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The canonical smooth muscle cell marker TAGLN is present in endothelial cells and is involved in angiogenesis

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MS TITLE: SM22, a canonical marker of smooth muscle cells, is expressed in endothelial cells and negatively regulates angiogenesis

AUTHORS: Kiyomi Tsuji-Tamura, Saori Morino-Koga, Shingo Suzuki, and Minetaro Ogawa
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. The concerns are quite extensive, and therefore I would understand if you decide to send the manuscript elsewhere, particularly in light of possible lab slow downs due to COVID.

However, if you think you can address the concerns, I would be amenable to considering a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This study describes the expression of SM22, an actin crosslinking protein normally expressed in SMCs, in elongated EC. This was observed in EC from ESC and HUVECs and SM22 knockout (all three isoforms) increased HUVEC´s cord formation ability, suggesting that SM22 is a negative regulator of angiogenesis.
SM22 was detected in an E10.5 EC cluster.

Comments for the author

1. The authors use a reporter mouse to detect SM22 in elongated ESC-derived EC. They must verify SM22 by RNA and protein.

2. In knockout HUVECs, cord formation is increased. They must determine cell migration under similar conditions.

3. Fig 1, show the gate for Flk1+ sorting and how many of these were EGFP +, Dsred.T4 + and EGFP+/DsRed.T4+ double positive at time of sort and after 7 days culture. The impression given is that hardly any EC express SM22 promoter activity (we have no data showing that the SM22 gene actually is expressed yet). How will VEGF during culture change these cell populations?

4. Fig 2 is hard to understand. In D, why are there two DsRed columns after VEGF, one 100% SC and one 100% C? In E, what are the proportions, EGFP or DsRed or double positive or only morphology? In J, VEGF+, are all cobblestone cells DsRed? That is inconsistent with the notion that SM22 becomes expressed in elongated cells?

5. Foxo data are not convincing and should be removed.

Reviewer 2

Advance summary and potential significance to field

Comments to authors: Tsuji-Tamura and colleagues present data investigating the role of SM22 in angiogenesis. The authors show definitively in embryonic stem cell lines bearing an SM22 reporter that Flk1+ progenitors can differentiate into both SMCs and endothelial cells with various culture conditions. The remainder of the experimental studies focus on correlations between SM22 promoter activation and an elongation phenotype in differentiated endothelial cells. While this reviewer recognizes that this is an interesting question and the role of SM22 in endothelial elongation and angiogenic sprouting is unresolved, the following concerns are identified in the data as described below.

Comments for the author

Major concerns:
1. The antibodies used to confirm SM22 alpha expression in endothelial cells cross-react with other isoforms (SM22b and transgelin3), which makes it difficult to appreciate specific upregulation of SM22 alpha in wild-type cell and mouse embryos.
This discrepancy is reinforced by single cell RNA Seq data (Supplemental Figure 2B) showing that SM22 expression is barely detectable and highly sparse in VE-cadherin-positive endothelial cells. Expression in Supplemental Figure 2B does not match robust SM22 staining shown in Figs 8B, 8C, and 8D, reinforcing that it is imperative to perform staining in mouse embryos with an SM22-

specific antibody. This discrepancy makes it difficult to appreciate how impactful SM22 expression is on angiogenesis.

2. Data in Figures 6 and 7 are contradictory. Figure 6 shows that SM22 gene expression and promoter activity increase in a 3D sandwich culture compared to gelatin-coated culture and this correlates with elongation. Yet, SM22 overexpression decreases angiogenic parameters in Figure 7, including # of cords, # of branch points, and length of cords. A better assay to test CRISPR and siRNA-silenced cells may be the collagen sandwich assay to determine elongation for consistency, as these discrepancies could be due to the differences in the experimental systems shown in Figures 6 and 7.

3. The concern above is underscored by the title, which states “SM22 … negatively regulates angiogenesis,” while the discussion states “expression of SM22 in elongating ECs must be interpreted carefully.” Thus, the conclusions to be drawn are unclear in the current text.

4. CRISPR cells are of concern for two reasons. First puromycin selection was only carried out for 3 days. Typically, 2 weeks of selection is necessary to ensure widespread antibiotic resistance. Second, evidence of gene excision should be provided in a supplemental file. To alleviate these concerns, the authors should confirm results with the CRISPR HUVEC using another method, such as siRNA or ASO-induced gene silencing.

5. Discrimination between SM22 isoforms is a concern. Western blots should be performed to confirm immunofluorescence staining in Figure 6. Also in Figure 6, SM22 knockdown should be performed with siRNA to confirm that the loss of SM22 leads to a decrease in elongation.

6. SM22 should be overexpressed in wild-type cells to determine if SM22 plays a direct role in reducing (or enhancing) elongation or sprouting. If no change in morphology or behavior is seen, one interpretation of the data enclosed suggests that endothelial elongation stimulated by VEGF activates a transcriptional factor complex that drives SM22 expression in the ESC lines studied here, along with other genes that are responsible for elongation in endothelial cells.

7. This manuscript relies heavily on the use of embryonic stem cell lines.
Figure 1 legend indicates multiple clones were used to confirm results. Some description of how clones were generated (from individual mice or the same mouse) should be included, as well as whether they are male/female in origin.

8. The relevance of the Foxo data are unclear and don’t provide insights because no SM22 expression is shown. Consider removing from this manuscript.

Minor concerns:
1. Reverse transcriptase PCR studies should be replaced with qPCR.
2. Colors used in figure labels should match figures. As one example, Figures 2F and 2G depict VE-cadherin staining in yellow but labels are orange.

Reviewer 3

Advance summary and potential significance to field

In their manuscript, Tsuji-Tamura and colleagues report the expression of SM22 (Tagln), which is commonly applied as a marker for smooth muscle cells, in endothelial cells when activated to take an elongated cell shape that is induced by either stimulation with the growth factor VEGF-A, or by treatment with inhibitors of the PI3K/AKT, or mTORC pathways. The authors demonstrate further the expression of SM22 in primary human endothelial cells (e.g. HUVEC) and that these cells exhibit an increased angiogenic behavior when SM22-isoforms (Tagln, Tagln2, Tagln3) are depleted in vitro. The authors demonstrate the expression of SM22 in endothelial cells also in mouse embryonic tissues by immunostaining (limb and aorta) as well as single cell RNA transcriptomics (public dataset of embryonic mouse aorta), and show the persistence of SM22 protein in abnormal endothelial cells (Foxo1-/-). Based on their observations, the authors suggest an involvement of SM22 as negative regulator in endothelial angiogenesis.

Overall, the study is well designed, data analysis is solid and statistical analysis performed. The study provides new insight into detailed regulatory mechanisms of endothelial behavior; the up-regulation of SM22 in endothelial cells when elongated. It has been shown before (Varberg KM et al. 2018 10.1152/ajpcell.00137.2018) that SM22 is expressed in endothelial colony forming cells, however, the postulated involvement as negative regulator of angiogenesis adds novelty to the current study. The manuscript is carefully written and the results presented in a coherent form. Nevertheless, several concerns are necessary to be addressed prior to a publication of the manuscript.

Comments for the author

The authors show convincingly the expression of SM22 (Tagln) in endothelial cells, by reporter gene expression under the control of the Tagln promoter element. However, the mechanistic insides, protein presence and functional relevance remain unclear. Conceptual, the authors should discuss why the expression of Tagln as negative regulator of angiogenesis is induced in response to stimulation with VEGF-A, a potent pro-angiogenic growth factor.

The authors claim that SM22 (Tagln) is a negative regulator of angiogenesis, however, they show in Figure 7 that only the knock-out of all three members of the Tagln family (Tagln, Tagln2, Tagln3) leads to increased cord length and branch point formation of endothelial cells in vitro. Thus, the specific functional relevance of Tagln for the reported cellular phenotype remains unclear. In turn, this observation raises the question, whether not only Tagln but all members of the family are similarly regulated.
How does the expression of Tagln2 and Tagln3 change in the experimental set-up presented in Figure 2-6? Since little is known about the functions of Tagln2 and Tagln3 a more detailed mechanistic information of the contribution of the different Tagln family members is important.

According to the Tabula Muris database (https://tabula-muris.ds.czbiohub.org/) Tagln2 is widely expressed in mature endothelial cells and other cell types.

To strengthen the impact of the mechanistic study around the expression of Tagln and its function a series of experiments should be added
- Dose-response effect of VEGF-A
- Dose-response effect of the small molecule inhibitors (In the manuscript, the authors use all inhibitors at the same concentration (5 µM), however, the most specific working concentrations of the inhibitors might vary.)
- Test of specificity of small molecule inhibitors, e.g. ERK1/2 – inhibition (counterpart to AKT signalling, in the current manuscript all inhibitors exhibit the same effect, why?)
- Effect of inhibition / blocking of VEGF-A receptors (e.g. VEGFR2/FLK1)
- Specificity of effect for certain VEGF-A splice isoforms (e.g. VEGF-A121, VEGF-A165, VEGF-A189)

It is difficult to draw any conclusion about the expression of SM22 from the tissue staining presented in Figure 8, since the authors show themselves that isolated endothelial cells do express all three isoforms (Tagln, Tagln2 and Tagln3), and the used antibody apparently binds to all three isoforms (western blots in Figure 7A, and manufacturers description). If possible more specific antibodies should be used, and/or the expression of Tagln2 and Tagln3 should be investigated in the other experimental set-up to understand if the expression of all Tagln family members follows the same pattern, or if there might be a specific affect for Tagln.

Additional minor points that should be addressed:
Figure 2C and 2D contain seemingly data from the same experimental set-up (according n-values); could these two graphs be combined into one graph.

Figure 2D shows an increase in SMC colonies after stimulation with VEGF-A, is this expected and how does this relate to the increase in SM22+ endothelial cell colonies in response to VEGF treatment.

Figure 2I and 2J contain seemingly data from the same experimental set-up (according to n-values); could these two graphs be combined into one graph. See also point (2). The authors could reconsider their classification of endothelial colonies, since there appear to be very few colonies of the intermediate (SC) type.

The authors are highly encouraged to adhere to the standard nomenclature of gene- and protein-names. E.g. Tagln (use italics) is the gene name of the protein SM22. For FLK1, the gene name Kdr should be used and the capital abbreviation for the protein. For VE-cadherin the gene Cdh5 should be used, and for alpha SMA, the gene name Acta2 should be used.
Also, the difference between human (TAGLN) and mouse (Tagln) should be applied.

The relevance of the data from the Foxo1-deficient cells (Supplementary Figure 3) for the main hypothesis of the manuscript is not clear, which in turn raises the question why the data is included in the manuscript. This should be clarified by the authors.
The scRNA-seq data presented in Supplementary Figure 2 and Supplementary Table 1-2 could be expanded and more detailed analysis be performed.
How many endothelial cells are positive for Tagln expression, and which genes are co-enriched in the Tagln+ population compared to the other endothelial cells. Supplementary Table 1 lists Acta2 as the second most enriched gene in the Tagln+ endothelial cell population, however, does this imply that these cells do express Acta2 or can this be due to contamination / doublets in the single cell preparation?
Other datasets to explore, such as adult endothelial cells, or those with pathological angiogenesis?

The authors should state which isoform and splice variant of VEGF they use. Different splice isoforms of VEGF-A have different receptor and extracellular matrix binding characteristics that can have an effect on downstream signaling.

Check y-axis labels in Figure 7D, and 7G

First revision

Author response to reviewers' comments

Response to all reviewers

We wish to express our appreciation to the reviewers for their insightful comments on our paper.

The following Figures and Supplementary figures/tables have been added or modified in the revision. The newly added or changed lines are displayed in red in Text and Supplementary text.We upload a formatted PDF of the Response as Supplementary Information.

Panel D (Model of ESC differentiation) was added in Figure 1.
Figure 2C and 2D were combined into new Figure 2C.
Figure 2I and 2J were combined into new Figure 2H.
Figure 4C and 4D were combined into new Figure 4C.
Figure 6B (semi-quantitative PCR data) was changed new Figure 6C (real-time quantitative PCR data).
Data of promoter activity of TAGLN2 and TAGLN3 were added in new Figure 6D.
Data of western blots in HUVECs were added in new Figure 6E.

Supplementary figure 1B and 1C were combined into new Supplementary figure 1B.
Data of real-time quantitative PCR of ESC-derived ECs were added as new Supplementary figure 2.
CRISPR/Cas9-generated mutation detection data were added as new Supplementary figure 3.
Data of migration in TAGLN knockout-HUVECs were added as new Supplementary figure 4.
Data of 3D sandwich culture in TAGLN siRNA-HUVECs were added as new Supplementary figure 5 (A-F).

Data of 3D sandwich culture in Tagln overexpression-HUVECs were added as new Supplementary figure 5 (G-K).
Supplementary figure 2 (scRNAseq analysis) was new Supplementary figure 6.
Data of new scRNAseq analysis were added as new Supplementary figure 7, 8.
Data of new scRNAseq analysis were added as new Supplementary table 3, 4.
Supplementary figure 3 (Foxo1 data) was deleted.

New scRNA-seq data (NCBI GEO accession no. GSE167932) in new Supplementary figure 7, 8 and Supplementary table 3, 4 is currently private. Reviewers can access to the private data by the following information.

To review GEO accession GSE167932:
Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167932
Enter token sfgxsaoqnxwlvkx into the box

Response to the reviewer 1

1. The authors use a reporter mouse to detect SM22 in elongated ESC-derived EC. They must verify SM22 by RNA and protein.

-The results of additional experiments about mRNA expression levels of *Tagln* isoforms in ESC-derived ECs showed at Supplementary figure 2. To analyze the original characteristics, we used two ESC strains (the parent strain and the other). ESC-derived ECs showed the increased expression of *Tagln*, but not *Tagln2* and *Tagln3* in the presence of VEGF.

-We agree that analysis of TAGLN proteins would be valuable. Regrettably, however, we are unable to do western blotting, because it is difficult to collect a sufficient number of cells for detecting protein due to very low EC-differentiation rate.

2. In knockout HUVECs, cord formation is increased. They must determine cell migration under similar conditions.

-The results of additional experiments about scratch-wound assay in *TAGLN* isoforms-knockout HUVECs showed at Supplementary figure 4. *TAGLN*, *TAGLN2* or *TAGLN3* single-knockout HUVECs showed that increased migration activity. *TAGLN* isoforms triple-knockout HUVECs had higher migration activity, which was attenuated by rescue-expression of *Tagln*.

3. Fig 1, show the gate for Flk1+ sorting and how many of these were EGFP +, Dsred.T4 + and EGFP+/DsRed.T4+ double positive at time of sort and after 7 days culture. The impression given is that hardly any EC express SM22 promoter activity (we have no data showing that the SM22 gene actually is expressed yet). How will VEGF during culture change these cell populations?

- Figure 1 shows that Flk1+ (KDR+) common precursor cells differentiated into EGFP+ DsRed.T4- ECs and EGFP- DsRed.T4+ SMCs at re-culture 7days. As the reviewer mentioned, EGFP+ ECs did not show *Tagln* promoter activity in routine ESC differentiation culture (no-exogenous VEGF). Figure 2 shows that the treatment with VEGF induced morphology change to elongation, and DsRed.T4 expression in VE-cadherin+ EGFP+ ECs. FACS analysis in Figure 5 also suggests that VEGF increased the DsRed.T4 expression in ECs. To clarify the results of Figure 1, we have added “a schematic model for activation of the F10-44 enhancer and Tagln promoter during differentiation of ECs and SMCs from ESCs” as Figure 1D.

4. Fig 2 is hard to understand. In D, why are there two DsRed columns after VEGF, one 100% SC and one 100% C? In E, what are the proportions, EGFP or DsRed or double positive or only morphology? In J, VEGF+, are all cobblestone cells DsRed? That is inconsistent with the notion that SM22 becomes expressed in elongated cells?

-We agree that this needs to be improved, and the two related graphs were combined into new one graph.

Figure 2C and 2D were combined into new Figure 2C.
New Figure 2C shows that the sheet-like EC colonies (Type S) accounted for about 70% of EGF+ EC colonies in VEGF(-), and did not express DsRed.T4. While, the cord-like EC colonies (Type C) accounted for about 90% of EGF+ EC colonies in VEGF(+), and expressed DsRed.T4.

The sheet-and- cord-like EC colonies (Type SC) are mixed types, and expressed DsRed.T4, and accounted for about 29% in VEGF(-) or 8% in VEGF(+).

Figure 2I and 2J were combined into new Figure 2H.
New Figure 2H shows that about 98% of VE-cadherin+ EGFP+ ECs were cobblestone-like (Type cob) in VEGF(-), and most of these cells did not express DsRed.T4. In VEGF(+), about 96% of VE-cadherin+ EGFP+ ECs were elongation-like (Type elong), and most of these cells expressed DsRed.T4. Therefore, these data suggest that *Tagln* promoter activity becomes induced in elongated ECs.

5. Foxo data are not convincing and should be removed.
-We agree that Foxo1 data and related lines were unnecessary, and deleted it.

Response to the reviewer 2

1. The antibodies used to confirm SM22 alpha expression in endothelial cells cross-react with other isoforms (SM22b and transgelin3), which makes it difficult to appreciate specific upregulation of SM22 alpha in wild-type cell and mouse embryos. This discrepancy is reinforced by single cell RNA Seq data (Supplemental Figure 2B) showing that SM22 expression is barely detectable and highly sparse in VE- cadherin-positive endothelial cells. Expression in Supplemental Figure 2B does not match robust SM22 staining shown in Figs 8B, 8C, and 8D, reinforcing that it is imperative to perform staining in mouse embryos with an SM22-specific antibody. This discrepancy makes it difficult to appreciate how impactful SM22 expression is on angiogenesis.
-We searched for the other antibody that specifically recognizes TAGLN (SM22) protein. However, unfortunately, the new antibody (Boster Biological Technology A03962-1) also seems to cross-react with other isoforms, TAGLN2 and TAGLN3, similar to the antibodies (abcam ab14106, Sigma-Aldrich HPA019467) used in our text. The results show at “Figure to reviewer-1”. As the reviewer indicated, it is difficult to appreciate levels of specific TAGLN protein, and the data of immunostaining with the antibody may contain the expression of all TAGLN isoforms. We have deleted the lines “TAGLN proteins were co-localized with the filamentous actin cytoskeleton at the cell periphery of the elongated ECs.” in abstract, and changed the lines 560-561 in Discussion.

To examine the detailed mRNA expression levels of *Tagln* isoforms, we have added the results of real- time PCR analysis on ESC-ECs and HUVECs in Supplementary figure 2 and new Figure 6C (Previous data of semi-quantitative PCR has been deleted). *Tagln* expression increased in stimulation of EC elongating, while *Tagln2* and *Tagln3* expression did not change.

To identify the expression of *Tagln* gene in *in vivo* ECs, we have added the results of new single cell RNA seq analysis of potent hematopoietic sites, the aorta-gonad-mesonephros (AGM) and fetal liver (FL) in a mouse embryo (Supplementary figure 7, 8, Supplementary table 3, 4). These results more clearly indicate the presence of *Tagln*-positive ECs.

2. Data in Figures 6 and 7 are contradictory. Figure 6 shows that SM22 gene expression and promoter activity increase in a 3D sandwich culture compared to gelatin-coated culture and this correlates with elongation. Yet, SM22 overexpression decreases angiogenic parameters in Figure 7, including # of cords, # of branch points, and length of cords. A better assay to test CRISPR and siRNA-silenced cells may be the collagen sandwich assay to determine elongation for consistency, as these discrepancies could be due to the differences in the experimental systems shown in Figures 6 and 7.
-We regret that the explanation of Figure legends was insufficient. To clarify the content, we have added and changed to lines in Figure legends section of Figure 7
(the lines 1013-1016, 1022).

Figure 6A, C, D shows that ECs elongation, the increased expression and promoter activity of *TAGLN* gene were shown in HUVECs in a 3D sandwich culture. The 3D sandwich culture was used in Figure 7B-E. The knockout of *TAGLN* in HUVECs by CRISPR gene editing increased cord length in a 3D sandwich culture. Moreover, triple-knockout of *TAGLN*, *TAGLN2* and *TAGLN3* increased cord length more than *TAGLN* single-knockout, which suggesting that *TAGLN* isoforms have

redundant function and inhibit cord-like structure formation. Rescued expression of *Tagln* attenuated the increased effect of *TAGLN* isoforms triple-knockout in number of branch points and cord length (Figure 7D and 7E), although it did not recover completely it. These results suggest that TAGLN is induced in EC elongation, and acts as negative regulator for angiogenesis.

The results of *Tagln*-overexpression experiments were added at Supplementary figure 5G-K. Excess- expression (up to 67-fold) of TAGLN unaffected the cord-like formation of HUVECs in a 3D sandwich culture, which suggesting that TAGLN alone may be not enough to regulate EC elongation.

3. The concern above is underscored by the title, which states "SM22 … negatively regulates angiogenesis," while the discussion states "expression of SM22 in elongating ECs must be interpreted carefully." Thus, the conclusions to be drawn are unclear in the current text.

-Our data suggest that Tagln may be transcriptionally induced in EC elongation, and negatively regulate angiogenesis. Figure 2 and 3 show that activation of the *Tagln* promoter is associated with elongation of ESC-derived ECs. *TAGLN* mRNA expression and promoter activity were increased in elongated HUVECs in a 3D sandwich culture in Figure 6. *TAGLN* knockout-HUVECs exhibited the increased cord length in Figure 7E. We have added the results of *TAGLN* knockdown-HUVECs using siRNA in Supplementary figure 5A-F. Consistent with the results of *TAGLN* knockout, *TAGLN* knockdown increased cord-length of HUVECs in a 3D sandwich culture, which suggesting that TAGLN partially inhibits cord-like formation.

ECs expressing TAGLN has been widely recognized to have undergone endothelial-to-mesenchymal transition (EndMT) in pathological process, because TAGLN is a canonical marker of SMCs. EndMT has been induced in *in vitro* culture of ECs under the stress such as a cyclic strain or prolonged culture (Cevallos et al., 2006) (Frid et al., 2002). However, our data suggest the presence of *Tagln* expression in ECs during physiological embryo development in Figure 8 and Supplementary figure 6-8. These findings imply that TAGLN may have the unique role in not only pathological but also physiological process. Thus, the expression of TAGLN in ECs must be interpreted carefully. To clarify these points, we have added and changed to lines in Discussion section (the lines 566-567, 573-575).

4. CRISPR cells are of concern for two reasons. First puromycin selection was only carried out for 3 days. Typically, 2 weeks of selection is necessary to ensure widespread antibiotic resistance. Second, evidence of gene excision should be provided in a supplemental file. To alleviate these concerns, the authors should confirm results with the CRISPR HUVEC using another method, such as siRNA or ASO-induced gene silencing.

-We agree that the results of CRISPR require to be confirmed, and the results of siRNA experiments were added at Supplementary figure 5A-F. In general agreement with results of CRISPR, *TAGLN* isoforms single-knockdown HUVECs showed the increased cord length in a 3D sandwich culture, and triple-knockdown of *TAGLN* isoforms further promoted it.

To provide the evidence of gene excision, data on mutagenesis of the *TAGLN* isoforms were added at Supplementary figure 3.

5. Discrimination between SM22 isoforms is a concern. Western blots should be performed to confirm immunofluorescence staining in Figure 6. Also in Figure 6, SM22 knockdown should be performed with siRNA to confirm that the loss of SM22 leads to a decrease in elongation.

-We searched for the other antibody that specifically recognizes TAGLN (SM22) protein. However, unfortunately, the new antibody (Boster Biological Technology A03962-1) also seems to cross-react with other isoforms, TAGLN2 and TAGLN3, similar to the antibodies (abcam ab14106, Sigma-Aldrich HPA019467) used in our text. The results show at "Figure to reviewer-1". We have deleted the lines "TAGLN proteins were co-localized with the filamentous actin cytoskeleton at the cell periphery of the elongated ECs." in abstract, and changed the line 560-561 in Discussion.

To examine the detailed mRNA expression levels of *Tagln* isoforms, we have added the results of real- time PCR analysis on ESC-ECs and HUVECs in Supplementary figure 2 and Figure 6C. *Tagln* expression increased in stimulation of EC elongating, while *Tagln2* and *Tagln3* expression did not change.

Although it is difficult to identify specific TAGLN isoforms, the results of additional western blot in HUVECs showed at Figure 6E. Proteins recognized by the antibody were increased in HUVECs in a 3D sandwich culture.

The results of siRNA experiments were added at Supplementary figure 5A-F. *TAGLN*-knockdown HUVECs caused the increased cord length in a 3D sandwich culture.

6. SM22 should be overexpressed in wild-type cells to determine if SM22 plays a direct role in reducing (or enhancing) elongation or sprouting. If no change in morphology or behavior is seen, one interpretation of the data enclosed suggests that endothelial elongation stimulated by VEGF activates a transcriptional factor complex that drives SM22 expression in the ESC lines studied here, along with other genes that are responsible for elongation in endothelial cells.

-We agree that analysis of *Tagln*-overexpression in EC elongation would be valuable, and the results of *Tagln*-overexpression experiments were added at Supplementary figure 5G-K. Excess-expression (up to 67-fold) of *Tagln* unaffected the cord-like formation of HUVECs in a 3D sandwich culture. As the reviewer suggested, the regulation of EC elongation may need the cooperation of TAGLN and other factors. We have added the new section on the interaction between TAGLN and other factors to Discussion (the lines 616-622)

7. This manuscript relies heavily on the use of embryonic stem cell lines. Figure 1 legend indicates multiple clones were used to confirm results. Some description of how clones were generated (from individual mice or the same mouse) should be included, as well as whether they are male/female in origin.

-We agree that this point requires to be defined, and have added the new lines to Method section (the lines 84-87).

8. The relevance of the Foxo data are unclear and don't provide insights because no SM22 expression is shown. Consider removing from this manuscript.

-We agree that Foxo1 data and related lines were unnecessary, and deleted it.

Minor concerns:

1. Reverse transcriptase PCR studies should be replaced with qPCR.

-We agree that analysis of the detailed expression levels of *Tagln* isoforms would be valuable, and have added the results of real-time PCR analysis in new Figure 6C (Data of semi-quantitative PCR has been deleted). The results of RT-PCR in Figure 6B and 8A were retained to reveal the presence of *TAGLN* isoforms in HUVECs, and ECs isolated from mouse embryos.

2. Colors used in figure labels should match figures. As one example, Figures 2F and 2G depict VE- cadherin staining in yellow but labels are orange.

-In these Figures, label colors were changed to yellow.

Response to the reviewer 3

The authors show convincingly the expression of SM22 (Tagln) in endothelial cells, by reporter gene expression under the control of the Tagln promoter element. However, the mechanistic insides, protein presence and functional relevance remain unclear. Conceptual, the authors should discuss why the expression of Tagln as negative regulator of angiogenesis is induced in response to stimulation with VEGF-A, a potent pro-angiogenic growth factor.

-We agree that it is important why Tagln (SM22) expression is induced in response to stimulation with VEGF, and have added the new section to Discussion (the lines 624-644)

The authors claim that SM22 (Tagln) is a negative regulator of angiogenesis, however, they show in Figure 7 that only the knock-out of all three members of the Tagln family (Tagln, Tagln2, Tagln3) leads to increased cord length and branch point formation of endothelial cells in vitro. Thus, the specific functional relevance of Tagln for the reported cellular phenotype remains unclear. In turn, this observation raises the question, whether not only Tagln but all members of the family are similarly regulated.
How does the expression of Tagln2 and Tagln3 change in the experimental set-up presented in Figure 2-6? Since little is known about the functions of Tagln2 and Tagln3 a more detailed

mechanistic information of the contribution of the different Tagln family members is important.

-Figure 7B-E show that the single-knockout of *TAGLN* isoforms in HUVECs increased the cord length, although not the number of cords and branch points. The triple-knockout of *TAGLN* isoforms caused a longer cord length than the single-knockout, and increased the number of cords and branch points.

Thus, *TAGLN* isoforms appear to have redundant function and negatively regulate cord-like structure formation. We regret that the text was confusing. To clarify it, we have changed the subtitle and text (the lines 486, 500, 1011), and asterisks in Figures were changed to a large size.

We agree that analysis of *TAGLN2* and *TAGLN3* expression in EC elongation would be valuable, as the reviewer suggested. The results of additional experiments about *TAGLN2* and *TAGLN3* showed at Figure 6C and 6D. HUVECs have similar mRNA expression and promoter activities of *TAGLN2* and *TAGLN3* in both gelatin-coated and a 3D sandwich cultures.

According to the Tabula Muris database (https://tabula-muris.ds.czbiohub.org/) Tagln2 is widely expressed in mature endothelial cells and other cell types.
It is difficult to draw any conclusion about the expression of SM22 from the tissue staining presented in Figure 8, since the authors show themselves that isolated endothelial cells do express all three isoforms (Tagln, Tagln2 and Tagln3), and the used antibody apparently binds to all three isoforms (western blots in Figure 7A, and manufacturers description). If possible more specific antibodies should be used, and/or the expression of Tagln2 and Tagln3 should be investigated in the other experimental set-up to understand if the expression of all Tagln family members follows the same pattern, or if there might be a specific affect for Tagln.

-Although we searched for the other antibody for TAGLN protein detection, unfortunately the other antibody (Boster Biological Technology A03962-1) also seems to cross-react with all of TAGLN isoforms, similar to the antibodies used in our text. The results of additional experiments showed at “Figure to reviewer-1”. As the reviewer indicated, the data of immunostaining with the antibody may contain the expression of all TAGLN isoforms. We have deleted the lines “TAGLN proteins were co- localized with the filamentous actin cytoskeleton at the cell periphery of the elongated ECs.” in abstract, and changed the line 560 in Discussion.

In addition, to examine the detailed expression levels of *TAGLN* isoforms, we have added the results of real-time PCR analysis on ESC-derived ECs and HUVECs in Supplementary figure 2 and Figure 6C, and the results of promoter activity in Figure 6D. ESC-ECs and HUVECs under angiogenic culture condition increased the expression of *Tagln*, but not *Tagln2* and *Tagln3*. The promoter activity of *TAGLN2* and *TAGLN3* were unaffected by the culture condition.

To strengthen the impact of the mechanistic study around the expression of Tagln and its function a series of experiments should be added.
-Dose-response effect of VEGF-A
-Dose-response effect of the small molecule inhibitors (In the manuscript, the authors use all inhibitors at the same concentration (5 µM), however, the most specific working concentrations of the inhibitors might vary.)
-Test of specificity of small molecule inhibitors, e.g. ERK1/2 – inhibition (counterpart to AKT signalling, in the current manuscript all inhibitors exhibit the same effect, why?)
-Effect of inhibition / blocking of VEGF-A receptors (e.g. VEGFR2/FLK1)
-Specificity of effect for certain VEGF-A splice isoforms (e.g. VEGF-A121, VEGF-A165, VEGF-A189)

-The concentrations of VEGF-A and small molecule inhibitors used in the present study referred to our previous reports (Matsukawa et al., 2009; Tsuji-Tamura and Ogawa, 2016). Elongation of ESC-derived ECs was caused by the treatment with more than 2 ng/mL of VEGF-A, and sufficiently induced by VEGF-A (10 ng/ml) (Matsukawa et al., 2009; Tsuji-Tamura and Ogawa, 2016). Small molecule inhibitors (LY294002, Akt Inhibitor, everolimus and rapamycin) were identified as potent inducers which caused EC elongation without an overdose of VEGF-A by screening a library of chemical inhibitors (Tsuji-Tamura and Ogawa, 2016). Although the optimal concentrations of the inhibitors might vary, as the reviewer pointed out, our previous report show that these inhibitors at the concentration of 5µM significantly induced EC elongation in the absence of exogenous VEGF-A (Tsuji-Tamura and Ogawa, 2016). In present study, these inhibitors were used to exclude the possibility that the *Tagln* promoter was

artificially activated by over-treatment VEGF-A. Figure 4 and 5 show that these inhibitors sufficiently induced EC elongation, and elongated ECs exhibited increased promoter activity of *Tagln* in the absence of exogenous VEGF-A. These inhibitors-induced EC elongation were completely suppressed by the blockade of VEGF signaling by the addition of Flt1 chimera (Flt1-Fc) (Tsuji-Tamura and Ogawa, 2016). Thus, we believe that EC elongation is negatively regulated by PI3K- AKT and mTOR signaling in the absence of exogenous VEGF.
In our present study, recombinant mouse VEGF-A165 was used. Regrettably, it was not described in the previous manuscript, so we have added to lines in Methods (the lines 145-146). In general, research in the field of vascular development has tended to be focused on the VEGF-A165. On the other hand, other isoforms of VEGF-A have been reported to induce differential signal transduction, and differential endothelial responses such as cell permeability and proliferation (Fearnley et al. (2016). Biology Open 5, 571-583). Thus, it is likely that other isoforms of VEGF-A may differently effect on EC elongation.

Additional minor points that should be addressed:
Figure 2C and 2D contain seemingly data from the same experimental set-up (according n-values); could these two graphs be combined into one graph.

-We agree that this needs to be improved, and the two related graphs in following Figures were combined into new one graph.
Figure 2C and 2D were combined into new
Figure 2C. Figure 2I and 2J were combined
into new Figure 2H. Figure 4C and 4D were
combined into new Figure 4C.
Supplementary figure 1B and 1C were combined into new Supplementary figure 1B.

Figure 2D shows an increase in SMC colonies after stimulation with VEGF-A, is this expected and how does this relate to the increase in SM22+ endothelial cell colonies in response to VEGF treatment.

-We regret the confusing previous Figures. New Figure 2C shows the EC colony type-dependent DsRed.T4 expression. The sheet-like EC colonies (Type S) are major in the absence of VEGF, and did not express DsRed.T4. While, the cord-like EC colonies (Type C) are major in the presence of VEGF, and expressed DsRed.T4. The sheet-and-cord-like EC colonies (Type SC) are mixed types and expressed DsRed.T4 regardless of VEGF treatment.

New Figure 2D shows that proportion of EGFP+ EC and EGFP- DsRed.T4+ SMC colonies were hardly affected by VEGF treatment.

Figure 2I and 2J contain seemingly data from the same experimental set-up (according to n-values); could these two graphs be combined into one graph. See also point (2). The authors could reconsider their classification of endothelial colonies, since there appear to be very few colonies of the intermediate (SC) type.

-We agree that this needs to be improved. Figure 2I and 2J were combined into new Figure 2H.

The authors are highly encouraged to adhere to the standard nomenclature of gene- and protein-names.
E.g. Tagln (use italics) is the gene name of the protein SM22. For FLK1, the gene name Kdr should be used and the capital abbreviation for the protein. For VE-cadherin the gene Cdh5 should be used, and for alpha SMA, the gene name Acta2 should be used.Also, the difference between human (TAGLN) and mouse (Tagln) should be applied.

-Thank you for pointing out it. The names were corrected.

The relevance of the data from the Foxo1-deficient cells (Supplementary figure 3) for the main hypothesis of the manuscript is not clear, which in turn raises the question why the data is included in the manuscript. This should be clarified by the authors.

-We agree that Foxo1 data and related lines were unnecessary, and deleted it.

The scRNA-seq data presented in Supplementary figure 2 and Supplementary Table 1-2 could be expanded and more detailed analysis be performed. How many endothelial cells are positive for Tagln expression, and which genes are co-enriched in the Tagln+ population compared to the other endothelial cells. Supplementary Table 1 lists Acta2 as the second most enriched gene in

the Tagln+ endothelial cell population, however, does this imply that these cells do express Acta2 or can this be due to contamination / doublets in the single cell preparation? Other datasets to explore, such as adult endothelial cells, or those with pathological angiogenesis?

-To identify the *in vivo* expression of *Tagln* gene in ECs, we have added the analysis of new single cell RNA seq data of potent hematopoietic sites, the aorta-gonad-mesonephros (AGM) and fetal liver (FL) in a mouse embryo (Supplementary figure 7,8 and Supplementary table 3,4). These results more clearly indicate the presence of *Tagln*-positive ECs.
The expression of ▫SMA, encoded by *Acta2* gene, is abundant in SMCs, and widely used as a marker for SMC differentiation. However, it has been reported that non-SMCs such as fibroblasts and aortic ECs also express ▫SMA under certain stimuli. Two analysis of scRNA-seq shows *Acta2* as related gene in the *Tagln*+ ECs in Supplementary table 1, 3. Thus, it is quite possible that *Acta2* also may be involved in control of physiological functions of ECs. We have added the new lines in Discussion section (the lines 593-599).

-We agree that analysis of *Tagln* gene in adult or pathological ECs would be valuable. Regrettably, however, we were unable to use the related datasets.

The authors should state which isoform and splice variant of VEGF they use. Different splice isoforms of VEGF-A have different receptor and extracellular matrix binding characteristics that can have an effect on downstream signaling.
-Thank you for pointing out it. “Recombinant mouse VEGF-A165” was added in Methods (the lines 145-146).

Check y-axis labels in Figure 7D, and 7G
-Thank you for pointing out it. The labels were corrected.

Figure to reviewer-1

***Note**: We have removed unpublished data that had been provided for the referees in confidence.*

Second decision letter

MS ID#: JOCES/2020/254920

MS TITLE: TAGLN, a canonical marker of smooth muscle cells, is present in endothelial cells and negatively regulates angiogenesis

AUTHORS: Kiyomi Tsuji-Tamura, Saori Morino-Koga, Shingo Suzuki, and Minetaro Ogawa
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

In particular, referee #2 requests that real-time PCR be included for all lines to rule out compensatory expression of all other isoforms. They also suggest that conclusions be softened and the title reconsidered to more accurately reflect the data.
We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us

to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This is a substantially revised manuscript that has taken the reviewers' comments into serious consideration.

Comments for the author

No further comments.

Reviewer 2

Advance summary and potential significance to field

These findings suggest SM22 (TAGLN) may be upregulated in elongating endothelial cells to limit migration.

Comments for the author

Inclusion of genomic sequencing to confirm CRISPR-mediated knockout of individual TAGLN isoforms is helpful. Real-time PCR should be included for all lines to rule out compensatory expression of all other isoforms.

Overexpression data for TAGLN1 in Supplemental Figure 5 are directly opposed to the author's main conclusions in the abstract and title. The authors continue to conclude that TAGLN is anti-angiogenic (abstract and title), but despite a 67-fold increase in TAGLN expression, cell morphology in sandwich assays does not change. Thus, while TAGLN knockdown accelerates cord formation, it's overexpression has no effect suggesting a more subtle function than 'negative regulation of angiogenesis'. This conclusion (and title) needs refining.

Reviewer 3

Advance summary and potential significance to field

Tsuji-Tamura and colleagues substantiated the data of their manuscript with additional analyses. They now show convincingly the expression and contribution of Tagln in endothelial cell behavior. The manuscript has value for the field of cell (especially vascular) biology.

Comments for the author

The authors have addressed my comments and concerns and I have no further remarks.

Second revision

Author response to reviewers' comments

Reviewer 2 Comments for the author
Inclusion of genomic sequencing to confirm CRISPR-mediated knockout of individual TAGLN isoforms is helpful. Real-time PCR should be included for all lines to rule out compensatory expression of all other isoforms.
-The results of additional experiments about the quantification of mRNA expression of Tagln isoforms in CRISPR-mediated knockout cells showed at Supplementary figure 3C.

Overexpression data for TAGLN1 in Supplemental Figure 5 are directly opposed to the author's main conclusions in the abstract and title. The authors continue to conclude that TAGLN is anti-angiogenic (abstract and title), but despite a 67-fold increase in TAGLN expression, cell morphology in sandwich assays does not change. Thus, while TAGLN knockdown accelerates cord formation, it's overexpression has no effect, suggesting a more subtle function than 'negative regulation of angiogenesis'. This conclusion (and title) needs refining.
- We have changed the title, abstract and text (the lines 80, 521-524, 650).

Third decision letter

MS ID#: JOCES/2020/254920

MS TITLE: TAGLN, a canonical marker of smooth muscle cells, is present in endothelial cells and involved in angiogenesis

AUTHORS: Kiyomi Tsuji-Tamura, Saori Morino-Koga, Shingo Suzuki, and Minetaro Ogawa
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.