EMBO reports

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LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 03 August 2011

Thank you for the submission of your manuscript to EMBO reports and my apologies for the delay in getting back to you. We have just now received the second report from the referees. As you can see below, both reviewers are quite positive but some minor concerns need to be addressed prior to acceptance.

While reviewer #2 is very positive, reviewer #1 points out to several issues that will need your attention. Besides several minor points regarding corrections and clarifications, this referee has three major points of concern. First, s/he is concerned with the consistency of the data presented in Figs 1 and S2, in relationship with Rspo and Wnt synergy. Second, the findings in regard to LGR KD in 293T cells should be discussed taking into account the results shown in Lau et al. (Nature, 2011). Finally, S/he believes that the results depicted in Fig. 4 concerning PCP phenotypes are in clear contradiction with results published in Ohkawara et al. (Dev Cell, 2010).

After additional discussion with the referees we believe that these issues can be solved without providing more experimental evidence unless, of course, you wish to add further support to your conclusions. However, experimental issues and the comparison with previously published studies need to be discussed in the manuscript. Also, browsing through the manuscript myself, I have noticed that statistical analysis is not properly described for all the figures, including supplementary data. Statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply. Please include a definition of the error bars used and, when

necessary, state the statistical significance of the results and the method used to calculate it. I have also noticed, and this is one of the minor points mentioned by referee #2 as well, that Figs. 3 and 4 do not have scale bars and they should be added for comparison.

I look forward to seeing final version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

This manuscript describes an identification of LGR4/5 receptors as novel R-spondin receptors and activation of both canonical and non-canonical Wnt signaling by R-spondins using a combination of biochemical, cell culture and in vivo Xenopus embryo experiments. As these authors noted, two articles recently published in other scientific journals with basically the same conclusion that LGR4/5 are novel R-spondin receptors for Wnt/beta-catenin signaling. However, this manuscript uniquely showed two sets of additional data; i) R-spondin activation of non-canonical Wnt /PCP signaling through LGR4/5 and ii) Clathrin-specific endocytosis of R-spondin-LGR4/5 complex, providing additional insights into the R-spondin signaling mechanism. Even though there are two published articles, the reviewer feels that this manuscript is appropriate for publication in the EMBO reports if the authors address the following issues.

1. Combined treatment of Rspo3 and Wnt3a conditioned medium, which generally induces strong synergistic activation of Topflash reporter, produced inconsistent results in the first and second panels of Figures1F and S2. They need to provide a better data set that shows the synergy more convincingly.

2. Data published by de Lau et al (Nature, 2011) showed that siRNA knockdown of LGR4/5 in 293T cells did not cause any diminished beta-catenin activation by Wnt3a, which is different from the data presented in this manuscript. This is a key difference that needs to be clarified for better understanding of Rspo-Wnt signaling, not necessarily in this manuscript but perhaps in the future. Therefore, the authors should explain this discrepancy in the text.

3. The reviewer is less convinced with the data presented in Figure 4C and D. First, Rspo and Fz7 cooperation in ATF2 reporter activation is not clear, unlike the data presented in their earlier publication (Ohkawara etal., developmental cell, 2011). Therefore, blocking LGR4/5 expression in Fz7/Rspo3 co-injected embryos reduced the reporter activity to the level of Fz7-injected embryo. Second, data in Figure4 D is not consistent with the data presented in Ohkawara et al. While Rspo3 MO almost caused no effect on endogenous PCP activity in this manuscript, a similar Rspo3 Mo causes approximately 40% inhibition in prior publication. These inconsistencies significantly hamper any conclusions drawn from these data. Reviewer wonders whether this issue is related to the particular assay. Other additional PCP assay data such as a convergent extension of animal caps are necessary to further clarify this issue.

Minor issues

1. The incorrect citations were used for the sentence "They are involved in embryonic patterning and differentiation to frogs and mice" in the introduction.

2. In the second sentence of second paragraph of introduction, "Wnts" should be deleted for better reading.

3. Figure 1F (panel1 and 2) and Figure S2 are basically the same experimental data. The reviewer recommends presenting only one.

4. The authors should distinguish the use of Rspo (full-length) vs Rspo-deltaC proteins or conditioned medium by properly labeling them in the figures. A possibility that Rspo full-length and Rspo-delta C proteins may not behave identically remains.

5. Figure 3 is too crowded. Selection of key images combined with quantitative data from Fig S5 would be more informative for the readers.

6. Beta-catenin accumulation data in Figure S5C did not include the critical one from Rspo3 stimulation.

7. Scale bars in the microscopic images in Figures 3 and S4 are required.

8. In Figure 4, quantitative data for gastrulation defects of whole embryos (Fig S6C) should be presented along with panel A for the readers.

9. In Figure 4B, the labeling of the second and third bars are the same. Needs to be corrected.

Referee #2:

In this manuscript, Glinka and colleagues show clearly that R-spondin is the ligand for the Lgr4 and 5 receptors and medate Wnt signalling.The data is consistent with 2 recent reports in Nature and PNAS. The data by Glinki is well controlled and definitive. Moreover they build on these 2 previous studies by showing R-spondin internalisation is imporant for signalling and that Lgr4/5 also mediates PCP polarity in vivo in xenopus.
Overall these are important new findings and are again performed elegantly with all the appropriate controls. I am therefore very supportive of this study.

1st Revision - authors' response 08 August 2011

Response to Referees

Referee #1:

This manuscript describes an identification of LGR4/5 receptors as novel R-spondin receptors and activation of both canonical and non-canonical Wnt signaling by R-spondins using a combination of biochemical, cell culture and in vivo Xenopus embryo experiments. As these authors noted, two articles recently published in other scientific journals with basically the same conclusion that LGR4/5 are novel R-spondin receptors for Wnt/beta-catenin signaling. However, this manuscript uniquely showed two sets of additional data; i) R-spondin activation of non-canonical Wnt /PCP signaling through LGR4/5 and ii) Clathrin-specific endocytosis of R-spondin-LGR4/5 complex, providing additional insights into the R-spondin signaling mechanism. Even though there are two published articles, the reviewer feels that this manuscript is appropriate for publication in the EMBO reports if the authors address the following issues.

1. Combined treatment of Rspo3 and Wnt3a conditioned medium, which generally induces strong synergistic activation of Topflash reporter, produced inconsistent results in the first and second panels of Figures1F and S2. They need to provide a better data set that shows the synergy more convincingly.

The referee is right that combined treatment of Wnt3a and Rspo3 conditioned medium can induce strong synergistic activation of Topflash reporter. However, in our Wnt reporter assays (Fig. 1, Fig S2) we used limiting doses of Wnt3a and Rspo3 or Wnt3a and Rspo1 conditioned medium, which do not strongly synergize in reporter activation. This was done because under these conditions the synergy with LGR4/5 is best seen. What seems an inconsistency between assays in fact reflects different dilutions optimized for what we aimed to demonstrate in the particular assay. Thus, there are no inconsistencies because the different panels are not meant to be compared.

2. Data published by de Lau et al (Nature, 2011) showed that siRNA knockdown of LGR4/5 in 293T cells did not cause any diminished beta-catenin activation by Wnt3a, which is different from the data presented in this manuscript. This is a key difference that needs to be clarified for better understanding of Rspo-Wnt signaling, not necessarily in this manuscript but perhaps in the future. Therefore, the authors should explain this discrepancy in the text.

First, de Lau et al. did not inhibit LGR5 but only LGR4. Second, they siRNA-targeted the 3' UTR of *LGR4* while we used a pool of 4 siRNAs targeting the entire ORF, which might be more effective and explain the discrepancies. We included this explanation in the main text.

3. The reviewer is less convinced with the data presented in Figure 4C and D. First, Rspo and Fz7 cooperation in ATF2 reporter activation is not clear, unlike the data presented in their earlier publication (Ohkawara etal., developmental cell, 2011). Therefore, blocking LGR4/5 expression in Fz7/Rspo3 co-injected embryos reduced the reporter activity to the level of Fz7-injected embryo.

We agree that Rspo and Fz7 act additive than synergistic in Fig. 4D, but what is important is that LGR4/5 Mos block the Rspo3 induced signal.

Second, data in Figure4 D is not consistent with the data presented in Ohkawara et al. While Rspo3 MO almost caused no effect on endogenous PCP activity in this manuscript, a similar Rspo3 Mo causes approximately 40% inhibition in prior publication. These inconsistencies significantly hamper any conclusions drawn from these data. Reviewer wonders whether this issue is related to the particular assay. Other additional PCP assay data such as a convergent extension of animal caps are necessary to further clarify this issue.

This a misunderstanding by the referee: He over-read that in the present experiments we deliberately used a lower Rspo3 Mo dose than in Ohkawara et al., which by itself does not yield signal inhibition. This was done in order to demonstrate synergy with Lgr4 Mo. So there is no discrepancy.

Minor issues

1. The incorrect citations were used for the sentence "They are involved in embryonic patterning and differentiation to frogs and mice" in the introduction.

Corrected

2. In the second sentence of second paragraph of introduction, "Wnts" should be deleted for better reading.

Corrected

3. Figure 1F (panel1 and 2) and Figure S2 are basically the same experimental data. The reviewer recommends presenting only one.

Done, we removed panel 1 from Figure 1.

4. The authors should distinguish the use of Rspo (full-length) vs Rspo-deltaC proteins or conditioned medium by properly labeling them in the figures. A possibility that Rspo full-length and Rspo-delta C proteins may not behave identically remains.

Done

5. Figure 3 is too crowded. Selection of key images combined with quantitative data from Fig S5 would be more informative for the readers.

We chose to leave the figure as is, it contains important cell biological data that the reader needs to see.

6. Beta-catenin accumulation data in Figure S5C did not include the critical one from Rspo3 stimulation.

The referee must have overlooked that Rspo3 stimulation is shown in lane 6 in what is now Fig S4C.

7. Scale bars in the microscopic images in Figures 3 and S4 are required.

We included scale bars in Figures 3,4, S3.

8. In Figure 4, quantitative data for gastrulation defects of whole embryos (Fig S6C) should be presented along with panel A for the readers.

We rearranged Fig4.

9. In Figure 4B, the labeling of the second and third bars are the same. Needs to be corrected.

This was a mistake and was corrected in what is now Fig 4C.

Referee #2:

In this manuscript, Glinka and colleagues show clearly that R-spondin is the ligand for the Lgr4 and 5 receptors and medate Wnt signalling.The data is consistent with 2 recent reports in Nature and PNAS. The data by Glinki is well controlled and definitive. Moreover they build on these 2 previous studies by showing R-spondin internalisation is imporant for signalling and

that Lgr4/5 also mediates PCP polarity in vivo in xenopus.
Overall these are important new findings and are again performed elegantly with all the appropriate controls. I am therefore very supportive of this study.

2nd Editorial Decision 10 August 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

