Bone morphogenetic protein and retinoic acid synergistically specify female germ cell fate in mice

Hidetaka Miyauchi, Hiroshi Ohta, So Nagaoka, Fumio Nakaki, Kotaro Sasaki, Katsuhiko Hayashi, Yukihiro Yabuta, Tomonori Nakamura, Takuya Yamamoto, Mitinori Saitou

Corresponding author: Mitinori Saitou, Graduate School of Medicine, Kyoto University

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(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 07 April 2017

Thank you for the submission of your manuscript (EMBOJ-2017-96875) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from two of them, which I copy below. The report from the third referee got delayed and is still to be submitted to us, but we decided, in the interest of time to proceed with this. As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. I judge the comments of the referees to be generally reasonable and we are the in principle happy to invite you to revise your manuscript experimentally to address the referees' comments, pending no substantial technical robustness concerns by the third referee to come. I will inform you about referee #3's comments as soon as possible.

REFEREE REPORTS

Referee #1:

The authors demonstrate how PGCLCs, which are derived and maintained using a protocol raising intracellular cAMP levels with Rolipram and Forskolin, described in a separate manuscript, undergo female sex differentiation when stimulated with a combination of RA and BMP2. The authors provide extensive transcriptional, IHC and epigenetic data to support their conclusions, and provide convincing evidence that the combination of BMP and RA signaling induced meiocyte formation in
vitro. I think this manuscript is of great interest to the readership of the EMBO Journal.

Comments:

The use of many acronyms makes the paper difficult to read. I understand the need for constraining space, but at the same time I favor clarity over brevity. This is particularly important in the figures, where annotation of FACS plots would be a lot clearer if instead of BV, Blimp1-Venus is used. Same for all the other reporter genes. In figure 1A, Forskolin, Rolipram and Scf are better written out.

The authors provide a schematic of the culture conditions used to screen for factors that induce female germ cell formation in figure 1A. I think at the end of the manuscript, a similar figure should be included, clearly outlining the timing and conditions that robustly induce female germ cell formation. As is, this is not entirely clear. For example, are Rolipram and Forskolin continually included in the culture when female germ cell differentiation is induced with RA and BMP2?

Referee #2:

The group of Mitinori Saitou have been at the forefront of work to produce gametes from pluripotent stem cells in vitro. Previous work has described production of primordial germ cells that could be differentiated by exposure to undefined gonadal cues to mature germ cells. The work presented in Miyauchi et al. represents a further step in defining an in vitro process to produce more differentiated germ cells. The study identifies a heretofore unknown role for the growth factor Bmp2, in combination retinoic acid, to induce specification of female germ cell identity. The quality of the data is high and the conceptual insights important enough to warrant publication in EMBO J.

Points:

• Both Dazl and Ddx4 are expressed in both male and female germ cells prior to the onset of sexual differentiation (and prior to germ cell exposure to retinoic acid). Data in this paper argue that further induction of these genes occurs later in differentiation in response to retinoic acid. This biphasic expression pattern should be discussed within the Discussion section.

• The interconnection of cultured germ cells into cyst-like structures could be further elucidated by immuno-staining the culture cells with an antibody against Tex14, which marks inter-cytoplasmic bridges connecting cysts of germ cells in fetal ovary (Greenbaum et al. 2009).

• In Figure 2 the authors show very nice staining for meiotic chromosomes in cultured germ cells. They observe the expected global down-regulation of gH2AX signal in pachytene as double strand breaks are repaired. Given that the cells used to induce germ cells fate are XY in origin, did the authors observe any examples of retained gH2AX on the un-synapsed sex chromosomes in these cells?

• The RNA-Seq analysis of SK1 cells should be compared to published data (Soh et al. 2015) on Stra8-deficient embryonic ovaries. How many of the Stra8 dependent genes observed to be mis-expressed in SK1 cells also fail to be induced in Stra8 knockouts?

• The author propose a very interesting model for the development of germ cells, integrating extracellular signaling and DNA methylation. This model posits a developmental window (between E11.5-E13.5), when germ cells have lost their DNA methylation, but still do not express Stra8 or markers of female differentiation. A role for chromatin in this process seems likely, given the results of Yokobayashi et al. 2013. In this study Polycomb Repressive Complex 1 (PRC1) was found to be necessary to prevent precocious retinoic acid-dependent activation of Stra8 in fetal female germ cells. This work and the broader role of chromatin (which is covered in greater detail in the Ohta et al. manuscript, currently under consideration) in primordial germ cell like cells during the phase of DNA demethylation needs to be discussed in relation to the new findings reported in the current manuscript (assuming that the Ohta et al. manuscript would be published prior to or concurrently with this study). Microarray data from Yokobayashi et al. 2013 examining PRC1-dependent changes...
in gene expression in fetal ovarian germ cells should also be compared to the RNA-Seq generated here to identify overlapping requirement sets of genes.

1st Revision - authors’ response 17 July 2017

Referees’ Comments:

Referee #1:

The authors demonstrate how PGCLCs, which are derived and maintained using a protocol raising intracellular cAMP levels with Rolipram and Forskolin, described in a separate manuscript, undergo female sex differentiation when stimulated with a combination of RA and BMP2. The authors provide extensive transcriptional, IHC and epigenetic data to support their conclusions, and provide convincing evidence that the combination of BMP and RA signaling induced meiocyte formation in vitro. I think this manuscript is of great interest to the readership of the EMBO Journal.

Response 1. We would sincerely like to thank the Referee for the encouraging comments on our manuscript.

Comments: The use of many acronyms makes the paper difficult to read. I understand the need for constraining space, but at the same time I favor clarity over brevity. This is particularly important in the figures, where annotation of FACS plots would be a lot clearer if instead of BV, Blimp1-Venus is used. Same for all the other reporter genes. In figure 1A, Forskolin, Rolipram and Scf are better written out.

Response 2. In response to the Referee’s comment, we spelled out the acronyms for the reporters, chemicals and cytokines described in the Figures. We also defined such acronyms more explicitly upon their first appearance in the main text, as well as in the legend to each Figure (p 5, “A system for analyzing the sex-determination mechanism of germ cells” section, the second paragraph, in the revised manuscript).

The authors provide a schematic of the culture conditions used to screen for factors that induce female germ cell formation in figure 1A. I think at the end of the manuscript, a similar figure should be included, clearly outlining the timing and conditions that robustly induce female germ cell formation. As is, this is not entirely clear. For example, are Rolipram and Forskolin continually included in the culture when female germ cell differentiation is induced with RA and BMP2?

Response 3. In response to the Referee’s comment, we provided a scheme of the culture condition used to induce the female germ-cell fate in PGCLCs in Fig. 7A in the revised manuscript. We included Forskolin and Rolipram during the entire culture period and we clarified this point in the revised manuscript and in Fig. 1A (its legend) and 7A (p 5, “A system for analyzing the sex-determination mechanism of germ cells” section, the third paragraph, in the revised manuscript).

Referee #2:

The group of Mitinori Saitou have been at the forefront of work to produce gametes from pluripotent stem cells in vitro. Previous work has described production of primordial germ cells that could be differentiated by exposure to undefined gonadal cues to mature germ cells. The work presented in Miyauchi et al. represents a further step in defining an in vitro process to produce more differentiated germ cells. The study identifies a heretofore unknown role for the growth factor Bmp2, in combination retinoic acid, to induce specification of female germ cell identity. The quality of the data is high and the conceptual insights important enough to warrant publication in EMBO J.

Response 1. We would sincerely like to thank the Referee for the encouraging comments on our manuscript.
Points:
• Both Dazl and Ddx4 are expressed in both male and female germ cells prior to the onset of sexual differentiation (and prior to germ cell exposure to retinoic acid). Data in this paper argue that further induction of these genes occurs later in differentiation in response to retinoic acid. This biphasic expression pattern should be discussed within the Discussion section.

Response 2. According to the Referee’s comment, we have added a discussion on the expression dynamics of late germ-cell genes, including Dazl and Ddx4, and fetal oocyte genes in response to RA and BMP in the DISCUSSION section of the revised manuscript (p 13, “DISCUSSION” section, the third paragraph, in the revised manuscript).

• The interconnection of cultured germ cells into cyst-like structures could be further elucidated by immuno-staining the culture cells with an antibody against Tex14, which marks inter-cytoplasmic bridges connecting cysts of germ cells in fetal ovary (Greenbaum et al. 2009).

Response 3. In response to the Referee’s comment, we immuno-stained the cultured PGCLCs with RA and BMP2 at culture day 9 (c9) and an embryonic ovary at E15.5 by an anti-TEX14 antibody. As shown in Fig. 1F in the revised manuscript, the cells within the cyst-like structures exhibited TEX14-positive inter-cytoplasmic bridge-like structures highly similar to those among fetal oocytes, providing further evidence that the combined action of RA and BMP signaling leads cultured PGCLCs into the female pathway. We provided the relevant data and statements in the revised manuscript (Fig. 1F, p 5, “A system for analyzing the sex-determination mechanism of germ cells” section, the fifth paragraph, in the revised manuscript).

• In Figure 2 the authors show very nice staining for meiotic chromosomes in cultured germ cells. They observe the expected global down-regulation of gH2AX signal in pachytene as double strand breaks are repaired. Given that the cells used to induce germ cells fate are XY in origin, did the authors observe any examples of retained gH2AX on the un-synapsed sex chromosomes in these cells?

Response 4. In response to the Referee’s comment, we performed a spread analysis for male PGCLCs cultured with RA and BMP2 at c9, and as shown in Appendix Fig. S2E in the revised manuscript, we identified cells at the pachytene stage that appeared to retain the gH2AX signal on the un-synapsed sex chromosomes. We provided the relevant data and statements in the revised manuscript (Appendix Fig. S2E, p 13, “DISCUSSION” section, the fifth paragraph, in the revised manuscript).

• The RNA-Seq analysis of SK1 cells should be compared to published data (Soh et al. 2015) on Stra8-deficient embryonic ovaries. How many of the Stra8 dependent genes observed to be mis-expressed in SK1 cells also fail to be induced in Stra8 knockouts?

Response 5. In response to the Referee’s comment, we examined the expression of genes mis-regulated in Stra8-deficient embryonic ovaries reported by Soh et al. (Soh, Junker et al., 2015) in SK1 cells. As shown in Fig. EV5E in the revised manuscript, all twelve genes reported to be dependent on Stra8 were down-regulated in c9 SK1 cells. We provided this data and a relevant statement in the revised manuscript (Fig. EV5E, p 10, “Role of STRA8 in fetal primary oocyte development” section, the second paragraph, in the revised manuscript).

• The author propose a very interesting model for the development of germ cells, integrating extra-cellular signaling and DNA methylation. This model posits a developmental window (between E11.5-E13.5), when germ cells have lost their DNA methylation, but still do not express Stra8 or markers of female differentiation. A role for chromatin in this process seems likely, given the results of Yokobayashi et al. 2013. In this study Polycomb Repressive Complex 1 (PRC1) was found to be necessary to prevent precocious retinoic acid-dependent activation of Stra8 in fetal female germ cells. This work and the broader role of chromatin (which is covered in greater detail in the Ohta et al. manuscript, currently under consideration) in primordial germ cell like cells during the phase of DNA demethylation needs to be discussed in relation to the new findings reported in the current manuscript (assuming that the Ohta et al. manuscript would be published prior to or concurrently with this study). Microarray data from Yokobayashi et al. 2013 examining PRC1-dependent
Response 6. In response to the Referee’s comment, we analyzed the 524 genes aberrantly up-regulated in female germ cells deficient in Rnf2, a key component of PRC1, at E12.5 (Yokobayashi, Liang et al., 2013). In good agreement with the notion that PRC1 plays a key role in coordinating the timing of sexual differentiation of female germ cells, we found that while a majority of the 524 genes were those involved in basic cellular functions (“cell cycle”, “protein dephosphorylation”, etc.), as many as 97 genes (18.5%) were late germ-cell (Mael, Mov10l1, Tdrd7, Tex11, Tex14, etc.) or fetal oocyte genes (Stra8, Rec8, Sycp1, Sycp3, Smc1b, Hormad2, Sohlh2, etc.). We provided the relevant data and discussion in the revised manuscript (Appendix Fig. S2A, p 13, “DISCUSSION” section, the fourth paragraph, in the revised manuscript).

Referee #3:

In this study, Miyauchi et al have developed a system to direct PGC-LCs cultured in vitro (and derived from ESCs) towards female germ cell fate. They use reporter cell lines, immunofluorescence staining and transcriptome analysis to compare their cells developed in vitro (in the presence of BMP2 and retinoic acid) to PGCs ex-vivo. Interestingly, both XX and XY ESCs are capable of female germ cell specification.

This paper is obviously the product of a lot of work and the authors use a variety of techniques to verify their system. The authors do well to convince me that the system recapitulates the in vivo situation. The transcriptional analysis in particular is very good evidence that their system is working as they say.

Overall, this is an interesting study which will allow more thorough investigation into the mechanisms of female sex-determination in PGCs. It also highlights the important role of BMP signalling in PGC specification, and reveals that BMP signalling at different stages of PGC specification is required for the activation of different sets of genes.

Response 1. We would sincerely like to thank the Referee for the encouraging comments on our manuscript.

General comments on the paper:
The whole manuscript would benefit from some tightening up. Sometimes the point being made is not clear because of the language used. One of the most interesting points here is that BMP signalling is required in a different context in late PGC specification compared to early PGC specification. This point is a little bit lost in the text.

Response 2. In response to the Referee’s comment, we tightened up the manuscript where possible and re-edited the entire manuscript with the assistance of a professional English editing service. We also emphasized the differential function of BMP signaling between PGC specification and female germ-cell induction in the ABSTRACT of the revised manuscript. We sincerely hope that the revision will meet with the Referee’s approval.

The manuscript refers to one paper (Ohta et al, 2017) in several places, and yet this manuscript is still in revision and not yet published. In light of this, the authors could add further detail to their explanation of the expansion culture conditions in Fig 1A.

Response 3. The manuscript by Ohta et al., 2017 has now been published in the EMBO Journal (Ohta, Kurimoto et al., 2017), and we cited this work in the relevant passages of the revised manuscript. Moreover, according to the Referee’s comment, we elaborated the scheme for the expansion culture in Fig. 1A of the revised manuscript.

Issues for further consideration:
The authors base their entire manuscript around the requirement for BMP signalling to induce female germ cell specification, as opposed to other cytokines. Yet, they do not show anywhere in the manuscript that the BMP signalling pathway is actually active in the cells. They could do this either
by staining the cells for P-smad 1/5, or even just by mining their mRNA-seq data and showing that BMP pathway components and target readout genes are expressed. Similarly, they do not verify the specificity of the BMP inhibitor used by checking that the pathway is inactive. This could be done by similar means.

Response 4. We would like to thank the Referee for this comment. In response to the Referee’s suggestion, first, by Western blot analyses, we measured the level of phosphorylated (p) SMAD1/5/8 in d4c3 PGCLCs in response to the stimulation that induces the female germ cell fate, i.e., BMP2 and RA. As shown in Fig. EV3A in the revised manuscript, the stimulation by BMP2 and RA specifically elevated the pSMAD1/5/8 level, which was in turn blocked by the administration of LDN193189, which selectively inhibits the BMP signaling through the ALK2/3 receptors.

Second, by qPCR, we quantified the expression levels of Id1 and Id2, immediate targets of the BMP signaling (Hollnagel, Oehlmann et al., 1999, Korchnytskyi & ten Dijke, 2002, Lopez-Rovira, Chalaux et al., 2002), in d4c3 PGCLCs by BMP2 and RA, and found that both Id1 and Id2 were specifically up-regulated in response to BMP2 and RA, and their up-regulation was blocked by LDN (Fig. EV3B in the revised manuscript).

Third, we re-analyzed the RNA-seq data during the female germ cell specification in vivo and in vitro for the expression levels of key components of the BMP signaling pathway (Fig. EV4A). This analysis revealed the following. 1) Consistent with the UHC and PCA analyses (Fig. 4A, B), the expression profiles/dynamics of the components of the BMP signaling pathway are highly similar during the female germ cell specification in vivo and in vitro. 2) In vitro, genes such as Id1 and Id2 showed acute up-regulation in response to BMP2 and RA, but not RA alone, and critically, in vivo, they were sharply up-regulated between E11.5 and E12.5 in female, but not male, germ cells (Fig. EV4A in the revised manuscript). Collectively, these findings demonstrate that the BMP signaling is indeed active during the female germ cell specification in vivo and in vitro. We provided these data and relevant statements in the revised manuscript (Fig. EV3A, B, 4A, p 7, “BMP and RA commit PGCLCs to the female fate” section, the first paragraph, in the revised manuscript).

The authors base their study around the use of two reporter ESC lines which they generated (to report blimp1/mVenus (BV) and Stella-ECFP (SC) transgenes recapitulate the expression of Blimp1 and Stella, respectively, has already been described in our previous publications (Ohinata, Sano et al., 2008). The faithful expression of the Ddx4/mVH-RFP (VR) transgenes has also been reported previously (Imamura, Aoi et al., 2010). As shown in Fig. EV1C in the original manuscript (Fig. EV1C, D), we have verified that the Dazl-tTomato (DT) and Ddx4/mVH-RFP (VR) transgenes recapitulate the expression of Dazl and Ddx4, respectively, in that they initiate weak expression at around E11.5 and show robust expression after E12.5 both in the testes and ovaries. Note that the Dazl-tTomato transgenic mouse lines have been established from the BVSCDT ESCs.

To further verify the specific expression of DT and VR at a cellular level, we immuno-stained the sections of the embryonic gonads of DT and VR mice at E12.5/E13.5, which revealed specific expression of DT and VR in DDX4-positive germ cells (Fig. EV1E in the revised manuscript). Please also note that d4c7 PGCLCs, which bear a transcriptome similar to that of migrating PGCs (Fig. 4A, B) (Ohta et al., 2017), express BVSC robustly, but not DR or VR (Fig. EV1F, G in the revised manuscript).

These findings/data, together with the fact that the cells sorted based on reporter expression recapitulate the transcriptome dynamics of the female germ-cell specification pathway as appreciated by the Referee “The transcriptional analysis in particular is very good evidence that their system is working as they say”, demonstrate that the BVSCDT/VR reporters recapitulate the expression of their endogenous genes. We provided these data and relevant statements in the
revised manuscript (Fig. EV1, p 5, “A system for analyzing the sex-determination mechanism of germ cells” section, the second paragraph, in the revised manuscript).

It is not always clear in the manuscript why one ESC reporter cell line is used over the other for different experiments. Perhaps this could be clarified.

Response 6. We used BVSCDT cells for the first screening and used BVSCVR cells for the verification of the results. We subsequently used BVSCVR cells to evaluate the signaling requirements for the female germ-cell fate in a detailed fashion, since VR exhibits a more specific response to RA and BMPs. We used BVSC cells for the experiments involving IF analyses to secure one fluorescence channel. We explained these approaches in the revised manuscript (p 5, “A system for analyzing the sex-determination mechanism of germ cells” section, the third, fourth, fifth paragraphs, p 7, “BMP and RA commit PGCLCs to the female fate” section, the first paragraph, in the revised manuscript).

Specific points:
Figures 1 & 2: The FACs plots in these figures are very small, and this makes it more difficult to evaluate the data. The authors should consider re-organising their figures so these plots can be made larger.

Response 7. In response to the Referee’s comment, we provided larger FACS plots in Appendix Fig. S1 in the revised manuscript.

Some of the IF staining seems over-exposed. The authors should consider decreasing the image saturation.

Response 8. In response to the Referee’s comment, we re-acquired some of the IF images with an appropriate exposure and replaced the original images with them in the revised manuscript [Fig. 2B (left: BVSC channels), EV2C, EV3D, in the revised manuscript].

Figure 2F: Did the authors do the same FACs analysis on cell cycle stage in control conditions? How can we be sure it is a specific effect of this treatment and not just an effect of increased time in the expansion culture?

Response 9. We would like to thank the Referee for this comment. To address this, we performed a cell-cycle analysis of the PGCLCs cultured under the control condition, those cultured with RA, and those cultured with BMP2 and RA at culture day (c) 5, 7, and 9. As shown in Fig. 2F in the revised manuscript, consistent with our original data as well as with those shown for wild-type and Stra8 KO cells (Fig. 5C), a majority of the cells (~58.7%) at c9 cultured with BMP2 and RA were found to be in the G2/M phase (4C state), whereas less than 20% of the cells cultured under the control condition or with RA alone were in the G2/M phase. We provided these data and relevant statements in the revised manuscript (Fig. 2F, p 7, “BMP and RA commit PGCLCs to the female fate” section, the third paragraph, in the revised manuscript).

Figure 3A-C: The authors observe that BMP2 alone can induce female germ cell fate and conclude that this is because there is RA in the serum and KSR in the culture media. But isn’t there also BMP in serum? If so, why doesn’t RA alone work?

Response 10. We would like to thank the Referee for this comment. We added a passage explaining that the concentration of BMP present in the serum may have been too low (negligible) to induce the female fate (p 7, “BMP and RA commit PGCLCs to the female fate” section, the fourth paragraph, in the revised manuscript).

Figure 3E & H: Ddx4 is still there in BMP inhibited conditions?

Response 11. This might be because we provided the BMP inhibitor from E11.5 onwards, at which time the up-regulation of Ddx4 had already begun. Please also see Response 12 below.

Figure 3G: What happened to the germ cells of the male counterparts of the females in the litters exposed to the BMP inhibitor? These would be an important internal control group.
Response 12. In response to the Referee’s comment, we quantified the expression levels of key genes by qPCR in VR (+) cells administered LDN, as performed in the original manuscript. As shown in Fig. 3J in the revised manuscript, first, we found that the levels of VR expression in females, but not males, administered LDN appeared to decrease compared to those in the controls.

Second, consistent with the IF data that we showed in the original/revised manuscript (Fig. 3H, I), the expression levels of genes such as *Id1, Ddx4, Dazl, Syep3* and *Prdm9*, but, interestingly, not *Stra8*, appeared to be down-regulated in VR (+) cells in female embryos with LDN administration compared to those in the control mice (Fig. 3K). In contrast, the expression levels of genes such as *Ddx4, Dazl, Nanos2* and *Dnmt3l* did not show substantial change in VR-positive cells in male embryos with LDN administration (Fig. 3K in the revised manuscript). It is also of note that we did not detect expression of *Id1* and *Id2* in male VR (+) cells (Fig. 3K in the revised manuscript). These findings strongly suggest that the BMP signaling would be dispensable/not active in the male germ cell specification pathway. We provided these data and relevant statements in the revised manuscript (Fig. 3J, K, p 8, “BMP and RA commit PGCs to the female fate” section, the second paragraph, in the revised manuscript).

Figure 4: One panel showing the expression of BMP-related genes would be good supporting data, for in vitro and in vivo.

Response 13. Please see Response 4. We provided such a panel in Fig. EV4A of the revised manuscript.

Response 14. We now state that STRA8 is essential for triggering meiosis in the INTRODUCTION (the second paragraph), as well as in the sections entitled “BMP and RA commit PGCLCs to the female fate” (the second paragraph), and “Role of STRA8 in fetal primary oocyte development” (the first paragraph) in the revised manuscript.

Figure 7B: This schematic does not really seem to add anything we don't already know about methylation status as PGCs develop.

Response 15. According to the Referee’s comment, we provided a revised scheme that illustrates the findings of our study more clearly (Fig. 7A in the revised manuscript).

REFERENCES


Thank you for submitting your revised manuscript for consideration by The EMBO Journal, and your patience with our response. Your revised study was sent back to referees #2 and #3 for re-evaluation. Please note that while referee #3 was not able to look back into the manuscript this time, we have editorially assessed his/her criticism and found that all concerns were adequately addressed. Please find the comments of referee #2 enclosed below.

As you will see, referee #2 finds that his/her concerns have been sufficiently addressed and is in broadly favour of publication, pending minor revisions.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues on material & methods and formal formatting as outlined below, which need to be adjusted at re-submission.

REFEREE REPORT

Referee #2:

We again carefully looked at the paper and think that the paper is ready for acceptance. One minor point is that the "materials and methods" information related to figure 6E appears to us to be missing.

Response 1. We provided more detailed information related to Figure 6E in the legend to Figure 6E and in “Analysis of the DNA methylation of promoters” in “MATERIALS AND METHODS” in the revised 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.
- The 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=3, the individual data points from each experiment should be plotted and any statistical test employed should be justified.

Source data should be included to repart the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:
- A description of the experimental system investigated (e.g. cell line, species name).
- The assay(s) and methods used to carry out the reported observations and measurements.
- An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- An explicit mention of the biological and chemical entity(ies) that are measured.
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- Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

For all figures, the figure legend should be included in the methods section and/or with the source data.

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Corresponding Author Name: Mitomori Satou
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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

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For all figures, the figure legend should be included in the methods section and/or with the source data.

B- Statistics and general methods

1. a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

See figure, then legend, and “Materialand Methods”.

1. b. Did you perform more than one experiment and, if so, how was the data pooled?

See figure, then legend, and “Materialand Methods”.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

NA

3. Were any steps taken to minimize the effects of adaptive bias when analyzing animal samples or treatments (e.g. randomization procedure)? If yes, please describe.

NA

4. Are animal studies, include a statement about randomization even if no randomization was used.

NA

5. Were any steps taken to minimize the effects of adaptive bias during group allocation when arriving at (e.g. blocking of the investigator)? If yes, please describe.

NA

6. a. For animal studies, include a statement about blinding even if no blinding was done.

NA

6. b. For every figure, are statistical tests performed as appropriate?

See figure, then legend, and “Materialand Methods”.

7. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.

Within performing trend (two groups), we examined the conformity and equal variance of the samples tested. Normality was confirmed by Kolmogorov-Smirnov test and equality was confirmed by F-test.

8. a. Is there an estimate of variation within each group of data?

Described above

8. b. Is the variance similar between the groups that are being statistically compared?

Described above

C- Reagents

1. a. What antibodies were picked for use in the system under study? (Please provide antibody manufacturer, antibody number and/or clone name, if available)

See “Supplementary Table 1”, “Supplementary Table 2”, “Supplementary Table 3”, “Supplementary Table 4”, “Supplementary Table 5”, and “Supplementary Table 6” for antibody information.

1. b. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

See “Supplementary Table 1”, “Supplementary Table 2”, “Supplementary Table 3”, “Supplementary Table 4”, “Supplementary Table 5”, and “Supplementary Table 6” for cell line information.

D- Animal Models

1. a. What were the endpoints of the experiments in vivo and in vitro?

See “Supplementary Table 1”, “Supplementary Table 2”, “Supplementary Table 3”, “Supplementary Table 4”, “Supplementary Table 5”, and “Supplementary Table 6” for animal model information.
### E- Human Subjects

11. Identify the committee(s) approving the study protocol.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiment conforms to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability and/or on the use of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For phase I and II 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.

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.

### F- Data Accessibility

18. Provide a ‘Data Availability’ section at the end of the Materials and Methods. Listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq, Gene Expression Omnibus (GEO) GSE1942, EMBL/EBI with GEO accession numbers [see author guidelines for ‘Data Deposit’].

19. Data deposition in a public repository is mandatory for:
   - Proteins, DNA and RNA sequences
   - Macromolecular structures
   - Cryo-electron microscopy data
   - Functional genomics data
   - Proteomics and molecular interactions.

20. Deposition is strongly recommended for any datasets that are novel and integral to the study, please consult 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 author guidelines for ‘Data Deposit’).

21. 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 ethical issues. If practically possible and compatible with the individual consent agreement under the study, such data should be deposited in one of the major public access-controlled repositories such as BIORX forget (see link list at top right) or in a public repository (see link list at top right).

22. Computers and/or computer codes should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized formats (DDBJ, GenPept) 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 models 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.

### 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 (APHL/UKCDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

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*Please consult and follow ‘Reporting Guidelines’. Please confirm you have submitted this list.*