Rspo2 antagonizes FGF signaling during vertebrate mesoderm formation and patterning

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MS TITLE: Rspo2 antagonizes FGF signaling during vertebrate mesoderm formation and patterning

AUTHORS: Alice H. Reis and Sergei Y. Sokol

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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 attend to all of the reviewers' comments and 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. 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

The manuscript by Reis and Sokol examines interactions between the R-spondin2 (rspo2) protein and the FGF signaling pathway. R-spondins are historically known as activators of the Wnt signaling pathway, and this work, using Xenopus embryos shows that rspo2 can also interact with the FGF pathway. Here they show that rspo2 normally inhibits the FGF pathway and further show that the TSP domain of the rspo2 protein is essential for this inhibition.
There are issues regarding the novelty as stated in the manuscript, and there are additional experimental issues that should be addressed.

Comments for the author

1. The abstract and introduction is framed in such a way that suggests that there is little known about how R-spondins interact with other signaling pathways, and that there are bits and pieces of evidence in the literature that suggests there might be some connection between R-spondins and FGF signaling. But the work by Min Zhang et al. (Scientific Reports 2017), which is not cited in this manuscript, shows that Rspo3 acts as an inhibitor of FGF/Erk signaling during human osteogenesis. This study examines Rspo2, but as the authors point out the domain structure and function of the four R-spondin proteins are well conserved. So the central theme of the paper that this represents a novel discovery that R-spondins inhibit FGF/Erk signaling needs to be revised. This work confirms the interaction in another system and provides insight into the domain of R-spondins mediating this function.

2. Numbers should be provided for the experiment in figure S1 (total embryos injected and number that exhibit the phenotype)

3. The authors describe the elongation of FGF stimulated rspo2-MO injected explants as being more efficient than FGF stimulation alone. This is hard to see in the image. Can it be quantified to better illustrate the difference?

4. The change in tbxt expression after FGF stimulation in figure 1G is very different that the same condition in figure 2C. Why is there such a difference? The fold increase of FGF + rspo2 MO tbxta induction in 2C is the same as the FGF only stimulation in 2G.

5. Since the initial analysis of rspo2 manipulations on gene expression was performed with tbxta (in explants) it would be good to perform a whole embryo analysis of tbxta expression after loss of rspo2 function (as is done with cdx4 in fig. 2E).

6. The text states that an independent splice blocking rspo2 MO also causes cdx4 upregulation as the translation blocking MO does, but there is no data given to support this (or any phenotypic analysis of the splice blocking MO).

7. A standard control for MOs is to perform a rescue experiment with injected mRNA that is not targeted by the MO. This would be straightforward in the explant assays and is an important specificity control.

8. MO injected embryos are described as having anterior truncations but the data is not show. It would be useful to show this.

Minor points

9. Introduction page 2: part of rationale for a relationship between FGF and R-spondins in that they both can act as oncogenes in the formation of mammary tumors, but this implies a positive relationship between the two, which does not support the relationship between them reported here, and in the publication listed above.

10. Introduction page 3: Dorey reference is not formatted.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Reis and Sokol report a novel function for Rspo2, as a negative regulator of FGF signaling in Xenopus mesodermal development. Rspo2 gain-of-function was shown to inhibit FGF-dependent activation of Erk1 and downstream target genes. Rspo2 did not inhibit the response to ca-Mek1, indicating a function upstream of Mek1. Rspo2 depletion in explants and whole embryos resulted in an upregulation of FGF responses, consistent with a negative regulatory role. Structure-function studies indicated the TSP domain is required for Rspo2 regulation of the FGF pathway, and co-IP studies suggest that Rspo2 forms intramolecular and intermolecular complexes. These studies are of significance to those working in the fields of signal transduction, developmental signaling and pattern formation.

Comments for the author

These studies provide a convincing demonstration that Rspo2 can function as an antagonist of FGF signaling in Xenopus mesoderm induction. While the observations are novel and significant, this work is limited in scope and fails to sufficiently address the broader developmental role of this antagonistic function or the specific mechanisms mediating the antagonism. While valuable, in its current form this work is too preliminary and superficial to justify publication in Development. A revised and expanded manuscript that address the issues raised below is worth reconsideration.

1) The authors refer to the axial phenotype obtained with Rspo2 knockdown in the whole embryo, but data is not shown. Rspo2 knockdown embryos should be analyzed in detail to better define the developmental requirement of Rspo2 in relationship to FGF signaling, mesodermal patterning and axial development.

2) A mechanistic model is presented (Fig 4) that indicates a role for Rspo2 in sequestration of HSPGs required for FGF signaling. This model is speculative and is unsupported by the results presented. In depth biochemical and functional studies are needed to define the interaction of Rspo2 with FGF signaling pathway components.

3) The authors conclude that the Tsp domain of Rspo2 is required for the FGF antagonism, and the data shown in Fig 3 largely support this conclusion. However, the morphogenetic response to FGF in the presence of Rspo2 deltaT far more vigorous than with FGF alone, and it is unclear if this is simply a “stronger” FGF response. Perhaps Rspo2 deltaT is modulating a distinct signaling pathway under these conditions. The explant results should be examined in more detail to define the gene expression response, as well as tissues formed.

4) The authors conclude that Rspo2 deltaT functions as dominant negative via interaction with endogenous Rspo2. This conclusion is poorly supported, and alternative interpretations are not considered. For example Rspo2 deltaT may modulate the function of other signaling pathways that impact Erk1 activation. In supplemental data the interaction of Rspo2 Activin is examined and the authors conclude that induction of pSmad2 is unchanged. However, the data (Figure S4) could be interpreted as indicating a small elevation of pSmad2 in the presence of Rspo2 and Rspo2 deltaT.

5) Statistical analysis should be performed for the quantified results in Figures 1 and 2.

First revision

Author response to reviewers' comments

Responses to reviewer 1

Advance Summary and Potential Significance to Field:
The manuscript by Reis and Sokol examines interactions between the R-spondin2 (rspo2)

protein and the FGF signaling pathway. R-spondins are historically known as activators of the Wnt signaling pathway, and this work, using Xenopus embryos shows that rspo2 can also interact with the FGF pathway. Here they show that rspo2 normally inhibits the FGF pathway and further show that the TSP domain of the rspo2 protein is essential for this inhibition. There are issues regarding the novelty as stated in the manuscript, and there are additional experimental issues that should be addressed.

Reviewer 1 Comments for the Author:
1. The abstract and introduction is framed in such a way that suggests that there is little known about how R-spondins interact with other signaling pathways, and that there are bits and pieces of evidence in the literature that suggests there might be some connection between R-spondins and FGF signaling. But the work by Min Zhang et al. (Scientific Reports 2017), which is not cited in this manuscript, shows that Rspo3 acts as an inhibitor of FGF/Erk signaling during human osteogenesis. This study examines Rspo2, but as the authors point out the domain structure and function of the four R-spondin proteins are well conserved. So the central theme of the paper that this represents a novel discovery that R-spondins inhibit FGF/Erk signaling needs to be revised. This work confirms the interaction in another system, and provides insight into the domain of R-spondins mediating this function.

We apologize for inadvertently omitting the reference cited by the reviewer (Zhang et al., 2017). Prompted by the reviewer, we revised the text and added the missing reference. Although Zhang et al. show the upregulation of phospho-Erk1 levels after osteogenic differentiation of human adipose-derived stem cells depleted of Rspo3, the direct involvement of Rspo3 in the FGF signaling cascade has not been assessed. Rspo3 shRNA activated Erk1 probably because of the changes in FGF4 and FGFR2 transcription (Figs. 4E,F in Zhang et al.). Alternatively, any of the multiple pathways influencing Erk activity during the 14-day differentiation could have been affected by Rspo3 depletion. Of note, we define a role of a molecule in a signaling pathway in the narrow sense, as its physical involvement in the biochemical process starting at the cell surface and ending at target (e. g., specific gene) activation. We, therefore, feel that, although important, this previous paper does not decrease the novelty of our work.

2. Numbers should be provided for the experiment in figure S1 (total embryos injected and number that exhibit the phenotype)

We have added the numbers of embryos displaying morphological phenotype and total number of embryos to the figure S1. The legend to Fig. S1 was adjusted.

3. The authors describe the elongation of FGF stimulated rspo2-MO injected explants as being more efficient than FGF stimulation alone. This is hard to see in the image. Can it be quantified to better illustrate the difference?

Following the suggestion, we have conducted morphometric analysis of the explants and added the corresponding graph to Fig. 2B. To unambiguously illustrate the change in explant morphology, the results from an independent experiment are shown in Figure 1 (for reviewers). We did not show this figure in the manuscript because a part of this experiment has already been used in Fig. 3 of our paper.

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

4. The change in tbxt expression after FGF stimulation in figure 1G is very different that the same condition in figure 2C. Why is there such a difference? The fold increase of FGF + rspo2 MO tbxta induction in 2C is the same as the FGF only stimulation in 2G.

We thank the referee for pointing out to this apparent inconsistency. In Fig. 2C, we used suboptimal amount of FGF to avoid the saturation of the response and better visualize the upregulation of *tbxt* by Rspo2 MO. Figure legends and Methods have been revised to reflect the actual amount of added FGF.

5. Since the initial analysis of rspo2 manipulations on gene expression was performed with

tbxta (in explants) it would be good to perform a whole embryo analysis of tbxta expression after loss of rspo2 function (as is done with cdx4 in fig. 2E).

We are currently unable to carry out this experiment, however, we consider our qPCR data very compelling. In lieu of the suggested experiment, we added the qPCR analysis of *msgn1*, another known FGF gene target (new Fig. 2E). Our conclusions are now backed up with the analysis of three different FGF pathway targets.

6. The text states that an independent splice blocking rspo2 MO also causes cdx4 upregulation as the translation blocking MO does, but there is no data given to support this (or any phenotypic analysis of the splice blocking MO).

We point out that the modified Figure 2 contains the RT-qPCR data requested by the referee: *cdx4* expression is upregulated in the explants injected with two different MOs (Fig. 2D) and we added new data for *msgn1* expression to new Fig. 2E. Moreover, morphological phenotypes of Rspo2 morphants that lack anterior structures have been included in the revised manuscript (Fig. 2G).

7. A standard control for MOs is to perform a rescue experiment with injected mRNA that is not targeted by the MO. This would be straightforward in the explant assays and is an important specificity control.

We agree with the referee that the suggested experiment would be a good additional control. Nevertheless, the use of two independent MOs in our study strongly reduces the chances of off-target effects (Fig. 2D and E). Moreover, we present the result that Rspo2 knockdown with either MO leads to anterior deficiencies (Fig. 2G). This is the phenotype opposite to that of Rspo2 overexpression that causes posterior defects, supporting specificity. These observations have been discussed in the revised manuscript, because the rescue experiment is currently not practical to carry out due to the imposed restrictions on wet lab work.

8. MO injected embryos are described as having anterior truncations but the data is not shown. It would be useful to show this.

As already pointed out in response to point 7, we have added the phenotypes of embryos depleted of Rspo2 to new Fig. 2G.

Minor points
9. Introduction page 2: part of rationale for a relationship between FGF and R-spondins in that they both can act as oncogenes in the formation of mammary tumors, but this implies a positive relationship between the two, which does not support the relationship between them reported here, and in the publication listed above.

We agree with the reviewer that it is unclear why Rspo2 promotes rather than inhibits MMTV-induced mammary tumors (Lowther et al., 2005; Theodorou et al., 2007). This argument has been removed from the introduction in the revised manuscript.

10. Introduction page 3: Dorey reference is not formatted.

This was corrected.

Responses to reviewer 2

Advance Summary and Potential Significance to Field:
In this manuscript, Reis and Sokol report a novel function for Rspo2, as a negative regulator of FGF signaling in Xenopus mesodermal development. Rspo2 gain-of-function was shown to inhibit FGF-dependent activation of Erk1 and downstream target genes. Rspo2 did not inhibit the response to ca-Mek1, indicating a function upstream of Mek1. Rspo2 depletion in explants and whole embryos resulted in an upregulation of FGF responses, consistent with a negative regulatory role. Structure-function studies indicated the TSP domain is required for Rspo2

regulation of the FGF pathway, and co-IP studies suggest that Rspo2 forms intramolecular and intermolecular complexes. These studies are of significance to those working in the fields of signal transduction, developmental signaling and pattern formation.

Reviewer 2 Comments for the Author:

These studies provide a convincing demonstration that Rspo2 can function as an antagonist of FGF signaling in Xenopus mesoderm induction. While the observations are novel and significant, this work is limited in scope and fails to sufficiently address the broader developmental role of this antagonistic function or the specific mechanisms mediating the antagonism. While valuable, in its current form this work is too preliminary and superficial to justify publication in Development. A revised and expanded manuscript that address the issues raised below is worth reconsideration.

1) The authors refer to the axial phenotype obtained with Rspo2 knockdown in the whole embryo, but data is not shown. Rspo2 knockdown embryos should be analyzed in detail to better define the developmental requirement of Rspo2 in relationship to FGF signaling, mesodermal patterning and axial development.

Prompted by the reviewer, we have added the morphological phenotypes of whole embryos that have been depleted of Rspo2 with two different morpholinos (new Fig. 2G). As expected for an FGF inhibitor, Rspo2 knockdown causes loss of anterior structures. To study developmental signaling, we chose FGF-dependent induction assays in embryonic ectoderm due to low background in ectodermal cells and strong response to FGF ligands. In addition to ectoderm explants and whole embryos, Figs. 2D, E present marginal zone analysis of FGF target genes, which is further supported by wholemount *in situ* hybridization (Fig. 2F). We hope that this is sufficient for the initial characterization of the role of Rspo2 in FGF signaling.

2) A mechanistic model is presented (Fig 4) that indicates a role for Rspo2 in sequestration of HSPGs required for FGF signaling. This model is speculative and is unsupported by the results presented. In depth biochemical and functional studies are needed to define the interaction of Rspo2 with FGF signaling pathway components.

We acknowledge that our model is primarily proposed based on the existing knowledge, rather than our experimental data. To better understand the underlying mechanism, we assessed the binding of Rspo2 to FGF pathway upstream components. We did not detect any physical interactions of Rspo2 with FGFR1 and FGFR4 or FGF8 (data not shown). On the other hand, R-spondins have been demonstrated to bind HSPGs, including syndecans and glypicans (Ohkawara et al., 2011). Since HSPGs function as coreceptors for FGF (Garcia- Garcia and Anderson, 2003; Rapraeger et al., 1991; Yayon et al., 1991), we propose the model presented in Fig. 4. In-depth analysis requested by the referee warrants additional experiments that are not currently feasible due to the limitations imposed by our institution in connection with Covid-19. Prompted by the reviewer, we discuss the alternative possibilities in the revised manuscript, page 8.

3) The authors conclude that the Tsp domain of Rspo2 is required for the FGF antagonism, and the data shown in Fig 3 largely support this conclusion. However, the morphogenetic response to FGF in the presence of Rspo2 deltaT far more vigorous than with FGF alone, and it is unclear if this is simply a “stronger” FGF response. Perhaps Rspo2 deltaT is modulating a distinct signaling pathway under these conditions. The explant results should be examined in more detail to define the gene expression response, as well as tissues formed.

We interpreted the stimulatory effect of RspoΔT on FGF signaling as its interference with the inhibitory activity of Rspo2. In principle, we agree with the referee that RspoΔT may synergize with FGF by affecting yet unknown signaling pathways. In response to this comment, we revised discussion to acknowledge the existing alternative explanations. Because of our limited ability to carry out new experiments, the analysis of how RspoΔT affects other pathways will be accomplished in future studies. Please, also see our response to point 4.

4) The authors conclude that Rspo2 deltaT functions as dominant negative via interaction with

endogenous Rspo2. This conclusion is poorly supported, and alternative interpretations are not considered. For example, Rspo2 deltaT may modulate the function of other signaling pathways that impact Erk1 activation. In supplemental data the interaction of Rspo2 Activin is examined and the authors conclude that induction of pSmad2 is unchanged. However, the data (Figure S4) could be interpreted as indicating a small elevation of pSmad2 in the presence of Rspo2 and Rspo2 deltaT.

As we replied in response to point 3, in the revised text we acknowledge that the mechanism of RspoΔT synergy with FGF is unknown. We discuss an alternative possibility that RspoΔT may cooperate with FGF by promoting Wnt signaling, consistent with the known synergy of FGF and Wnt proteins (Christian and Moon, 1992). In support of this hypothesis, Rspo2 can promote Wnt signaling via its FU-like domains (preserved in RspoΔT) by interfering with ZNRF3/RNF43, an inhibitor of Wnt signaling (Hao et al., 2012; Koo et al., 2012) and we observed that RspoΔT RNA can trigger partial secondary axis formation in Xenopus embryos (data not shown).

We also considered that RspoΔT might influence the Activin/Nodal pathway, but could not find significant changes in pSmad2 after stimulation with Rspo2 constructs. Supporting this conclusion, we show an independent experiment (Figure 2 for reviewers) that can be presented as a supplementary Figure. We chose to use the current version because it contains duplicate biological samples.

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

5) Statistical analysis should be performed for the quantified results in Figures 1 and 2. The statistical analysis has been provided as suggested by the referee.

Second decision letter

MS ID#: DEVELOP/2020/189324

MS TITLE: Rspo2 antagonizes FGF signaling during vertebrate mesoderm formation and patterning

AUTHORS: Alice H. Reis and Sergei Y. Sokol
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.