THE EMBO JOURNAL

Manuscript EMBO-2012-80964

The F-BAR protein NOSTRIN participates in FGF signal transduction and vascular development

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Review timeline:

Submission date:	02 February 2012
Editorial Decision:	02 March 2012
Revision received:	31 May 2012
Accepted:	01 June 2012

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 02 March 2012

Thank you for submitting your research manuscript on the F-BAR protein nostrin in endothelial cell function for consideration to The EMBO Journal editorial office.

The attached comments indicate scientific merits of your interesting contribution. You will also recognize that every single referee offers constructive suggestions aimed at increasing the overall impact of your results. Particularly, ref#1 suggests assessing possible proliferation defects and complement the quantitative data on vascular outgrowth. Ref#2 adds a rather valuable point by inquiring about potential specific F-Bar domain functions in the context of membrane dynamics. Lastly, ref#3 asks for some improvements on image presentation, potential Rac-rescue experiments and more elaborate discussions with regard to possible signal specificity.

Based on these, I am happy to offer you the chance to revise and modify the current dataset.

I do have to formerly remind you that The EMBO Journal only allows one round of revisions and that the ultimate decision will depend on content and strength of the final manuscript. Please do not hesitate to contact me directly (preferably via E-Mail) in case of further questions.

I am very much looking forward to your revised paper and keep the fingers crossed for efficient proceedings.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Kovacevic et al. report that Nostrin, an F-BAR protein, controls FGF2-mediated Rac1 activation in endothelial cells and is therefore required for angiogenesis in the zebrafish embryo and the mouse retina.

Overall, the manuscript is very interesting and well written, and the data is of good quality and presented in a logical order.

While the function of Nostrin appears to be critical for the sprouting of intersegmental vessels in the zebrafish, the viability of knockout and comparably weak phenotype in the retinal vasculature would argue that the F-BAR protein is a minor player in the mouse. The authors acknowledge this point in the Discussion and suggest that other F-BAR proteins might act in a redundant fashion.

Much of the manuscript deals with tip cell function and the extension of filopodia. However, it is by no means clear that Nostrin is not required in the rest of the angiogenic vasculature. This could be easily addressed by looking at endothelial cell proliferation (e.g. BrdU incorporation), a function that is more confined to stalk cells and the freshly formed vessel plexus behind the sprouting front. In fact, some of the data (Figures 3A and S5) hint strongly at reduced endothelial proliferation so that this important question should not be overlooked. Rac1 and Sos1 are also involved in the regulation of proliferation in numerous cell types and tissues.

In Figure 3, the authors provide quantitation for vascular outgrowth (radius) and the number of tip cells and filopodia. Key parameters such as vessel density and the number of branch points are missing, which should be easy to address.

The authors argue that Nostrin is required for FGF-induced vessel growth. A very obvious question is whether or not the same applies to VEGF, the most important pro-angiogenic growth factor. It would be straightforward to stimulate the cultured control and Nostrin KO endothelial cells with VEGF and analyze whether or not Rac1 activation is altered.

The manuscript does not address whether Nostrin is required in a cell-autonomous fashion in the endothelium in vivo. In fact, changes in other cell populations might contribute substantially to the defects seen in the morphant fish and knockout mice. This important caveat should be at least discussed.

Molecular weight markers are not indicated in the Western blots.

Referee #2:

I the present article, Kovacevic et al. identifies a new function for the F-BAR domain containing NOSTRIN in vascular development in vivo. Using an elegant combination of approaches including genetic loss-of-function both in zebrafish and NOSTRIN knockout mice, time-lapse imaging, biochemical and cell biological approaches, the authors present a strong set of results showing that NOSTRIN is required for the FGFR1-mediated angiogenic response of FGF2/bFGF. They also provide a potential molecular mechanism underlying the function NOSTRIN in angiogenesis which involves the ability of NOSTRIN to bind activated Rac1 through its HR1 domain and the exchange factor Sos1 through its SH3 domain thereby providing a functional link between FGFR1 activation and tip cell membrane dynamics.

Overall, the paper is of good quality and presents very interesting new evidence providing novel molecular insights into the molecular mechanisms underlying angiogenesis in vivo. However, probably the most interesting and novel aspect of the paper is not tackled which is the role of the F-BAR domain in this process. The author basically treat the F-BAR domain as a membrane targeting domain but emerging evidence in the literature demonstrated that these domain are involved in membrane deformation (either membrane invagination/endocytosis or membrane protrusion in the

forms of lamelipodial or filopodial dynamics). The authors should at least address the basis minimal activity of this F-BAR domain in some cell lines (test what membrane deformation properties the isolated F-BAR has) and even better, provide rescue experiment in the tip cell assay rescuing NOSTRIN LOF by re-introducing mutated form of NOSTRIN abolishing the F-BAR membrane-binding and/or membrane deformation activities.

Other specific comments to be addressed:

1- Figure 1: A few controls are missing. Need to be included: a direct comparison of WT and control MO in panels A-C, control MO in panel D, WT in panel E and quantifications of control MO and ATG MO in panel F.

2- Figure 5: The control in panel B is missing too. A Rac1-GTP pulldown in cells expressing GFP or a control vector needs to be included so that the results can be compared to the basal level of Rac1 activation. The delta SH3 lane is not described in the results.

3- Please explain in the text how the vasculature is visualized in mice (figures 3 and 7).

4- Functional validation of the pathway would require knock-down analyses of SOS-1.

5- It would also be important for the non-specialized audience to clarify what is known about the involvement of FGFR1 in vascular development relevant to the phenotype described in this paper.

Referee #3:

The manuscript by Kovacevic and co-workers investigates the role of the F-BAR protein Nostrin in vascular development using a combination of zebrafish morpholino knockdown, mouse mutant studies invivo, demonstrating that loss of function impairs vascular development and guided sprouting. A detailed biochemical analysis identifies binding of Nostrin to active forms of Cdc42 and Rac1, involving the HR1 motif. The authors further identified that this activation is mediated by the recruitment of the RhoGEF Sos1.
The authors further perform Y2H screen to search for interactors and identify FGFR1, the interaction of which they confirm in primary endothelial cells. Finally, using the matrigel plug assay and FGF stimulation, the authors identify reduced vascular formation in Nostrin KO animals induced by FGF2.

Overall, this is an interesting study into the function and molecular interaction of the F-BAR protein Nostrin in vascular development. The text is well written and illustrated. The strong combination of in vivo and in vitro methods and the high quality of the data provide confidence in the proposed mechanism. However, there are a number of questions that should be addressed for further improvement.

1. The images of retinal filopodia and ISV filopodia in figures 2 and 3 are heavily oversaturated. It would be preferable to present images with full dynamic range in order to appreciate the structural details.

2. Page 6 and Figure 3: The authors state the retinal analysis confirms a crucial role of Nostrin in directed tip cell migration and filopodia formation. This seems somewhat overstated considering that the phenotype in the retina is very mild. Also, the authors do not analyse or comment on any possible embryonic vascular phenotype. Given that the animals are born and survive, and the retinal phenotype is mild, clearly Nostrin plays a more modulatory role than an essential or crucial role. This may be due to the fact that also Rac1 deletion does not in itself produce such a strong phenotype in endothelial tip cells. Please comment.

3. Can the authors rescue the vascular defects in the zebrafish using Rac1 activation in the endothelium?

4. How do the authors propose Rac1 controls filopodia and tip cell migration? And would this necessarily be FGF specific? Given that endothelial activation can be mediated by FGF, VEGF, S1P etc involving different receptors, it would be important to discuss why and how a downstream component that regulates membrane dynamics is linked to a specific pathway.

1st Revision - Authors' Response 31 May 2012

We thank the three referees and the EMBO Journal editorial office for their constructive criticism and their encouraging response. Please find below the point-by-point reply to the referees' comments.

Referee 1

Referee #1: Kovacevic et al. report that Nostrin, an F-BAR protein, controls FGF2-mediated Rac1 activation in endothelial cells and is therefore required for angiogenesis in the zebra fish embryo and the mouse retina. Overall, the manuscript is very interesting and well written, and the data is of good quality and presented in a logical order.

Referee #1: While the function of Nostrin appears to be critical for the sprouting of intersegmental vessels in the zebra fish, the viability of knockout and comparably weak phenotype in the retinal vasculature would argue that the F-BAR protein is a minor player in the mouse. The authors acknowledge this point in the Discussion and suggest that other F-BAR proteins might act in a redundant fashion.

We have re-phrased the respective sentence on the results section (page 7, line 3-5) and acknowledged this point in the discussion section (page 11, line 8-12).

Referee #1: Much of the manuscript deals with tip cell function and the extension of filopodia. However, it is by no means clear that Nostrin is not required in the rest of the angiogenic vasculature. This could be easily addressed by looking at endothelial cell proliferation (e.g. BrdU incorporation), a function that is more confined to stalk cells and the freshly formed vessel plexus behind the sprouting front. In fact, some of the data (Figures 3A and S5) hint strongly at reduced endothelial proliferation so that this important question should not be overlooked. Rac1 and Sos1 are also involved in the regulation of proliferation in numerous cell types and tissues.

We have we determined the number of cells stained positive for phosphorylated Histone H3 and Ki67 by immunofluorescence on mouse retina at P5 as a measure of the proliferation and have found a significant reduction of proliferating cells in retinas of NOSTRIN knockout mice in comparison to WT control animals. These data are included in the manuscript as Figure 3G/H and Supplementary Figure S4 and described in the Results (page 6, line 31-33) and Material and Methods (page 16, line 9-11) section. Introduction and discussion have been adjusted accordingly.

Referee #1: In Figure 3, the authors provide quantitation for vascular outgrowth (radius) and the number of tip cells and filopodia. Key parameters such as vessel density and the number of branch points are missing, which should be easy to address.

We have analysed vessel density and the number of branch points at the vascular front and the central area as suggested and have found all three parameters significantly reduced in NOSTRIN knockout mice. These new data are included in the manuscript as Figure 3C/D and described in the Results (page 6, line 23-28). Introduction and discussion have been adjusted accordingly.

Referee #1: The authors argue that Nostrin is required for FGF-induced vessel growth. A very obvious question is whether or not the same applies to VEGF, the most important pro-angiogenic growth factor. It would be straightforward to stimulate the cultured control and Nostrin KO endothelial cells with VEGF and analyze whether or not Rac1 activation is altered.

We have performed the suggested experiment and the results indicated that VEGF-C induced Rac1 activation both in WT and NOSTRIN KO cells, suggesting that at least in this experimental setting

the function of NOSTRIN is specific for the FGFR1 and does not apply to the VEGF receptor. The respective data are included in the Supplementary Information as Figure S7 and described in the Results section (page 10, line 6-10) and Discussion section (page 12, line 14-18).

Referee #1: The manuscript does not address whether Nostrin is required in a cell-autonomous fashion in the endothelium in vivo. In fact, changes in other cell populations might contribute substantially to the defects seen in the morphant fish and knockout mice. This important caveat should be at least discussed.

To address the question of cell-autonomous function of NOSTRIN in endothelial cells *in vivo* we have repeated the analysis of angiogenic parameters in NOSTRIN knockout mice with Tie2driven expression of Cre recombinase in endothelial cells. The analysis indicates that as in the complete NOSTRIN KO, vascular spreading, vascular density, tip cell number, tip cell filopodia number and length, the number of branch points and endothelial cell proliferation are significantly reduced in comparison to littermate controls (NOSTRIN floxed/floxed without Cre recombinase expression). This strongly argues for an endothelial cell autonomous function of NOSTRIN in vascular development. These new data are included in the manuscript as Supplementary Figure 6 and described in the Results section (page 7, line 8-13). A minimal description of the respective conditional NOSTRIN knockout mice, which will be described in detail independently of this study, is included in the Supplementary Information as Figure 5)

Referee #1: Molecular weight markers are not indicated in the Western blots.

Molecular weights have now been indicated throughout the manuscript.

Referee 2

Referee #2: In the present article, Kovacevic et al. identifies a new function for the F-BAR domain containing NOSTRIN in vascular development in vivo. Using an elegant combination of approaches including genetic loss-of-function both in zebra fish and NOSTRIN knockout mice, time-lapse imaging, biochemical and cell biological approaches, the authors present a strong set of results showing that NOSTRIN is required for the FGFR1-mediated angiogenic response of FGF2/bFGF. They also provide a potential molecular mechanism underlying the function NOSTRIN in angiogenesis which involves the ability of NOSTRIN to bind activated Rac1 through its HR1 domain and the exchange factor Sos1 through its SH3 domain thereby providing a functional link between FGFR1 activation and tip cell membrane dynamics. Overall, the paper is of good quality and presents very interesting new evidence providing novel molecular insights into the molecular mechanisms underlying angiogenesis in vivo. However, probably the most interesting and novel aspect of the paper is not tackled which is the role of the F-BAR domain in this process. The author basically treat the F-BAR domain as a membrane targeting domain but emerging evidence in the literature demonstrated that these domain are involved in membrane deformation (either membrane invagination/endocytosis or membrane protrusion in the forms of lamelipodial or filopodial dynamics). The authors should at least address the basis minimal activity of this F-BAR domain in some cell lines (test what membrane deformation properties the isolated F-BAR has) and even better, provide rescue experiment in the tip cell assay rescuing NOSTRIN LOF by re-introducing mutated form of NOSTRIN abolishing the F-BAR membrane-binding and/or membrane deformation activities.

We perfectly agree with the statement made by the reviewer that the function of the F-BAR domain itself in the process of membrane dynamics and angiogenesis is of great interest. To address this important question, we have already commenced a series of biochemical, biophysical and structural biology experiments including an analysis of the function of the isolated F-BAR domain, as suggested above. At the current state we prefer not to include these data into this manuscript, but to analyse the membrane dynamics in more detail (e.g. by life cell imaging) and combine the results with the biophysical and structural analysis to address this question thoroughly.

Referee #2: 1- Figure 1: A few controls are missing. Need to be included: a direct comparison of WT and control MO in panels A-C, control MO in panel D, WT in panel E and quantifications of

control MO and ATG MO in panel F.

We have included the respective controls and quantifications. In summary, we could not detect any difference between WT (uninjected) zebra fish embryos and those injected with Control morpholino. The quantification of the vascular defects following injection of the ATG morpholino was comparable to those seen after injection of the SH3 morpholino, confirming our previous results. The additional controls are shown as Fig. 1A WT (former Fig. 1A and 1B now combined as 1A); Fig. 1B Control MO (former Fig. 1C); Fig. 1C Control MO (former Fig. 1D); Fig. 1D WT (former Fig. 1E). The quantification for the frequency of the vascular phenotype in Control MO and ATG MO injected embryos is included in Fig. 1E (former Fig. 1F) Results section, legend and Material and Methods section have been adjusted accordingly.

Referee #2: 2- Figure 5: The control in panel B is missing too. A Rac1-GTP pulldown in cells expressing GFP or a control vector needs to be included so that the results can be compared to the basal level of Rac1 activation. The delta SH3 lane is not described in the results.

We have included a Rac1-GTP pulldown from control cells as suggested to compare Rac1-GTP activation achieved by overexpression of NOSTRIN and the respective mutants with basal levels of Rac1 activity. This data is shown as Fig. 5A and replaces Figs. 5A and 5B. Results section, legend and material and methods section have been adjusted accordingly.

The information on the delta SH3 lane is included in the Result section (page 9, line 4-6).

Referee #2: 3- Please explain in the text how the vasculature is visualized in mice (figures 3 and 7).

This information is included in the Material and methods section (page 16, line 7-9 and line 2227)

Referee #2: 4- Functional validation of the pathway would require knock-down analyses of SOS 1.

We agree that a knock-down of Sos1 would allow validation of the functionality of the proposed pathway. Unfortunately the primary character of the mouse lung endothelial cells we have used in our study to demonstrate the FGF2-dependent activation of Rac1 and its dependence on the presence of NOSTRIN prohibits the establishment of stable knock down cell lines e.g. after lentiviral-mediated transduction. Several attempts to transiently transfect these primary cells with siRNA did not allow the generation of cell populations with reproducible and comparable reduction of Sos1 levels. This prevented the functional validation of the pathway by the means of a Sos1 knockdown.

Referee #2: 5- It would also be important for the non-specialized audience to clarify what is known about the involvement of FGFR1 in vascular development relevant to the phenotype described in this paper.

We have included basic information on the role of FGFR1 in vascular development and briefly discussed this in the context of the observed phenotype in the discussion section (page 11, line 21-31). Due to space limitations the functions of the FGF/FGFR family in vascular development could not be addressed in more detail but we have included a number of additional references directing the audience to the respective literature reviews and original articles (page 11, line 2227).

Referee 3

Referee #3: The manuscript by Kovacevic and co-workers investigates the role of the F-BAR protein Nostrin in vascular development using a combination of zebra fish morpholino knockdown, mouse mutant studies in vivo, demonstrating that loss of function impairs vascular development and guided sprouting. A detailed biochemical analysis identifies binding of Nostrin to active forms of Cdc42 and Rac1, involving the HR1 motif. The authors further identified that this activation is mediated by the recruitment of the RhoGEF Sos1. The authors further perform Y2H screen to search for interactors and identify FGFR1, the interaction of which they confirm in primary endothelial cells. Finally, using the matrigel plug assay and FGF stimulation, the authors identify reduced vascular formation in Nostrin KO animals induced by FGF2.

Overall, this is an interesting study into the function and molecular interaction of the F-BAR protein Nostrin in vascular development. The text is well written and illustrated. The strong combination of in vivo and in vitro methods and the high quality of the data provide confidence in the proposed mechanism. However, there are a number of questions that should be addressed for further improvement.

Referee #3: 1. The images of retinal filopodia and ISV filopodia in figures 2 and 3 are heavily oversaturated. It would be preferable to present images with full dynamic range in order to appreciate the structural details.

We agree that the cell bodies in the high magnification images in Fig. 2 and 3 are highly oversaturated. The 3-dimensional character of filopodia with extension above or below the acquisition plane requires the compilation of several Z-stacks in order to visualize the protrusions in their entire length, enhancing the signal from the cell body. In addition, for the zebra fish we use fish with endothelial cell specific expression of eGFP, but eGFP is diffusely distributed and not targeted to the membrane or to cellular protrusions. Therefore long exposures and high gain acquisition settings during image acquisition are unavoidable to visualise filopodial membrane protrusions.

Referee #3: 2. Page 6 and Figure 3: The authors state the retinal analysis confirms a crucial role of Nostrin in directed tip cell migration and filopodia formation. This seems somewhat overstated considering that the phenotype in the retina is very mild. Also, the authors do not analyse or comment on any possible embryonic vascular phenotype. Given that the animals are born and survive, and the retinal phenotype is mild, clearly Nostrin plays a more modulatory role than an essential or crucial role. This may be due to the fact that also Rac1 deletion does not in itself produce such a strong phenotype in endothelial tip cells. Please comment.

We have re-phrased the respective sentence on the results section (page 7, line 3-5) and acknowledged this point in the discussion section (page 11, line 8-12 and page 12, line 20 - page 13, line 5).

Referee #3: 3. Can the authors rescue the vascular defects in the zebra fish using Rac1 activation in the endothelium?

The information on the activity state of Rac1 in endothelial cells in developing vasculature in vivo is limited. The current notion that Rac1 activity is crucial for the proper ISV growth and trajectory in zebra fish is derived from experimental data obtained in other experimental systems, e.g. the observation that treatments leading to altered ISV development in zebra fish *in vivo*, induce changes in Rac activity in cultured cells *in vitro* (Epting et al., 2010, Wang et al., 2010). Jointly, these data strongly suggest an important function of Rac, but its spatial activity and consequence of loss or gain of function of Rac1 activity in developing ISVs, especially on the trajectory and the filopodia formation of tip cells *in vivo*, has not been shown to our knowledge.

Referee #3: 4. How do the authors propose Rac1 controls filopodia and tip cell migration? And would this necessarily be FGF specific? Given that endothelial activation can be mediated by FGF, VEGF, S1P etc involving different receptors, it would be important to discuss why and how a downstream component that regulates membrane dynamics is linked to a specific pathway.

We have addressed this question experimentally and compared Rac1 activity in primary mouse lung endothelial cells from WT and NOSTRIN KO mice after stimulation with VEGF-C. We observed, that VEGF-C induced Rac1 activation both in WT and NOSTRIN KO cells, suggesting that at least in this experimental setting the function of NOSTRIN is specific for the FGF receptor and does not apply to the VEGF receptor. The respective data are included in the Supplementary Information as Figure S7 and described in the Results section (page 10, line 6-10) and Discussion section (page 12, line 14-18).