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Endothelial Basement Membrane Laminin 511 is Essential for Shear Stress Response

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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 14 June 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your work. However, they also think that the analysis is too preliminary to make your manuscript a good candidate for publication here. They provide constructive input on how to extend your analysis. More in vivo data and further insight into the differences between lam511 versus lam411 and lam11 is required. Furthermore, additional controls are needed to better support your conclusions.

Given the referees' constructive input and positive recommendations, I would like to invite you to submit a revised version of the manuscript, should you be able to address the points noted above. I can extend the reviewing time to 6 months, should this be helpful. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses to the referees' comments in this revised version. Please let me know in case you want to discuss the revision further.

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, please visit our website: http://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS

Referee #1:

This MS challenges an important question in vascular biology: Are membrane basal molecular determinants relevant for the vascular mechano-response? And if it is the case, what are they? By mouse genetic models and in vitro experiments the authors provide evidences that laminin 511 is necessary for arterial hemodynamic response to shear stress. However the MS needs some important controls to support the general hypothesis

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Furthermore, besides analysing mesenteric arteries , it should be important to study afferent and efferent glomerular arterioles.

Then the most crucial in vitro evidences need to be validated by submitting cell cultures to share stress as shown in Fig 1.

OTHER POINTS
Fig 1. In my opinion Lama 4 null mice not only do not show any vasodilating response but they show vasoconstriction . This point need comments and it is difficult to recapitulate with other results shown in the paper

Fig 2. To better understand the staining in Fig 2, it is necessary to have information on how and when the Cre was activated. The authors compare an endothelia specific null model (Lam 5) with a whole KO (Lam 4) Therefore can the authors exclude that these genetic differences have impacts on their results? In particular, does the presence of Lam 5 in smooth muscle cells allow a correct comparison with mice that do not express both Lam 4 in endothelial and SMC basal membrane? This point needs to be clarified with appropriate controls and needs to be commented on.

Fig 3B. Besides the transcript, it is necessary to show the protein expression of COX2.

In some experiments, most of them important (e.g Fig 4), the authors use s.end murine cell line. This cell line was firstly stabilized by Wagner's group (Cell. 1989 Jun 16;57(6):1053-63, pls quote this more appropriate reference) by using polyoma virus, which constitutively activates src. Because this kinase is involved in FA dynamics, caution is required and more controls are needed.

Fig 5C. This experiment shows that laminin 511 modulates VE-cadherin homotypic cell adhesion by a direct effect on this protein. The experiment needs a neutralization of integrin receptors for laminin 511.

Referee #2:

The paper by Di Russo et al. shows that Lama5 is required for a correct vasodilation of resistance mesenteric arteries while Lama4 limits vasodilation.
Not surprisingly, the authors show that endothelial cell adhesion to Laminin 511 is blocked by integrin beta1 antibodies and bring some evidence that VE cadherin internalization is reduced when the cells are seeded on laminin 511 as compared to laminin 411 and 111.
In general, this paper adds to our knowledge of endothelial cell interaction with laminin and to our

understanding of the role of matrix in modulating endothelial cell response to shear stress.

However, some important conclusions are not fully supported by the data presented.

More specifically:
- Figure 1 shows that vasodilation of isolated resistance arteries requires the presence of lama5 since vessels derived from lama5 -/- are unable to dilate under shear stress. The authors show that in quite a few experimental conditions laminin 411 is unable to sustain endothelial cell adhesion. It is therefore crucial to control that in absence of lama5 endothelial cells are still present on the vascular surface, otherwise these data can be simply interpreted by the lack of endothelial dependent vasodilation.

-The use of different types of endothelial cells (resistance arteries, human umbilical artery, bEnd) due to the difficulties in obtaining a substantial adhesion to laminin 411 confuses the picture and it is very difficult to draw a simple message. Endothelial cells are profoundly different in the different types of vessels. On the same line in few experiments the lack of comparison between laminin 511 and 411 complicates the interpretation of the differences between the different vascular associated laminins. This is particularly important since it is the starting point for the entire paper.

-Fig 4A, C,D the pictures reporting smaller but more abundant (+16%) focal contacts in absence of lama5 and an increase of 45% of focal contacts in lama 4-/- in comparison to WT cells do not fully support the quantification reported. The images support the idea of smaller focal contacts in absence of lama5, but it is difficult to detect the other differences, at least from the pictures shown.

-Dual pipette pulling assay. Fig 5 C: I understand that adhesion strength is identical if the beads are coated with laminin 511 or if 511 is given in solution and therefore not suitable as a solid substratum.

My interpretation of these data is that cell adhesion is mostly mediated by VE-cadherin independently from the substratum on which the cells are plated on. Indeed, VE-cadherin blocking antibodies inhibit in both conditions to the same extent.

-Figure 5 B . What do you mean for extracellular VE-cadherin? Released in the medium? Or peripheral membrane associated?

Referee #3:

The manuscript by Russo et al., studies the role of the basement membrane laminins in the regulation of the endothelial sheer stress responses. The focus is on the ECM components encountered by the endothelial cells under physiological situations in contrast to the previous work done on ECM components like collagen and fibronectin, which endothelial cells encounter only under pathological conditions. They find that b1-integrin mediated adhesion to lam511 is essential for sheer stress detection and stabilization of cell-cell junctions. They also demonstrate that adhesion to lam511 promotes cortical stiffening of cells in vivo and in vitro. This is an interesting study and the experiments are carefully conducted and support the conclusions drawn. Several techniques are used and investigation of cell behaviour has been assessed both in vitro and in vivo. This reviewer is not an expert in the in vivo physiological aspects of the study and hence will not be making any remarks regarding those experiments.

In the current form, the study remains somewhat descriptive and the impact could be increased by further studies addressing the underlying molecular mechanisms.

1) The distinct responses to lam111 and lam511 and the markedly poor adhesion to lam411 are interesting. Can the authors attribute this to the usage of a specific a/b1-integrin heterodimer on different laminins? Is there evidence for integrin heterodimer specific signalling that would begin to explain the differences?

2) Can the adhesion site differences observed in vivo be seen also in vitro on lam511, lam111 and lam411? If possible live cell imaging of focal adhesion dynamics and turnover on the different

laminins could reveal the underlying dynamics linked to these differences. There is abundant recruitment of vinculin to cell-cell junctions in the in vivo stainings. This appears to be increased in the lama5-/- mice (Fig 4b). Is this linked to integrins switching from focal adhesion to cell-cell junctions?

3) Does cortical stiffness changes in vitro on lam511 and lam411 correlate with changes in the actin cytoskeleton or recruitment of ERM proteins in vitro. Is increased junctional vinculin observed also in vitro

4) Does the soluble lam511 (or lam511 beads) trigger adhesion induced signalling or alterations in Rho activity? Are the effects of lam511 to cortical stiffening or increased VE-cadherin cell-cell adhesion strength dependent on RhoA signalling or signalling via integrins (FAK, Src?)

Minor:
The AFM experiments should be described in more detail. What was the probe used on the cantilever?

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1st Revision - authors' response 15 October 2016

Continued on next page

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The paper shows interesting in vitro data that need an in vivo counterpart by developing an vascular model to demonstrate the data relevance in dynamic conditions. The authors based their hypothesis by analysing mesenteric resistance arteries in vivo. This analysis (i.e. experiments shown in Fig 4) need to be extended in a model characterized by stressed vascular resistance (i.e. hypertension or diabetes or vascular shock).

We agree that it would be interesting to examine focal adhesion size and density and endothelial cortical stiffness in vessels of hypertensive or diabetic animals, where endothelial basement membrane laminins may also be changed. Although there have not been many studies, there are a few that have shown that the shear response of resistance arteries from hypertensive and diabetic rats is altered (Bouvet et al., 2007. Hypertension 50: 248-254; Yu-Jing et al., 2008. Can J Physiol Pharmacol 86:737-744; Dumont et al., 2014. Int J Hypertension 2014, ID859793; Matrougui, et al., 1998. Hypertension 30: 942-947). However, in such models it is not possible to distinguish between effects due to chronic increase in blood pressure and effects due to shear alone. In addition, it is not known whether changes in endothelial laminin isoform expression occur in these models. Hence, changes in endothelial focal adhesions may occur in the resistance arteries, but whether this is due to chronic high blood pressure and resulting increased shear (if the shear response is indeed defective) or the chronic high blood pressure alone.

In the original manuscript, we showed that the laminin knockout mice have no changes in blood pressure. The remodelling observed is therefore due to a long term defective response to changes in shear in the resistance arteries, which occurs to maintain constant blood pressure (pg. 9,19/20). The direction of the changes observed, i.e. smaller diameters in laminin alpha5 deficient mice which show no response to shear and larger diameters in the laminin alpha4 nulls which show hyper-responsiveness to shear, is consistent with the literature. We feel that examining hypertension and diabetes models is beyond the scope of this manuscript, which aims at providing the first evidence that endothelial laminins affect the normal physiological shear response and elucidating the molecular mechanism involved.

Nevertheless, on the basis of this reviewer's comment, we intensively reviewed mouse hypertension and diabetic models available, which reveal that there are very few well-accepted models, in particular for hypertension. Those that are most accepted are the BPN/3J mice with BPH/2J as normotensive controls or angiotensin II infused mice preferably with unilateral nephrectomy, and NOD mice for type I diabetes. The problem with such models is both the long time required for the hypertension and diabetes (NOD) to develop and for detectable changes in the extracellular matrix to develop; in the case of NOD mice this would require the implantation of insulin pellets to keep the mice alive. In addition, many extracellular matrix molecules could be changed in hypertension and diabetes, hence, ideally these models should be employed on the laminin deficient backgrounds.

We therefore now refer to the studies dealing with defective shear response in diabetic and hypertensive rats in the revised manuscript and discuss the possibility that such defects could be associated with laminin isoform changes in the endothelium (either in expression levels or glycosylation states) (see page 19/20).

Furthermore, besides analysing mesenteric arteries, it should be important to study afferent and efferent glomerular arterioles.

En face staining for focal adhesions requires dissection of intact arterioles and their longitudinal opening for whole mount staining. While glomerular arterioles are definitely resistance arteries and shear may be relevant to glomerular artery function (rather than pressure alone), they are rarely analysed in such ex vivo analyses because of extreme difficulty in isolation. We have chosen mesenteric resistance arteries because most studies on shear response and mechano-transduction are performed on these arteries, they can be readily dissected and are classified as *bona fide* resistance arteries. They are also relevant to both small and large resistance arteries and therefore provide that largest body of data for comparison with our results. While we would like to be able to examine glomerular arteries, this will required collaboration with one of the few specialized groups that can excised glomerular arterioles and distinguish afferent from efferent arterioles.

Then the most crucial in vitro evidences need to be validated by submitting cell cultures to share stress as shown in Fig 1.

This was partially done by measuring COX2 expression in HUAECs plated on laminin 511 compared to laminin 111 under flow shown in Fig 3B of the original manuscript (laminin 411 could not be used as the cells detach from this substrate

under flow). Since COX2 expression is induced by shear stress and is upstream of release of prostacyclin, a well known vasodilator, the higher expression of COX2 on cells plated on laminin 511 substantiated the in vivo data of reduced vasodilation in the arteries lacking endothelial laminin alpha5.

As requested, we have now extended these in vitro analyses to include experiments examining the alignment of HUAECs plated on laminin 511, laminin 111 and fibronectin to the direction of flow. These experiments were performed after 120 min exposure to 10 dyn/cm^2. It was not possible to use laminin 411 as the cells detached from the substrate immediately upon commencement of flow, even at low flow rates. Fig. 3C and Supplementary Fig 4A of the revised manuscript show that HUEACs plated on laminin 511 align in the direction of flow, but cells on the non-endothelial laminin 111 do not and remain perpendicular to the direction of flow. Data from HUEACs plated on laminin 511 is comparable to that of cells on fibronectin, which is consistent with previous published data and supports the concept that laminin 511 is the physiologically relevant substrate in the endothelial basement membrane, while fibronectin is the pathologically relevant substrate (eg in artherosclerosis).

OTHER POINTS

Fig 1. In my opinion Lama 4 null mice not only do not show any vasodilating response but they show vasoconstriction. This point need comments and it is difficult to recapitulate with other results shown in the paper.

Figure 1A shows an enhanced vasodilatory response in *Lama4*$^{-/-}$ and a reduced vasodilatory response in TekCre::*Lama5*$^{-/-}$ mesenteric arteries to step-wise increases in shear stress levels. Due to differences in mesenteric artery diameters (Fig 2C), the experiments were standarized by changing the intraluminal flow-rate according to vessel diameters to obtain comparable shear stress levels (described in methods, page 27). This fact, together with the control experiments shown in Figures S1C and S1D, where different vasoconstrictors were employed, demonstrate that the differences in shear response can only be the result of an impaired vasodilation and are not due to any vasoconstriction differences. We clarify these experiments and discuss these points in pages 7-8 of the revised manuscript.

Fig 2. To better understand the staining in Fig 2, it is necessary to have information on how and when the Cre was activated . The authors compare an endothelia specific null model (Lam 5) with a whole KO (Lam 4) Therefore can the authors exclude that these genetic differences have impacts on their results? In particular, does the presence of Lam 5 in smooth muscle cells allow a correct comparison with mice that do not express both Lam 4 in endothelial and SMC basal membrane? This point needs to be clarified with appropriate controls and

needs to be commented on.

The Tie2-cre promoter is active at approximately embryonic day 9.5 in all endothelium. As *Lama5* expression in endothelial cells occurs only postnatally (Sorokin et al., 1997. Dev Biol 189: 285-300), there is no chance that laminin alpha5 protein exists in the endothelial BMs as also documented in Supplementary Fig 2 and Fig 2, and in one of our previous publications (Song et al., 2013. PNAS 110: E2915-2924). The information on the Tie2-cre strain is now included in the methods section on pg 22. In *Lama4*$^{-/-}$ mice, laminin alpha4 is indeed eliminated from both the endothelial and smooth muscle BMs; however, the absence of any defect in vSMC function was shown in the physiological studies. In particular, in Figure S1D the stimulation of mesenteric arteries with vasoconstrictors and vasodilators or by changes in intraluminal pressure, which is solely dependent on vSMC, shows no differences to control vessels. These data show that the lack of laminin alpha4 from the smooth muscle cell basement membrane cannot explain the defective flow induced dilation observed in these mice, as vSMC can dilate and contract when directly stimulated to do so. This is now made clearer on page 7 of the revised manuscript.

Fig 3B. Besides the transcript, it is necessary to show the protein expression of COX2.

The COX2 data was obtained from HUAECs seed in μ-Slide I$^{0.2}$ Luer from IBIDI chambers (cell area of 2.5 cm^2) coated with laminin 511 or laminin 111 and subjected to shear. As these chambers are small the total number of cells that can be analysed is extremely low, which is why we presented mRNA data in the original manuscript. We have now tried to supplement this mRNA data with protein data using a Western blot and a capture ELISA. Seven separate experiments were performed and analysed in both ways, revealing pico gram levels of protein, but the same direction of results. We now show the Western blot data in Fig 3B in addition to the mRNA data, which show significant upregulation of COX2 protein under shear conditions only in HUAECs plated on laminin 511 and not laminin 111, consistent with the mRNA data.

In some experiments, most of them important (e.g Fig 4), the authors use s.end murine cell line. This cell line was firstly stabilized by Wagner's group (Cell. 1989 Jun 16;57(6):1053-63, pls quote this more appropriate reference) by using polyoma virus, which constitutively activates src. Because this kinase is involved in FA dynamics, caution is required and more controls are needed.

We now quote Williams et al., 1989 Cell 57, 1053-63 for all references to sEND.1 cell line, and apologise for the incorrect reference.

We agree that to use only the endothelioma cells would not be appropriate, not only because of Src activation but also because they are not arterial endothelial cells. For this reason we used mainly human primary arterial endothelial cells (HUEACs) in in vitro assays. sEND.1 were used in the adhesion assays and AFM assays only, firstly to confirm that mouse endothelial cells show the same pattern of adhesion as HUAECs and secondly because of their stronger binding to laminin 411 and, hence less dislodgement of cells by the AFM cantilever. However, adhesion assays, adhesion blocking assays, duel pipette assays, VE-cadherin localization and immunoprecipitation of VE-cadherin, shear alignment assays and COX-2 expression assays were all performed with HUAECS. The focal adhesion analyses were performed only in vivo using excised mesenteric arteries in the original manuscript. We have now also quantified focal adhesion numbers and density in HUAECs plated on the laminin 411, 511 or 111, shown in Supplementary Fig 5C.

Fig 5C. This experiment shows that laminin 511 modulates VE-cadherin homotypic cell adhesion by a direct effect on this protein. The experiment needs a neutralization of integrin receptors for laminin 511.

As requested, we have repeated the dual pipette assay in the presence of function blocking antibody against the β1 integrin. The new data are included in Fig 5D and discussed in the discussion. The experiments were performed only with laminin 511 in solution since in the cell-bead experiment anti-integrin β1 would inhibit binding of the cells to the beads. These new experiments provide additional evidence that laminin 511 signalling via integrin β1 is required for enhanced VE-cadherin cell-cell adhesion.

Referee #2:

The paper by Di Russo et al. shows that Lama5 is required for a correct vasodilation of resistance mesenteric arteries while Lama4 limits vasodilation. Not surprisingly, the authors show that endothelial cell adhesion to Laminin 511 is blocked by integrin beta1 antibodies and bring some evidence that VE cadherin internalization is reduced when the cells are seeded on laminin 511 as compared to laminin 411 and 111.

In general, this paper adds to our knowledge of endothelial cell interaction with laminin and to our understanding of the role of matrix in modulating endothelial cell response to shear stress.

However, some important conclusions are not fully supported by the data presented.

More specifically:

- Figure 1 shows that vasodilation of isolated resistance arteries requires the presence of lama5 since vessels derived from lama5 -/- are unable to dilate under shear stress. The authors show that in quite a few experimental conditions laminin 411 is unable to sustain endothelial cell adhesion. It is therefore crucial to control that in absence of lama5 endothelial cells are still present on the vascular surface, otherwise these data can be simply interpreted by the lack of endothelial dependent vasodilation.

We agree that this is an important point and have indeed checked that endothelial cells are still present in blood vessels of *Tek-Cre::Lama5*$^{-/-}$ mice, as shown in Figs 2A, 4A and B, 7A and S4B. In the experiments shown in Fig 1A, endothelial cell viability was checked after every measurement by adding 10 μM acetylcholine to the bath. Acetylcholine directly acts on endothelium causing vasodilation, in the absence of this response excised vessels were not included in the final analyses. This is shown in Fig S1B and discussed in the methods section on page 27.

-The use of different types of endothelial cells (resistance arteries, human umbilical artery, bEnd) due to the difficulties in obtaining a substantial adhesion to laminin 411 confuses the picture and it is very difficult to draw a simple message. Endothelial cells are profoundly different in the different types of vessels. On the same line in few experiments the lack of comparison between laminin 511 and 411 complicates the interpretation of the differences between the different vascular associated laminins. This is particularly important since it is the starting point for the entire paper.

We agree that there are differences between endothelial cells from different vessels types, therefore, we have performed the majority of *in vitro* analyses using arterial endothelial cells (HUAECs) to better compare the findings with the *in vivo* data. We now restrict the use of sEND.1 to adhesion assays and AFM experiments to ensure that the data obtained with HUEACs is relevant to mouse endothelial cells.

In addition, we have modified our adhesion assays to be able to include laminin 411 adhesion blocking data to the manuscript (Fig 3A). This required the use of higher concentrations of the laminin substrates (which are extremely difficult to prepare and limited in amount) and increased time of adhesion. This information now appears in the methods section. However, even with these altered conditions it was not possible to use laminin 411 in the duel pipette experiments, nor in the AFM experiments because the number of bound HUEACs is so much lower than on laminin 511 (or laminin 111) and the binding is significantly weaker. We hope that by now focusing on the human arterial endothelial cells, HUEACS, we have made the picture clearer.

-Fig 4A, C,D the pictures reporting smaller but more abundant (+16%) focal contacts in absence of lama5 and an increase of 45% of focal contacts in lama 4-/- in comparison to WT cells do not fully support the quantification reported. The images support the idea of smaller focal contacts in absence of lama5, but it is difficult to detect the other differences, at least from the pictures shown.

Different immunofluorescence pictures have now been selected in order to better illustrate the quantified adhesion complexes number and size. In addition, to better illustrate the measured the range of adhesion complex numbers and sizes in the arteries of WT, *Lama4*$^{-/-}$ and *Tek-Cre::Lama5*$^{-/-}$ we express this data as a distribution plot in the new Fig 4D. We think that this more clearly illustrates the observed phenotypes. Quantification of adhesion complex numbers is now shown in Supplementary Fig 5C. This is described on pages 12 and 20.

-Dual pipette pulling assay. Fig 5 C: I understand that adhesion strength is identical if the beads are coated with laminin 511 or if 511 is given in solution and therefore not suitable as a solid substratum. My interpretation of these data is that cell adhesion is mostly mediated by VE-cadherin independently from the substratum on which the cells are plated on. Indeed, VE-cadherin blocking antibodies inhibit in both conditions to the same extent.

The comparison of cell-cell adhesion strength when cells are incubated with laminin 511 coated beads versus cell-soluble laminin 511 interactions was done to investigate the importance of laminin α5 induced signaling, independent of substrate adhesiveness. We realized this point was not properly explained in the original manuscript and therefore have rephrased this section on page 15.

The reviewer is right that the cell-cell adhesion is mediated by VE-cadherin. However, this can only occur when the cells are plated on laminin 511 coated beads or when laminin 511 is added to the cells in solution and, in both cases, the laminin 511 engages the b1 integrin. HUEACS bound to laminin 111, also a highly adhesive substrate as shown in Fig 3A, does not support strong cell-cell adhesion, even though this is also a b1-integrin mediated event. The comparison of soluble laminin 511 and laminin 511 on beads shows that it is not the strength of adhesion to laminin 511 that relocates VE-cadherin to junctions (thereby enhancing cell-cell adhesion) but rather that it can also occur by signaling via the right b1 integrin receptor.

-Figure 5 B. What do you mean for extracellular VE-cadherin? Released in the medium? Or peripheral membrane associated?

We apologise for the confusion, we have now changed 'Extracellular VE-cadherin' to 'Cell Surface VE-cadherin' in Fig 6A. This is described in methods session at page 29.

Referee #3:

The manuscript by Russo et al., studies the role of the basement membrane laminins in the regulation of the endothelial sheer stress responses. The focus is on the ECM components encountered by the endothelial cells under physiological situations in contrast to the previous work done on ECM components like collagen and fibronectin which endothelial cells encounter only under pathological conditions. They find that b1-integrin mediated adhesion to lam511 is essential for sheer stress detection and stabilization of cell-cell junctions. They also demonstrate that adhesion to lam511 promotes cortical stiffening of cells in vivo and in vitro. This is an interesting study and the experiments are carefully conducted and support the conclusions drawn. Several techniques are used and investigation of cell behaviour has been assessed both in vitro and in vivo. This reviewer is not an expert in the in vivo physiological aspects of the study and hence will not be making any remarks regarding those experiments.

In the current form, the study remains somewhat descriptive and the impact could be increased by further studies addressing the underlying molecular mechanisms.

1) The distinct responses to lam111 and lam511 and the markedly poor adhesion to lam411 are interesting. Can the authors attribute this to the usage of a specific a/b1-integrin heterodimer on different laminins? Is there evidence for integrin heterodimer specific signalling that would begin to explain the differences?

As requested, data on blocking of adhesion to the different substrates using specific integrin alpha chain blocking antibodies is now included in Fig 3D,E and the data are discussed on pages 11 and 20. The data show that binding to laminin 511 requires synergistic effects of mainly integrins a3b1 and a6b1, while binding to laminin 411 requires integrin a6b1 only, as does binding to the non-endothelial laminin 111. It is, however, not possible to confirm the existence of integrin a3b1 in mouse resistance arteries in vivo as an antibody that functions in stainings does not exist. This is discussed on page 20 of the discussion.

2) Can the adhesion site differences observed in vivo be seen also in vitro on lam511, lam111 and lam411? If possible live cell imaging of focal adhesion dynamics and turnover on the different laminins could reveal the underlying

dynamics linked to these differences.

We now provide data on focal adhesion numbers and sizes in HUAECS plated on the different laminins; focal adhesions are identified by anti-vinculin. Quantification of these data are now provided in Supplementary Fig 5C. Unfortunately, it is not possible to get optimal focal adhesion staining and junctional staining using the same experimental conditions. However, the in vitro data supports the larger and higher density of focal adhesions in HUEACS plated on laminin 511 compared to laminin 411. While we would very much like to be able to dynamically image focal adhesions in cells plated on the different laminins, this would be require transfection of fluorescently tagged vinculin into vinculin null HUAECs, which we feel is beyond the scope of this first paper.

There is abundant recruitment of vinculin to cell-cell junctions in the in vivo stainings. This appears to be increased in the lama5-/- mice (Fig 4b). Is this linked to integrins switching from focal adhesion to cell-cell junctions?

This is an interesting question but difficult to address quantitatively, mainly because it requires examining cells at the same density at different time points after plating on laminin 411 and 511; however, it is difficult to obtain equally confluent cells on laminin 511 and 411 (please see Fig 6A), due to the low binding to laminin 411. Ideally, it would require transfection of cells with a reporter-integrin and live imaging of the cells as they adhere and form monolayers on the different laminins. While we would like to be able to do this experiment and believe that it will be important for our future investigations on how the VE-cadherin mediated cell-cell adhesion is strengthen when HUAECs are bound to laminin 511, it is beyond our current possibilities. We also think that it will not add additional important information for the message that we wish to make here, i.e. that binding of HUAECs to laminin 511 can strengthen VE-cadherin mediated cell-cell adhesion.

3) Does cortical stiffness changes in vitro on lam511 and lam411 correlate with changes in the actin cytoskeleton or recruitment of ERM proteins in vitro. Is increased junctional vinculin observed also in vitro.

We stained for vinculin, phalloidin and pERM in HUAECs plated on laminin 511, 411 and 111 at 6h where we can see junctions (Supplementary Fig 5C). While this permitted quantification of focal adhesion size and density and tended to show more F-actin and vinculin at junctions in the cells plated on the more adhesive substrates, laminin 511 and laminin 111, there were no clear differences between HUAECs plated on the different laminin isoforms. This data is shown in Supplementary Fig 5C. Staining of pERM proteins in HUAECs plated on the different laminins tend to show more pERM at junctions on HUAECs plated on laminin 511, however, the signal for pERM is extremely low. Western

blot to quantify the signal also did not provide conclusive results. We provide the data for the reviewer but have decided not to include this information in the manuscript.

4) Does the soluble lam511 (or lam511 beads) trigger adhesion induced signalling or alterations in Rho activity? Are the effects of lam511 to cortical stiffening or increased VE-cadherin cell-cell adhesion strength dependent on RhoA signalling or signalling via integrins (FAK, Src?)

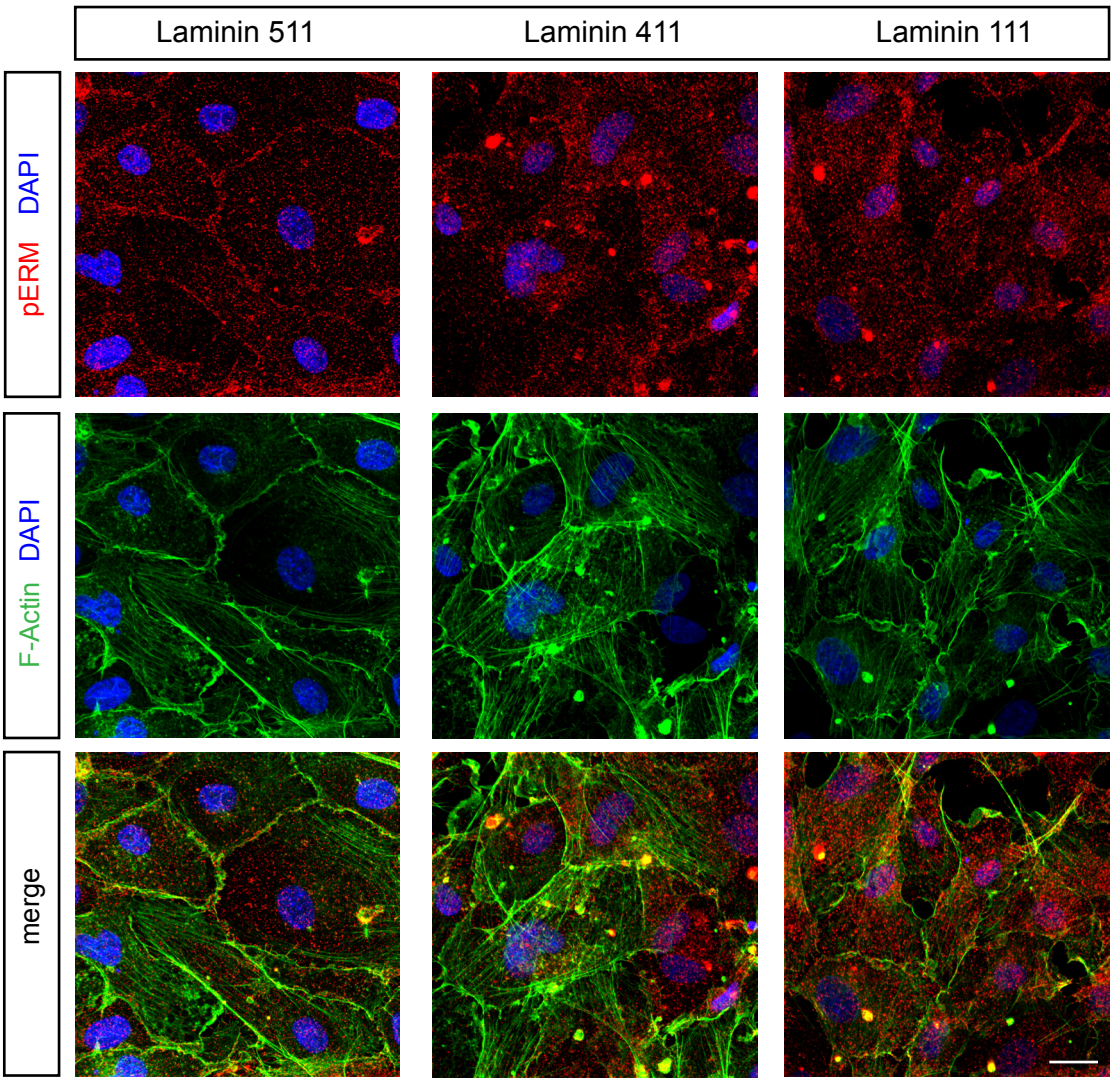
Dual pipette pulling assays have now been performed in the presence of ROCK, FAK and SRC inhibitors showing their involvement in the laminin 511 induced cell-cell adhesion strength. The data are shown in Fig 5D and are discussed on pages 14, 15 and 21.

Minor:
The AFM experiments should be described in more detail. What was the probe used on the cantilever?
There was no probe employed on the AFM cantilever, rather a non adhesive polystyrene spherical cantilever tip (10μm diameter; Novascan) was used to probe and indent the endothelial cell surface in order to record its cortical stiffness (50-150 nm depth). As requested, these details on the AFM experiments are included in the methods session of the manuscript on page 28/29)

Page 12-13 "HUAEC cortical stiffness was also higher on laminin 511 than on the same concentration of laminin 111 (Fig 4F)." should be 4G
We have corrected this mistake.

5F typo in the y-axis label
We have corrected this mistake.

5G-H also blots should be shown
As requested these data are now included in the new Fig 6.


Laminin 511
Laminin 411
Laminin 111
pERM DAPI
F-Actin DAPI
merge

2nd Editorial Decision 07 November 2016

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by the original referees (see comments below), and I am glad to inform you that they all appreciate the introduced changes. I am thus happy to accept your manuscript in principle for publication in The EMBO Journal.

Before sending you the official acceptance letter, I would like to ask you to address the following few editorial points:

- please check whether all figure files are of adequate resolution and quality for production, and upload improved versions if necessary.
- please combine the appendix figure legends and figures into a single pdf that also contains a TOC
- please suggest (in a cover letter) a one-sentence summary 'blurb' of your paper, as well as 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper; this will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.
- as you might know, we encourage our authors to provide original source data (such as excel spreadsheets etc) for the main figures of your manuscript. If you would like to add source data, we would welcome one file per figure for this information. These will be linked online as supplementary "Source Data" files.
- figure 8 would be ideal for the synopsis image for the HTML version of your manuscript. Could you please re-arrange the figure for that purpose? The dimensions of our synopsis images are 550px (width) x 150-400 px (hight).

I am therefore formally returning the manuscript to you for a final round of minor revision, only to allow easy alterations of the files. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE REPORTS

Referee #1:

The authors properly addressed my comments

Referee #2:

The authors answered to my criticisms in an adequate way

Referee #3:

The authors have now addressed satisfactorily the majority of the issues raised.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Lydia Sorokin
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-94756

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	A minimum of 4 mice per genotype were analyzed for a specific experiment and a minimum of 3 independent experiments were performed with cells in culture. In some cases, the sample size was increased due to high variability of the detected effect.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	A minimum of 4 mice per genotype were analyzed; higher sample sizes were employed due to the variability of the detected effect (specified in each figure legend).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In the physiological analyses (Fig 1 and S1) excited vessels were always tested for responsiveness using method-specific drug stimulations (see material and methods). Non-responsive vessels were excluded from the analyses. In other analyses, positive or negative controls were always included in the different settings to be able to exclude artifacts.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Mice from different breeding pairs were included in the analyses when possible to increase randomization.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No specific blinding was performed in animal studies
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. I case of the assumption of normal distribution the data were tested with Kolmogorov Smirnov test for normality.
Is there an estimate of variation within each group of data?	Yes, variation of each group of data was estimated calculating its standard deviation.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The name of the clone with reference is included in materials and methods section of the manuscript and the company and catalogue number are also included.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines employed in the work were previously characterized and their source and reference are stated in the materials and methods section of the manuscript. Mycoplasma testing is routinely performed in the laboratory.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We employed mice (Mus Musculus), C57BL/6, of different ages and genetic modifications as specified in the manuscript materials and methods section. Housing was performed according to the German and Swedish Animal Welfare guidelines.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Experiments were conducted according to German and Swedish Animal Welfare guidelines and were approved by the 'Landesamt fuer Natur-, Umwelt- und Verbraucherschutz Nordrhein-Westfalen' and by the 'Centrala föröksdjursnämnden (www.cfn.se)'.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA