an interesting paper on the function of fibronectin/alpha5beta1 integrin signaling complexes in TGF-beta1-dependent angiogenesis, highlighting important new insights on the functions of these proteins via endoglin and ALK1 pathways in vitro, and also in vivo."

1) Figure 2C: The authors state that fibronectin increases both basal and TGF-beta1 induced Smad1/5/8 phosphorylation, while collagen and laminin have little to no effect. However, western blot analysis clearly shows that collagen reduces TGF-beta1 induced Smad1/5/8 phosphorylation.

We have made a comment on collagen reducing TGF-beta1 induced Smad1/5/8 phosphorylation in the results section of the manuscript.

2) Figure 2C/D and Suppl. Figure 3: Whereas the western blot analysis in Figure 2C displays a strong increase of Smad1/5/8 phosphorylation following TGF-beta1 stimulation in the absence of exogenous fibronectin, there is no increase of the signal in Suppl. Figure 3 and only a weak increase in Figure 2D following TGF-beta1 stimulation as compared to unstimulated control. These assays should be repeated.

TGF-beta1, for the most part, modestly induced Smad1/5/8 phosphorylation in the absence of exogenous fibronectin, a result which was consistent in our experiments (Figures 2D, 3A, 3B, 3C, 3D, Supplementary Figures S3, S5, S6). However, the extent to which this is evident can vary based on the exposure and cell system utilized. In the revised manuscript we have tried to keep the exposure across figures as uniform as possible.

3) Figure 2D/E: The authors should comment about the different optimal concentrations of exogenous fibronectin to increase TGF-beta1 or BMP-9 induced Smad1/5/8 phosphorylation, respectively.

We have added related comments in the manuscript, as it appears that higher concentrations are required to augment BMP-9 mediated signaling.

4) Figure 5D and Suppl. Figure 12: Endogenous endoglin specifically co-precipitates endogenous integrin alpha5 (subunit of the fibronectin receptor alpha5beta1) but not integrin beta4 (subunit of the laminin receptor alpha6beta4). Does endoglin co-precipitate with beta3, beta7 or alphav, which are subunits of other fibronectin receptors?

To address this issue, we detected the endogenous interaction between endoglin and integrin avb3, which is another fibronectin receptor. Although alphav and beta3 are expressed in HMEC-1 cells with high abundance, endogenous endoglin cannot interact with them. This result has been added as new Supplementary Figure S14.

5) Figure 6B: How many cells were counted from how many independent Duolink assays? What is the reference parameter for quantification: number of Endo/ALK-1 complexes per cell?

42 cells were counted in Figure 6B from one Duolink assay. However, we repeated this experiment 3 independent times. Although fibronectin increased the ALK1/endoglin interaction every time, the relative fold increase was variable (for example, in Figure 6B, supplementary Figures 17,18), making averaging of this data difficult. We used the number of Endo/ALK-1 complexes per cell as the reference parameter for quantification.

6*) Figure 7A: The authors state that overexpressed HA-endoglin colocalizes with overexpressed GFP-alpha5 at the cell membrane and in intracellular vesicles. The colocalization is unconvincing. Which type of microscopical analysis method was used? For colocalization analysis, the authors should use confocal laser scanning microscopy. In addition, for exact allocation of intracellular vesicles co-stainings with endosomal/lysosomal markers are necessary. However, it is also possible, that endoglin and alpha5 are localized in plasma membrane clusters.*

Thank you for your suggestions. To improve this data, we co-stained for endoglin, integrin alpha5 and the endocytosis markers, Rab5 or EAA1. As the suitable antibodies for co-staining are unavailable, we co-transfected MEEC-/- cells with GFP-alpha5 and endoglin for these studies, and used confocal laser scanning microscopy to capture the data (revised Figure 7A, 7B). We also revised the related text.

Based on this data, in addition to co-localizing in endosomal vesicles, we believe endoglin and alpha5 colocalize in the plasma membrane. In further support, in Figure 7C (in the revised manuscript), biotinylated alpha5 was co-immunoprecipitated with biotinylated endoglin in cells maintained at 4^{o}C (the "PC" lane in Figure 7B), which should reduce internalization.

7) Figure 8A: What is the concentration of TGFbeta1 in the transwell migration assay? Were the cells pre-incubated with TGFbeta1 or is TGFbeta1 applied to the lower well compartment?

We used 100pM TGF-beta1 to pretreat endothelial cells, and then plated the cells on the top of upper well. No TGF-beta was added to lower compartment. To avoid confusion, we changed the description in figure legends from "……treated with or without 100pM TGF-beta1 and assessed" to "……pretreated with or without 100pM TGF-beta1……." in the figure legend.

8) Figure 8D: The authors assign apoptotic cells by the use of phase contrast microscopy (black arrows). This is not convincing, and the authors should use fluorescence based assays to detect apoptotic cells instead of transmitted light. What is the reference parameter for quantification of tubule degradation?

In the tubule formation experiments, we noticed endothelial cells formed tubules after 12h, with some tubules degrading after then, accompanied by endothelial cell blebbing, suggesting that the degradation may be due to apoptosis. We agree that fluorescence based assays would be quantitative, however, labelling or transfecting the cells with fluorescent reagents affects the ability of endothelial cells to form tubules. Therefore, to provide more direct and convincing evidence, we lysed all cells from the tube formation assay and detected pro-caspase 3 cleavage biochemically. This data (in Figure 8E), demonstrates that TGF-beta increases procaspase 3 cleavage, and fibronectin inhibits this effect in an endoglin dependent manner. Accordingly, we revised the related description from "TGF-b induced pro-caspase-3 cleavage (Figure 8E), apoptosis (black arrows in 24h panel in Figure 8E) and tubule degradation (Figure 8D) were all significantly decreased in the presence of fibronectin (Figure 8D, E)" to "TGF-b induced apoptosis as assessed using pro-caspase-3 cleavage (Figure 8E), and tubule degradation (Figure 8D) were all significantly decreased in the presence of fibronectin (Figure 8D, E)"

We quantified the tubule degradation by counting the tubule number per 4× microscopy field.

9) In some cases western blots appear to be in signal saturation which is problematic for subsequent quantification (especially Figure 2F, 4G).

We repeated the experiments in Figure 2F, 3B, 4F and 4G, and improved the exposure time. These results have been replaced with the shorter time exposures.

10) The paper is too long (especially the results section) and could be improved by focusing on the important findings.

To shorten the results section, we combined some related descriptions and deleted some descriptions which were repetitive with the Introduction or Discussion sections.

Referee #3:

We appreciate the reviewer for finding that the manuscript "give insights to the way TGF-beta1 changes from a promoter of endothelial cell migration to a suppressor of cell migration via the described crosstalk. The experiments are carefully performed and complementary approaches are being used to show the results. While the basic molecular studies are comprehensive, the angiogenic assays and in vivo validation using zebrafish are short but confirm the model presented at the end.The manuscript is well written and highlights the novel findings in the right context."

1. Figure 2C: while fibronectin indeed showed enhanced TGF-beta1 induced Smad1/5/8 phosphorylation, collagen shows decreased phosphorylation. Please comment on this.

We now comment on this finding in the revised manuscript.

2. Figure 2F: here and in many Figures to follow, some of the blots are overexposed and with that a quantification relative to e.g. total Smad1 and Smad 2 (as shown here) is impossible. Please show shorter exposure times of the same blot.

We repeated the experiments in Figure 2F, 3B, 4F and 4G, and improved the exposure time. These results have been replaced with the shorter time exposure. We quantified the western results relative to beta-actin instead of T-Smad1 and T-Smad2.

3. Fig 3D: this is a/the crucial experiment of this study. The data here are clear and well presented. How often has this been repeated? Please highlight this finding more in your text.

We appreciate the reviewer for identifying the significance of these results, which is very consistent (N=3). In addition to the data in Figure 3D, we also provide data in supplementary Figure 6 that SB-431542 inhibits both Smad1/5/8 and Smad2 phosphorylation on plastic or dishes coated with laminin and collagen, while in supplementary Figure 10 we demonstrate that SB-431542 only inhibits Smad2 but not Smad1/5/8 phosphorylation in dishes coated with fibronectin. We have now further highlighted this finding in the discussion section to discuss the potential mechanism and its implication in angiogenesis (From "Interestingly, while the ALK5 inhibitor, SB-431542, inhibited TGF-beta induced Smad2 and Smad1/5/8 phosphorylation in endothelial cells ……..," to "…..and would not be dependent on ALK5 signaling, consistent with what has been reported in murine models (Park et al, 2008)").

4. Figure 4A and B: in A (time course of stimulation) needs pbeta1 blot for completeness, while Figure 4B needs alpha5 blot. Please add data or comment on this.

We have added these data in revised Figures 4A and 4B.

5. Figure 4F and 4G: as mentioned already before here total beta1 is overexposed and therefore it is impossible to control equal loading or potential changes of total beta1 integrin levels. Please show shorter exposures.

We repeated these experiments and improved the exposure time. Revised Figures 4F and 4G have been replaced by the shorter time exposure.

6. Figure 5C: the reciprocal immunoprecipitation (i.e. immunoppt of endogenous alpha5- or beta1-integrin and detection of exogenous HA-tagged endoglin) is less convincing.

The reciprocal immunoprecipitation is less convincing due to the low expression level of integrin alpha5beta1 in HEK293 cells. Accordingly, we transiently co-transfected COS7 cells with HA-endoglin and alpha5 or beta1 integrin, and performed reciprocal immunoprecipitation experiments. Figure 5C has been replaced with this data.

7. The authors try to map the interaction sites between endoglin and alpha5 integrin using a number of endoglin extracellular deletion mutants (Figure 5H). None of those abolished binding even though the TMCT mutant (lacking the complete extracellular domain) failed to do so. It is very important here to check for cell surface expression of all mutants before a statement as done in the results and discussion section (alpha5beta1 interact with endoglin with the extracellular domain) can be placed.

To directly demonstrate cell surface expression, we expressed the mutants in COS7 cells, labeled the cell surface proteins with biotin, lysed the cells, immuneprecipitated with anti-HA antibody, and then probed with streptavidin-HRP to determine whether the endoglin mutants are expressed on cell surface. All endoglin mutants, including ECTM, TMCT, DEL26-173, DEL26-323 and DEL26-468 were all expressed on the cell surface (Fig.2 in this letter). This result has been added as supplementary Figure 16.

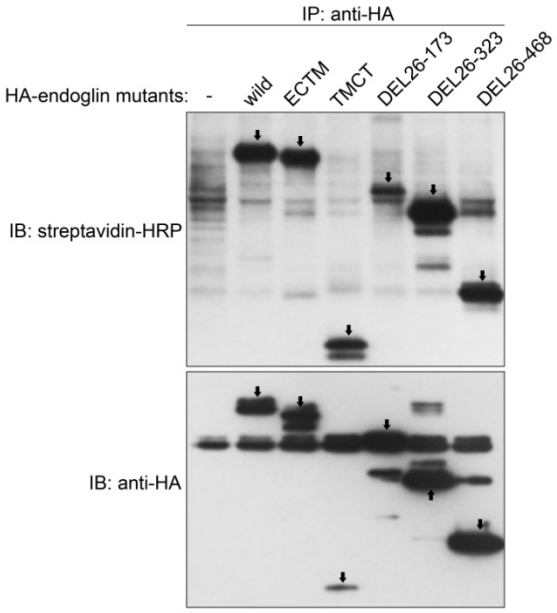

Fig.2 COS 7 cells were transfected with HA-endoglin, HA-endoglin ECTM, TMCT, DEL26-173, DEL26-323 and DEL26-468 mutants for 24h. Transfected cells were labeled with 0.5mg/ml Sulfo-NHS-LC-Biotin in 4^{o}C for 30min. Cells were lysed and subjected to immunoprecipitation with anti-HA antibody and probed using streptavidin-HRP and anti-HA antibody.

8. Where in endoglin and Alk1 are the tags (HA and myc) localized, extracellularly or intracellularly?

HA and myc tags are localized in N terminals of extracellular domains of endoglin and ALK1. This is now noted in the text.

9. Figure 7B: for completeness, please include here also the interaction with beta-arrestin.

We investigated the interaction between b-arrestin2 and endoglin and endoglin T650A mutants in co-transfected COS 7 cells. As we described in the manuscript, beta-arrestin2 interacted with wild type endoglin, but not with endoglin T650A mutants. We added this result as Supplementary Figure S19.

10. Figure 7D: the HA blot is difficult to see; what is the transfection efficiency in this experiment? How often has this been repeated? The statements of this experiment is too strong considering, how complex clathrin-mediated endocytosis is regulated. I strongly suggest to slow down on any endocytosis arguments in this study, since the data for this are incomplete and not really necessary. For this please also rewrite the paragraph in the discussion (Our data here demonstrate that endoglin T650A mutant, which cannot bind barrestin2 (Lee&Blobe, 2007), suppresses endoglin/integrin alpha5beta1 complex internalization and ....).

We replaced the HA blot. The transfection efficiency is around 50%. We repeat this result 3 times and obtained similar results.

We rewrote the paragraph in the discussion and decreased our emphasis and softened our conclusions with regards to endocytosis. The revisions are highlighted in the revised manuscript.

11. The authors mention in the discussion, that the data support a model in which fibronectin induces clustering of integrin alpha5beta1. This would be very interesting as a potential add-on study.

We investigated fibronectin inducing clustering of integrin alpha5beta1 using Duolink assay, and found that this is indeed the case. This result has been added as Supplementary Figure S17.

References for rebuttal letter

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Lee NY, Golzio C, Gatza CE, Sharma A, Katsanis N, Blobe GC (2012) Endoglin Regulates PI3-Kinase/Akt Trafficking and Signaling to Alter Endothelial Capillary Stability During Angiogenesis. *Molecular biology of the cell*

Park JE, Shao D, Upton PD, Desouza P, Adcock IM, Davies RJ, Morrell NW, Griffiths MJ, Wort SJ (2012) BMP-9 induced endothelial cell tubule formation and inhibition of migration involves Smad1 driven endothelin-1 production. *PLoS One* 7: e30075

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2nd Editorial Decision 03 August 2012

Thank you for the submission of your revised manuscript and please accept my apologies for the delay due to the holiday season. As the referee who was asked to evaluate your study considers that all the previous concerns have been properly addressed (see below), I am writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few more minor details have been addressed, as follows.

Browsing through the manuscript, I have noticed minor problems with the description of your statistical analyses, particularly with the definition of the error bars used in panel C in figure 2, panel B in figure 6 and panels B, D and E in figure 8. Same applies to the figures in the supplementary data section. As a guide, statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply and will include a definition of the error bars used and the number of independent experiments performed. As these are minor text additions, you do not need to upload a new version of your manuscript. Send me the corrected figure legends as an e-mail and we will introduce the necessary changes.

I would also like to mention that, as a novel initiative in The EMBO Journal, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this initiative do not hesitate to contact me.

After these remaining corrections have been introduced, you will then receive an official decision letter accepting your manuscript for publication in The EMBO Journal. This letter will also include details of the further steps you need to take for the publication process to continue.

Thank you for your contribution to The EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

Referee #2:

All issues raised before have been satisfactorily addressed by the authors. The current version of the manuscript should be published in the EMBO Journal.