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Molecular basis for antagonism between PDGF and the TGFß-family of signaling pathways by control of miR-24 expression

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Transaction Report:

(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 03 July 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated your manuscript and I enclose their reports below. As you will see from their comments overall the referees express potential interest in the crosstalk between PDGF and BMP signaling in vSMCs, however, it is clear that currently the study does not provide sufficient direct experimental evidence for the importance of the pathway to make the study suitable for publication in the EMBO Journal.

Although the referees find the main conclusions of the potentially interesting and important they require further experimental evidence for the mechanism by which miR-24 inhibits Trb3 expression and on Smad levels. They also request more evidence that the miR-24 pathway is important for the phenotypic modulation of vSMC and that Trb3 is directly involved in modulating marker gene expression. There is also concern about the non-physiological levels of ectopic expression of miR-24. Finally, they find that the studies on Trb3 also influencing TGF-beta signaling need to be expanded to show if Trb3 functions directly downstream of TGF-beta.

I realize that this is a lot of work to demonstrate the direct role of the PDGF-miR-24-Trb3 pathway as being important in vSMC but should you be able to address these criticisms, 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 included in the next, final version of the manuscript. I would like to point out that once you revise the manuscript and submit a revised version to the EMBO Journal,

please make sure you upload a letter of response to the referees' comments.

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

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

The manuscript reports interesting findings that PDGF-BB induces the expression of miR-24, represses the expression of Trb3, and inhibits BMP signaling in vascular SMCs. They have also shown that the effect of miR-24 is observed in BMP-induced osteoblast differentiation in C2C12 cells and TGF-b-induced SMC differentiation in PASMCs. In general the data showing the crosstalk between PDGF and BMP signaling on vascular SMCs is very interesting and important in the field. However, some of the data were overestimated, and more careful interpretation of the data may be required.

Critiques
1. Fig. 4. PDGF-BB induced 1.5- to 2-fold induction of miR-24. However, in most experiments using miR-24 mimic, they used 0.3 nM-3 nM miR-24 mimic, which results in very high expression of exogenous miR-24 compared to physiologically induced levels of miR-24 (see Fig. 3A). Thus, the results using miR-24 mimic should be carefully interpreted. In Fig. 6F, they used a very high amount of miR-24 mimic, so the effect of miR-24 on osteoblast differentiation by BMP4 may be not physiological.
2. They state that miR-24 inhibits the expression of Trb3, resulting in decrease in the expression of Smad1/5. However, they show the decrease of Smad1/5 protein only in Fig. 3C. It is possible that miR-24 may act on some other genes and exhibit its effects by alternative ways. To confirm that the effect of miR-24 is induced by repression of Trb3, they should show the decrease of Smad1/5 protein by miR-24 mimic in vascular SMC. In Fig. 6F and Fig. 9, they should also show the expression levels of Smad proteins by miR-24 in these cells.
3. In addition to the above experiments, they should show whether the effect of miR-24 is abolished by overexpression of exogenous Trb3.
4. The effect of miR-24 on TGF-b signal needs more data. In its present form, they just show that the effect of TGF-b can be regulated by miR-24 mimic and by anti-miR-24. It is unclear whether Trb3 is responsible for the effect of miR-24 on TGF-b signaling. Since there are some contradictory data published by other investigators, they can show these data in another paper in the future after doing more experiments.
5. Page 12, line 18 and 19. "Fig. 3C" should be "Fig. 3B". Page, 14, line 5. "Fig. 4D, green dotted line" should be "Fig. 4C, green dotted line".

Referee #2 (Remarks to the Author):

Chan et al. (Hata) EMBO J.

The authors present evidence that PDGF upregulates the expression of miR-24 and that this microRNA plays a key role, through the regulation of Trb3 expression, in the ability of PDGF to inhibit the BMP- and TGFß-induced contractile phenotype.

Overall, this manuscript makes an important set of observations that may be important for our understanding of the roles of PDGF and BMP/ TGF-ß in the phenotype of smooth muscle cells (SMCs). As specified below, some data need to be more convincing, whereas further substantiation of the role of this microRNA in SMC phenotype is needed. Also, additional data are needed to substantiate its effect in TGF-b signaling (Fig. 9) and better quality pictures are needed for the in vivo data in Fig. 8.

- Related to Fig. 1A: The authors state that there is an induction of Id3 mRNA in response to co-stimulation with BMP-4/PDGF at 6h, but that the result is different at 24 h. This makes me concerned that the 24 h data, as presented, may differ significantly from the 6h data that are not presented. Since the 6h data may be more indicative of direct responses to BMP-4 and/or PDGF, it would be better to show the 6h data in Fig. 1A.
- Related to Fig. 2B and 2D: These data are not publication quality. Furthermore, the GAPDH protein levels are not equal; therefore, the decrease in Trb3 or Smad1, when accurately normalized against GAPDH, may differ form what is now shown. Cleaner data are needed.
- page 12, first two sentences, related to suppl. Fig. S2: The possibility that there may be transcriptional regulation of Trb3 expression in response to PDGF is ruled out by the authors merely using a promoter-reporter construct. This is a poor indication, since one could easily say that this construct lacks the regulatory element. A better evaluation is needed. Nuclear run-on assays may be needed to evaluate this issue.
- page 15, end of the section, related to Fig. 5B: considering the importance of the conclusion that the "miR-24-Trb3 axis" is required for the phenotype modulation of SMCs by PDGF, it is disappointing that the readouts are only relative mRNA/miRNA levels for 2 marker genes. Please provide more evidence, related to the SMC phenotype.
- Related to Fig. 8A, the pictures of the staining are inadequate to illustrate what is concluded in the text (and I am used to seeing immunohistochemistry data). Better pictures are needed. In addition, Smad1/5 staining is required since Smad1/5 levels should be modulated by Trb3 and should serve as control for the phospho-Smad1/5 staining.
- Related to Fig. 9, the evidence for regulation of TGF-ß signaling by the "miR-24-Trb3 axis" is not as convincing as it should be, and as illustrated for BMP signaling. The authors should not necessarily repeat all the same experiments as for the regulation of BMP signaling, but more convincing evidence is needed. For example, how do the data in Fig. 9A, B relate to the Smad3 and phospho-Smad3 levels. As another example, in Fig. 9B is the difference between Trb3 and Trb3+UTR related to regulation of the Trb3 UTR by miR-24, or could it be just plain unequal expression levels, independent of the miRNA. Additional examples of how this section needs to be "tightened up" should be considered.

Minor points:
- Abstract, line 9: Isn't the use of "a block" somewhat of an exaggeration? Maybe "decrease" is better.
- page 12, 6th and 5th lines from bottom: Fig. 3C should be Fig. 3B.
- page 14, last line of first paragraph: miR-24-2 should be miR-24-1.
- page 16, 2nd line: replace "the" with "a". There are other E3 ligases that target Smads.

Referee #3 (Remarks to the Author):

In this manuscript authors analyzed the function of miR-24 in the response to PDGF-BB in SMCs. Results demonstrate that miR-24 levels are increased by PDGF-BB and miR-24 downregulates levels of Trb3. That knocking down Trb3 resulted in reduced expression of SMC differentiation markers, and that anti-miR24 eliminated the PDGF-BB-induced downregulation of Trb3 suggest that PDGF-BB may control Trb3 levels via miR-24. They also showed that Trb3 is involved in TGF/BMP-Smad signaling. Consequently, results suggest that miR-24 is a possible cross-talk mechanism that mediates the PDGF and TGF-Smad signaling in SMCs.

Major points.

In this study it appears that miR-24 downregulates Trb3 mRNA. It has not been formally tested, but authors suggest that miR-24 promotes degradation of Trb3 mRNA in Discussion. miRNA is known to regulate target gene expression primarily by both translation block and mRNA downregulation. Is translational block also important for the miR-24 dependent inhibition of Trb3?

Fig. 8A. There are a number of SMA-positive cells in the adventitia and surrounding tissues. What kind of cells are they? Also, higher magnifications views would clarify localization of the proteins in the vascular wall. Scale bars should be added.

In Fig 2A Trb3 levels were dramatically reduced by PDGF-BB and the inhibition sustained over 24

h. However, miR-24 levels were increased by only less than 2-fold and peaked within 4 h (Fig. 4C). In the miRNA-mimetic transfection experiments shown in Fig. 3A, in which 0.03 nM of miR-24 increased the level of miR-24 approximately 2-fold, Trb3 levels was decreased by only 40%. What could be the reason for this apparent discrepancy in the effects of miR-24?

In this study, the involvement of Trb3 in PDGF-BB-induced downregulation of SMC marker genes was not directly addressed. One possible experiment would analyze expression of endogenous SMC markers in SMCs overexpressing Trb3 and Trb3+3'UTR as Fig. 9B.

Minor points.

1. p12 (Fig. 3C, lanes 2 and 3) and (Fig. 3C, lanes 3 and 4) seem to be Fig. 3B.

2. p. 23 "Thus, PDGF-BB is able to inhibit expression of contractile markers by .... MRTF-A/B through induction of miR-24". This was not directly tested.

3. In Fig. 9B, luciferase activities in the cells transfected with control mimic and either Trb3 or Trb3+3'UTR should be shown.

1st Revision - authors' response 14 August 2009

Response to Referees

Referee #1:

We want to begin by thanking Referee#1 for writing that "the finding in our manuscript is generally very interesting and important in the field." We also appreciated the constructive criticism and advice. We addressed all the points raised by the reviewer, as summarized below.

1. According to the referee's suggestion, the experiment demonstrating the effect of miR-24 mimic on osteoblast differentiation in C2C12 cells (old Fig. 6F) was repeated with a decreased concentration of miR-24 mimic; in the new experiment, the expression level of the mimic is only ~2-fold above the endogenous level, and is comparable to the level obtained by induction with PDGF-BB in PASMCs. Despite the moderate increase in miR-24 expression, both Trb3 and the BMP-mediated induction of ALP and Id3 were significantly reduced in the presence of miR-24 mimic. This result is presented in the revised Fig. 6I.
2. The referee advises to demonstrate the effect of miR-24 mimic on the Smad protein level in vSMCs. Total-Smad, phospho-Smad, and Trb3 protein levels were examined by immunoblot and are presented in the new Fig. 6A (for BMP-specific Smad) and the new Fig. 9A (for TGFß-specific Smad).
3. The referee suggests demonstrating that the effect of miR-24 mimic is abolished by overexpression of exogenous Trb3. This experiment was performed in PASMCs by comparing the effect of the Trb3 expression construct deleted in the 3'UTR, which is resistant to miR-24, and the Trb3 construct containing the 3'UTR, which is sensitive to miR-24. This result is presented in the revised Fig. 6C.
4. The referee comments that it is unclear whether the effect of miR-24 on TGFß-signaling is due to downregulation of Trb3. To address the referee's comment, we revised Fig. 9B and demonstrated that the inhibitory effect of miR-24 on the TGFß signaling pathway can be rescued by overexpression of a form of Trb3 that is resistant to miR-24, but not by a Trb3 construct including the 3'UTR, which contains the miR-24 seed sequence, despite a similar level of expression of these two Trb3 constructs. To further confirm the miR24-Trb3-Smad axis, two new data have been added in the revised Fig. 9; (i) miR-24 mimic decreases the TGFß-specific Smad protein level (see the revised Fig. 9A), and (ii) the effect of downregulation of Trb3 by siRNA on the TGFß-mediated induction of the TGFß-Smad target genes, which mimics the effect of miR-24 (see the revised Fig. 9D). In summary, the results in Fig. 9 demonstrate that (i) the effect of miR-24 mimic on the TGFß signal is mediated by downregulation of Trb3 and Smad protein, and (ii) the miR-24 effect on TGFß signaling requires the 3'UTR of Trb3.
5. Thanks to the referee's comment, the wrong figure numbers were corrected in the revised

manuscript.

Referee #2:

We want to thank Referee#2 for constructive and insightful criticism and advice. We addressed all the points raised by the reviewer as summarized below.

1. The referee recommends to show the Id3 mRNA level after 6 hr treatment with BMP4/PDGF-BB in Fig. 1A. We performed the experiment and its result is included in the revised Fig. 1A.
2. According to the referee's suggestion, the experiments in Fig. 2B and 2D were repeated several times and representative data are included in the revised Fig. 2B and 2D.
3. Based on the referee's comment that the result of the Trb3-promoter-luciferase reporter assay (Supplementary Fig. S2) is not conclusive evidence to exclude the possibility that PDGF-BB may modulate Trb3 expression through transcriptional regulation, the text in p. 12 was revised. The revised text reads "... suggesting the possibility that PDGF-BB might modulate Trb3 expression through a mechanism other than transcriptional regulation."
4. The referee comments that the effect of the miR24-Trb3 axis on vSMC phenotype regulation by PDGF-BB should be examined by readouts other than contractile gene expression. The effect of si-Trb3 was examined in the PDGF-mediated stimulation of cell growth and the result is presented in the revised Fig. 5D. We would like to note that miR-24 has no effect on the PDGF-induced cell migration in PASMCs (see Supplementary Fig. S4).
5. Based on the referee's comment, the revised Fig. 8A includes total Smad1 staining.
6. The referee comments that the miR24-Trb3 axis in the context of the TGFß signaling pathway, presented in Fig. 9, is not convincing enough, echoing comment #4 of Referee#1, above. As stated above, we have included new results, which include: (i) miR-24 mimic decreases the TGFß-specific Smad protein (Smad2/3) level (see the revised Fig. 9A), (ii) rescue of the inhibitory effect of miR-24 on the TGFß signaling pathway (SBE-Luc reporter assay) by Trb3 without the 3'UTR (see the revised Fig. 9C), and (iii) the effect of downregulation of Trb3 by siRNA on the TGFß-mediated induction of the TGFß-Smad target genes, which mimics the effect of miR-24 (see the revised Fig. 9D). In summary, these results demonstrate that (i) the miR-24 mimic effect on the TGFß signal is mediated by downregulation of Trb3 and Smad protein, and (ii) the miR-24 effect on the TGFß is dependent on the 3'UTR of Trb3.
7. All minor points raised by the reviewer were corrected accordingly.

Referee #3:
We want to thank Referee#3 for constructive and thoughtful criticism and advice. We addressed all the points raised by the reviewer as summarized below.

1. It has been shown that the mechanism of silencing a target mRNA by miRNA is either by degradation of mRNA or translational repression, and it depends on a specific combination of target mRNA and miRNA. In the case of miR-24-Trb3, Trb3 mRNA is significantly downregulated by both PDGF treatment (see Fig. 2A) and overexpression of miR-24 (see Fig. 3A). It is believed that translational repression requires a stable association between miRNA and 3'UTR of the target mRNA; therefore, since miR-24 degrades Trb3 mRNA, we speculate that it may not be able to inhibit protein translation simultaneously. We agree with the referee that we cannot exclude the possibility that miR-24 might block protein translation; however, investigating the potential mechanism of translational inhibition of Trb3 by miR-24 is beyond the main scope of this manuscript, since a number of different mechanisms have been proposed for miRNA-mediated translational repression, such as (i) inhibition of recruitment of translation initiation factors (eIFs) to mRNA, (ii) inhibition of recruitment of ribosomes, or (iii) enhancement of dissociation of ribosomes from mRNA.
2. The referee advices to present a result with higher magnification in Fig. 8A, as well as staining with anti-Smad1 antibody. The revised results are now presented in the revised Fig. 8A. The referee also notes a positive staining in the adventitia. Adventitia is the outermost layer of the blood vessel and is composed of a loose matrix of elastin, vascular smooth muscle cells, fibroblasts and collagen. As the adventitia contains high level of extracellular matrix, it tends to absorb antibodies non-specifically, generating a positive staining. Therefore, it is currently inconclusive whether the positive staining in the adventitia is specific.
3. We agree with the referee's comment on a difference between PDGF-mediated downregulation of

Trb3 and the miR-24 mimic-mediated downregulation of Trb3. We speculate that the discrepancy is due to a difference in stability and silencing activity between miR-24 mimic and endogenous miR-24. We and other groups have noticed that miRNA mimics, which contain chemically modified ribonucleotides, exhibit different stability and silencing activity in vivo. One possibility is that a protein complex stabilizes the endogenous double-strand (ds) mature miRNA prior to separation of dsRNA into a single-strand miRNA. It is also possible that endogenous miRNA might be more efficiently incorporated into the RISC complex in comparison with miRNA mimic due to differential recognition by Argonaute proteins. Our previous work on miR-21 and miR-221 also indicated that miRNA mimics exhibit weaker silencing activity, so that higher levels of miRNA mimic expression are required for efficient silencing of targets. However, despite a moderate downregulation of Trb3 by miR-24 mimic, the inhibitory effect of miR-24 mimic on BMP-mediated vSMC contractile gene expression in PASMCs and osteoblastic differentiation in C2C12 is significant, as shown in Fig. 6B and 6I, respectively.
4. The referee comments that the involvement of Trb3 in PDGF-induced downregulation of vSMC marker genes is not directly addressed. We would like to draw attention to Fig. 5B and Fig. 5D (new result) demonstrating that PDGF is unable to inhibit vSMC marker genes or increase proliferation of cells when endogenous Trb3 is reduced by siRNA. We also included a new result which we overexpressed a Trb3 construct missing the 3'UTR and therefore resistant to miR-24 in rat pulmonary artery PAC-1 cells, and demonstrated that this construct is able to rescue the effect of PDGF on the contractile gene expression in vivo (Fig. 5C).
5. As a minor point, the referee comments that a sentence on p. 25 ("PDGF is able to inhibit contractile markers by inhibiting the function of MRTF-A/B through induction of miR-24") is not directly tested. We acknowledge the referee's point and revised the sentence. The revised text reads: "it is intriguing to speculate that PDGF-BB might inhibit..."
6. As a minor point, the referee comments that "luciferase activities" instead of "fold induction" should be presented in old Fig. 9B. The requested result is presented in the revised Fig. 9C.
7. The other minor point was corrected according to the referee's comment.

2nd Editorial Decision 07 September 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the three original referees whose comments are shown below. As you will see from their comments, both referee #2 and #3 find that some of their original comments have not been satisfactorily addressed, and require some further experimentation to resolve these issues. While it is normally EMBO Journal policy to only allow a single round of revision, based on the comments of the referees we are able to allow a second round of revision in order to allow an opportunity to address these remaining concerns.

Should you be able to address these criticisms we could consider a revised manuscript. I should point out that acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

The manuscript is very interesting in the field. The authors have properly revised the manuscript.

However, minor revision of the manuscript is required.

1. "Materials and Methods" should be placed after "Discussion".
2. page 17, line 6 from the bottom: This part should be "vSMC-specific BMP targets SMA and CNN".

Referee #2 (Remarks to the Author):

Chan et al. (Hata) EMBO J.
The authors present evidence that PDGF upregulates the expression of miR-24 and that this microRNA plays a key role, through the regulation of Trb3 expression, in the ability of PDGF to inhibit the BMP- and TGF-ß-induced contractile phenotype.

Overall, this manuscript makes an important set of observations that may be important for our understanding of the roles of PDGF and BMP/ TGF-ß in the phenotype of smooth muscle cells. In revising the manuscript the authors present some additional data in response to the reviewers' comments. Unfortunately, two of my previous comments (and one minor comments) were not appropriately addressed, even though this would have been very doable and is needed for the conclusions. Additionally, some new questions arise with the new data presented. My individual comments (some of them minor) are listed below in order of appearance.

- page 12, first few sentences, related to suppl. Fig. S2: The possibility that there may be transcriptional regulation of Trb3 expression in response to PDGF is tested using a promoter-reporter construct, which very well may lack the regulatory element(s). In my previous report, I requested that the authors address whether there is transcriptional regulation. The authors elected not to do so, but to rather change some wording related to this issue. This is disappointing, especially since they continue their story in the full assumption that there is no transcription regulation. Further, they did not change the legend to Fig. S2, concluding that PDGF does not alter the transcription of Trb3. As in my previous review, I re-request that the authors assess whether or not PDGF regulates Trb3 gene transcription, which can be done e.g. using nuclear run-on assays.
- Fig. 3A (right panel) and corresponding text: It is unclear how the authors come to the conclusion that 0.03, 0.3 and 3 nM of miR-24 corresponds to 2, 35 and 53 times the endogenous miR-24 level. I have a hard time understanding this assertion, especially considering the values in the y-axis of this plot, and also that e.g. 0.3 and 3 nM (a 10-fold increment) correspond to only a change from 35 to 53 times (i.e. a 1.5 increase) the endogenous level.
- page 17, 2nd line of new paragraph: replace "the" with "a". There are other E3 ligases that target Smads. (same comment as in previous review)
- page 17, line 11 of new paragraph (related to Fig. 6B): I am surprised by the statement that transfection of 0.3 nM of miR-24 corresponds to a 5-fold increase of the endogenous miR-24 level, since, related to Fig. 3A, the authors stated that transfection of 0.3 nM of miR-24 corresponds to a 35-fold increase of the endogenous miR-24 level in the same cells. Also in that same sentence (line 12) delete "efficiently", since a reduction to 30% does not seem efficient. In addition, the next sentence states "although not completely abolished", but the data in Fig. 6B show that the induction is completely abolished.
- Fig. 6C (new figure) and corresponding text. The authors make conclusions about the expression of Smurf1, but they never show the expression of Smurf1, and this should be shown. Also, why is this effect proposed to be restricted to Smurf1 without affecting both Smurf1 and 2?
- page 19, line 6 of 2nd paragraph, related to Fig. 6H: delete "efficiently", since the data do not show an efficient decrease.
- Fig. 9C and associated text: As requested in my previous review, the authors should show a correlation of this bar graph with the levels of Smad3 and phosphoSmad3. This is needed since the SBE reporter scores TGF-ß-induced Smad3 activity and the authors conclude that miR-24 acts through Trb3 on TGF-ß-activated Smad2/3.

Referee #3 (Remarks to the Author):

In this revised manuscript, authors added data that support their initial conclusions.

Fig 8. Immunostaining is unconvincing. This reviewer understands that staining of epithelial and endothelial layers are sometimes problematic due to high contents of ECMs and the edge effect. However, endothelial cells are negative for SMA-actin and many researchers have successfully obtained specific staining of SMCs. In figures presented in Fig. 8A, it is difficult to distinguish positive and negative cells, which suggest nonspecific overstaining. As this reviewer suggested to the original manuscript, higher magnifications might help differentiation of staining levels. Alternatively, Western analysis of the proteins would provide more convincing quantitative data. Quantification using immunostaining is generally problematic. If possible, quantification of miR-24 by other methods, such as Northern and QPCR, would strengthen the findings.

Fig. 5C. Along with Fig. 5D, these new results potentially support that Trb3 mediates at least a part of the PDGF-BB signaling. However, relative expression levels of exogenous human Trb3 against endogenous rat Trb3 are not clear. Although it might be difficult to directly compare expression levels of exogenous Trb3 and those of endogenous Trb3 due to the species difference, if possible Western analysis of those two species of Trb3 would provide clearer idea about the levels of exogenous Trb3. Also this reviewer feels that the graph showing relative levels of CNN and SM22A would show effects of overexpression of exogenous Trb3. This reviewer expects that Trb3 overexpression may increase expression of CNN and SM22.

2nd Revision - authors' response 05 October 2009

Response to Referees

We would like to thank the referees for their thoughtful review of our manuscript. We believe that the additional changes we have made in response to the reviewers comments have made this a significantly stronger manuscript. Below is our point-by-point response to the referees' comments.

Referee #1:

Referee #1 requests two minor editorial changes. Both changes have been made accordingly in the revised manuscript.

Referee #2:
We sincerely apologize to Referee#2 for not completely addressing all of the points raised in the previous response. We have done so below and added additional data in hopes that this reviewer will be supportive of publication.
1. Referee #2 requests evidence that the transcription rate of the Trb3 gene is not altered by PDGF-BB treatment. According to the referee's suggestion, a nuclear run-on assay was performed in PAC1 cells to demonstrate that the transcription rate of Trb3 is not altered by PDGF-BB treatment. The result is presented in Supplementary Fig. S2B.
2. The referee points to a discrepancy between the concentration of miR-24 mimic used in transfection experiments and the amount of miR-24 detected in cells. We repeated the experiment several times with increasing transfected amounts of miR-24 mimic. A representative result is presented in the revised Fig. 3A. The new result demonstrates that transfection of 0.1, 0.3, 0.6, and 1 nM miR-24 mimic produces an increase of miR-24 expression of 1.6-, 3-, 4-, and 7-fold over the endogenous level. Under these conditions, Trb3 mRNA level was reduced to 52%, 24%, 18%, and 16% of the basal level.
3. Page 17, "the" E3 ubiquitin ligase was changed to "an" E3 ubiquitin ligase.
4. Referee #2 comments that the level of Smurf1 is not shown in Fig. 6C. We feel that the effect of Trb3 expression and function on Smurf1 expression, ubiquitination, degradation and function has been thoroughly explored and documented in our previous publication (Chan et al, MCB 2007). In this manuscript, we showed that Trb3(WT) but not Trb3(ΔK) interacts with Smurf1 and promotes degradation of Smurf1 in PASMCs. Thus, we believe that the analysis of Smurf1 expression in response to exogenous expression of Trb3(WT) or Trb3(ΔK) in the present study is somewhat

redundant.
5. Referee#2 asks whether the effect of Trb3 is specific to Smurf1. We would like to note that we investigated potential regulation of Smurf2 by Trb3 in our previous study and found no evidence that Trb3 can modulate Smurf2 level in PASMCs. Therefore, in this manuscript we focused on Smurf1 as a downstream effecter of Trb3.
6. The request to demonstrate total and phospho-Smad3 levels after miR-24 expression in Mv1Lu cells in Fig. 9C has been satisfied. This result is presented in Supplementary Fig. S6.
7. A few minor editorial changes requested by the referee have been made in the revised manuscript.
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Referee #3:
We begin by apologizing sincerely to Referee#3 for not completely addressing all of the points raised in the previous response. We have done so below and added additional data in hopes that this reviewer will be supportive of publication.

1. Referee #3 comments that the immunostaining data are not convincing in terms of quantitative changes. Despite our best attempt to capture magnified images of rat pulmonary artery sections, we faced the inherent difficulties in obtaining clear staining of the endothelium and quantitative changes in levels of different proteins in very small pulmonary arteries. Therefore, we followed the reviewer's suggestion to employ qRT-PCR analysis to address changes in Trb3, SMA, or miR-24 expression quantitatively in rat lung samples after normoxia or hypoxia treatment. These data are presented in the new Fig. 8A, demonstrating a 2-fold induction of miR-24 while both vSMC-specific contractile markers and Trb3 are reduced about 50% in the hypoxia-treated lung in comparison with normoxia-treated lungs.
2. The referee advises to compare exogenous vs. endogenous Trb3 expression in Fig. 5C. Performing a western blot using two antibodies that specifically recognize human or rat Trb3 proteins is problematic because: (i) two antibodies have intrinsically different affinities to their substrate and it is difficult to compare the results of two separate western blots, and (ii) the western blot experiment is not quantitative. Thus, we generated PCR primers that specifically recognize a sequence evolutionarily divergent between human and rat Trb3, and performed a qRT-PCR analysis. The result is presented in the revised Fig. 5C, bottom panel.
3. Referee#3 is right in predicting that overexpression of Trb3 or Trb3+3'UTR should increase the basal expression of CNN and SM22 in Fig. 5C. However, the data presented in Fig. 5C were plotted as "% inhibition" of CNN or SM22 levels upon PDGF-BB treatment, normalized to basal levels. Therefore, potential changes in the basal levels of CNN or SM22 mRNA by exogenous Trb3 were not displayed. In response to the referee's comment, we added Supplementary Fig. S5 to show the levels of CNN and SM22 normalized to GAPDH with or without PDGF treatment. The result indicates significant increase in basal expression of CNN and SM22 upon transfection of Trb3 or Trb3+3'UTR construct.

3rd Editorial Decision 02 November 2009

Your manuscript has been re-reviewed once more. As you will see one of the referees finds that you have still not addressed some of earlier raised issues, at this point I suggest that you either address these concerns if you have the data or remove the discussion form the manuscript.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. 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 initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

Referee #2 (Remarks to the Author):

Chan et al. (Hata) EMBO J. re-revised
The authors present evidence that PDGF upregulates the expression of miR-24 and that this microRNA plays a key role, through the regulation of Trb3 expression, in the ability of PDGF to inhibit the BMP- and TGF-ß-induced contractile phenotype.

This is the second revision of a manuscript that I now reviewed for the third time. In the previous revision and its rebuttal, several important comments were not addressed or essentially dismissed. The authors have now addressed these comments as well as some additional ones that I had raised, but again did not address two of my comments. This is obviously aggravating, since I take reviewing manuscripts seriously and accordingly spend a substantial amount of time and effort on it. Accordingly, I would hope that the authors respect this commitment. Is this mere negligence or defiance? It certainly does not set a good tone, especially as I have plenty of other things to do (and frankly am getting tired of spending so much time on reviewing this manuscript).

- page 13, 2nd to last line related to Fig. 6B: the "5-fold" in the text does not correspond to what I see in Fig. 6B, which looks like 2-fold.
- page 14, end of last paragraph: How can one make conclusions about Smurf1 and that "miR-24 elevates the levels of Smurf1 through downregulating Trb3", if no data on Smurf1 protein levels are shown? The authors refer to some evidence reported in a previous paper, but this is just not not enough. If you want to have conclusions on Smurf1 levels in this manuscript, then the data need to show Smurf1 levels.

Referee #3 (Remarks to the Author):

All my critiques have been addressed satisfactorily.

3rd Revision - authors' response 02 November 2009

Response to Referees
Referee #1 and #3:
No further comments.

Referee #2:
We deeply apologize for failing to address all points that were raised by this reviewer previously. As you see below, remaining points have been addressed in the revised manuscript.

1. Referee#2 comments that Fig. 6B: the "5-fold" in the text does not correspond to the result in Fig. 6B, which looks like 2-fold. We apologize for our mistake of not fixing the text. As Referee#2 comments, the text should be stated "2-fold" increase. We corrected this sentence in the revised manuscript.
2. Referee#2 comments that p14, last paragraph, our manuscript states that "miR-24 elevates the levels of Smurf1 through downregulating Trb3" is not supported by experimental evidence. We agree with Referee#2 and revised this sentence to "these results support our hypothesis that miR-24 leads to inhibition of the BMP-Smad signaling pathway through downregulation of Trb3." We also revised the sentence in p.14, line 14; "To confirm that the miR-24-mediated inhibition of the BMP activity on SMA and Id3 is due to an increase in Smurf1 as a result of downregulation of Trb3,..." to "To confirm that the miR-24-mediated inhibition of the BMP activity on SMA and Id3 is due to a

result of downregulation of Trb3 and its function, ...". Thus, our speculative comment on the effect of miR-24 on Smurf1 protein is deleted from the revised manuscript.

