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Adaptive RSK-EphA2-GPRC5A signaling switch triggers chemotherapy resistance in ovarian cancer

Lidia Moyano-Galceran, Elina Pietilä, S. Pauliina Turunen, Sara Corvigno, Elisabet Hjerpe, Daria Bulanova, Ulrika Joneborg, Twana Alkasalias, Yuichiro Miki, Masakazu Yashiro, Anastasiya Chernenko, Joonas Jukonen, Madhurendra Singh, Hanna Dahlstrand, Joseph Carlson and Kaisa Lehti.

Editor: Jingyi Hou

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   16th September 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from 2 out of 3 reviewers whom we asked to evaluate your manuscript. Given that both reviewers provide similar recommendations, we prefer to make a decision now in order to avoid further delay in the process.

As you will see from the reports below, the referees acknowledge the potential interest of the study. However, they also raise a number of concerns regarding your work, which should be convincingly addressed in a major revision of the present manuscript. In particular, it will be important to strengthen the underlying mechanism of RSK inhibition and to examine the effect of RSKi in vivo (as recommended by referee #2) to improve conclusiveness and clarity.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

1. A good effort has been made to use patient-derived cells to validate the findings and the original observation was made with some primary cultures of patient-derived cells. However, as the EphA2 pathway can impact on cell-cell contact and ECM adhesion and the authors studied HGSC cells and fibroblasts independently, results co-cultures would have been interesting.

Referee #1 (Remarks for Author):

Overall this is a well-performed study and the results are of translational interest. The authors have validated some of their work in primary patient-derived cultures which is commendable.

As the EphA2 pathways alter malignant cell interactions with their microenvironment, the paper would be strengthened by studying the effects of cisplatin and the inhibitors in 3D co-cultures of

malignant cells and fibroblasts even if they only use the cell lines.

In the Discussion the authors suggest that as RSKi have not yet entered clinical trial and may have off-target or toxic effects, MEK inhibitors may be useful clinically in HGSC in combination with platinum compounds. Investigation of this possibility would strengthen the translational impact of the paper.

Were all the cell lines used validated as HGSC origin?

Referee #2 (Remarks for Author):

In this article, Moyano-Galceran L et al. investigated the effect of targeting RSK to overcome resistance to platinum-based therapy in high-grade serous ovarian cancers (HG-SOC). The current work provides new insight indicating that chemotherapy-induced RSK-EphA2-GPRC5A signaling switch is associated to resistance to the platinum and that pharmacological RSK inhibition prevented platinum-induced oncogenic EphA2-S897 phosphorylation and EphA2-GPRC5A co-regulation, and in combination with platinum can counteract HG-SOC drug resistance. Further studies should offer more definite insight into the specific mechanisms through which RSK inhibition might represent a possible novel therapeutic strategy for HG-SOC patients. These findings need to be strengthened by additional experiments.

1. The hypothesis that platinum treatment leads to EphA2 upregulation and EphA2-pS897 phosphorylation in patient-derived HG-SOC cells ex vivo and in HG-SOC cell lines is intriguing, but the data are insufficient to offer more definite insight into the specific mechanism that regulate oncogenic EphA2 phosphorylation switch by platinum chemotherapy in HG-SOC cells.

2. In order to evaluate the effect of platinum in HG-SOC cells, cell vitality experiments should be corroborated by apoptotis analysis (Annexin V, Cleaved caspase, etc). Similarly, in order to evaluate whether combination treatment of platinum with RSKi could affect key mechanisms that are important for HG-SOC cell survival, the authors should analyze apoptosis.

3. The authors should provide a better understanding of the supportive role of RSK-EphA2-GPRK5A in vivo. How does this work? Is this via a decrease in apoptosis (TUNEL) or an increase in proliferation rate (Ki67)?

4. The inhibitor studies by using the pharmacological RSK inhibition to demonstrate the relevance of RSK-EphA2-GPRC5A signaling, should be complemented by the use of RNAi approaches (knock-down of EphA2 or GPCR5A or RSK).

5. To demonstrate that targeting RSK could improve platinum therapy response, the authors should analyze the effect of RSKi in vivo, in HG-SOC xenografts and PDX, providing indication whether combination with platinum can counteract HG-SOC drug resistance. Similarly, as suggested in point 2-3, the combination of RSKi and platinum should be evaluated in terms of apoptosis.

6. Moreover what happens in the experiment where "sensitive" HG-SOC cells are used and EphA2 or GPCR5A or RSK are overexpressed? Would these GPCR5A overexpressing cells now be less sensitive to platinum? This experiment might add further support for targeting this signaling pathway in HG-SOC.

7. The acquisition of drug resistance can be explained in part by intrinsic properties of cancer cells, but could be dependent also by the tumor microenvironment (TME). In order to demonstrate that targeting RSK can disable critical signaling in HGSOC cells, but not alter cell vitality in cancer-associated fibroblasts (CAF), the authors should evaluate the heterogeneous expression of EphA2 or GPCR5A or RSK on tumor cells and CAF. Moreover, it should be interesting to evaluate host component effects in HG-SOC treated with RSKi in mono and combination therapy with platinum co-cultured in the absence and in the presence of CAF.

8. To better define GPRC5A as a predictive marker the authors should provide more details using larger cohort of platinum-sensitive and resistant HG-SOC patients. Moreover the authors should

evaluate the expression of RSK-EphA2-GPRC5A axis as predictor signature of poor platinum-based therapy responses and shorter survival in HG-SOC patients.

1st Revision - authors' response   16th January 2020

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

A good effort has been made to use patient-derived cells to validate the findings and the original observation was made with some primary cultures of patient-derived cells. However, as the EphA2 pathway can impact on cell-cell contact and ECM adhesion and the authors studied HGSC cells and fibroblasts independently, results co-cultures would have been interesting.

Response: We thank Reviewer #1 for the constructive comments and agree that co-cultures of HGSC cells and fibroblasts are relevant and of interest to test the impact of tumor microenvironment on the identified RSK-EphA2-GPRC5A signaling axis. To address this point, we have now established 3D co-culture models using OC patient-derived cancer associated fibroblasts (CAFs), and included the results from RSKi and platinum treatments of these co-culture experiments in the new **Fig 7** and **Appendix Fig S6** of revised manuscript. Please see more detailed description of the co-culture results below in the response to specific comment 1.

Referee #1 (Remarks for Author):

1. Overall this is a well-performed study and the results are of translational interest. The authors have validated some of their work in primary patient-derived cultures which is commendable. As the EphA2 pathways alter malignant cell interactions with their microenvironment, the paper would be strengthened by studying the effects of cisplatin and the inhibitors in 3D co-cultures of malignant cells and fibroblasts even if they only use the cell lines.

Response: We thank the reviewer for interest in our results and for this highly relevant suggestion to use co-cultures of cancer cells and fibroblasts. To study the effects of cisplatin and RSKi in OC-CAF co-culture, we first generated red fluorescent OVCAR8-RFP cells, and allowed them to form spheroids as mono- or co-cultures with green fluorescent BjhTERT-GFP fibroblast cell line in non-adherent conditions, followed by treatment with cisplatin and RSKi alone as well as in combination. Using RFP signal intensity at the starting point as well as after 48h and 72h of treatments as a measure of OC cell content, we validated the activity of RSKi, cisplatin and their combination in the 3D cell spheroids. In OVCAR8-RFP monocultures, single treatments with RSKi or cisplatin inhibited RFP signal relative to

untreated control, but most notably, combination treatment with RSKi and cisplatin reduced OC cells (RFP intensity) even relative to the treatment start, suggestive of effective cell killing. The combination treatment reduced OVCAR8-RPF most effectively also in co-culture with BjhTERT-GFP fibroblasts. Please see these results below in Figure 1 for review.

This data, although further supporting the efficacy of cisplatin-RSKi combination, did not allow us to separate apoptosis and proliferation. Moreover, the strong induction of OVCAR8 cell content by BjhTERT fibroblasts compared to monocultures appeared to contrast with the effect of OC patient-derived CAFs on OVCAR8 in our following experiments now included in the revised manuscript (see below). Therefore, we decided not to include this BjhTERT-OVCAR8 co-culture data in the manuscript. It is included here, however, to further support the suggestion that the cisplatin-RSKi combination can sensitize the cells to platinum-based therapy, as well as to show how differential impact different fibroblasts can have on the OC cells.
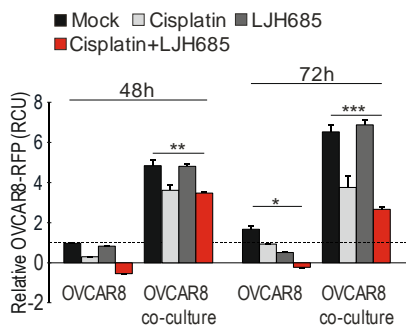
Figure 1 for Review. *Chart illustrates the relative changes in OVCAR8-RFP fluorescence signal over time in spheroid mono- and co-cultures with BjhTERT fibroblasts treated with cisplatin and LJH685 alone or as a combinatorial treatment (48 h and 72 h, N = 3).*

As suggested by both Reviewer#1 and #2, to analyze in a highly relevant 3D microenvironment the specific effects of platinum and RSKi treatments in OC apoptosis and proliferation, we prepared spheroids of OVCAR8-RFP and OC patient-derived CAFs under non-adherent conditions, embedded them in 3D collagen and allowed the cells to grow for 4 days. After subsequent treatments with cisplatin and RSKi alone and in combination for 20 h, apoptosis was assessed by immunofluorescence for cleaved caspase-3 (clCasp3). In OVCAR8-RFP 3D monocultures clCasp3/apoptosis was increased by cisplatin alone and by combining LJH685 with cisplatin (new **Fig 7A-B**).

In patient CAF monocultures, cisplatin alone or in combination with LJH685 did not affect clCasp3/apoptosis. Notably, in the 3D co-culture with CAFs, cisplatin or LJH685 alone also failed to enhance OVCAR8-RFP apoptosis significantly, whereas the combined LJH685-cisplatin treatment increased clCasp3 in OVCAR8-RFP (new **Fig 7A-B**; p = 0.022).

To validate these findings in an even more clinically relevant model, we generated co-cultures of patient-derived OCKI_p13 HGSC cells and OCKI_p22 CAFs and treated them as described above. Notably, combination of LJH685 with cisplatin further enhanced the platinum-induced cancer cell apoptosis (clCasp3) in the co-culture (p = 0.002). These results, indicating that in the relevant TME, RSKi-cisplatin combination can sensitize OC cells to platinum-based therapy, have now been included in the new **Fig 7A-D, Appendix Fig S6A-B** and described in Results on p. 12 of the revised manuscript.

2. In the Discussion the authors suggest that as RSKi have not yet entered clinical trial and may have off-target or toxic effects, MEK inhibitors may be useful clinically in HGSC in combination with platinum compounds. Investigation of this possibility would strengthen the translational impact of the paper.

Response: We appreciate the importance of providing experimental evidence to discuss and suggest such translational implications. In the originally submitted manuscript, we had only used MEKi UO126 (at a concentration of 10 µM), showing efficient inhibition of the cisplatin-induced EphA2-pS897 in TYK-nu cells (Figure 4E). To address this relevant point, we first investigated the *in vitro* efficacy of two additional MEKi, Refametinib and Trametinib, the latter being an FDA approved drug for solid tumors (1, 2). Both these MEKi showed good inhibition of the EphA2 oncogenic EphA2-pS897, while UO126 (now used at a lower concentration of 1 µM based on literature search) was inefficient, and thus not used for further experiments.

Please see new **Appendix Fig S3E-F** and Results on p. 10: "Coincident with ERK1/2-pT202/Y204 inhibition, Trametinib decreased viable OVCAR4 by over 40% compared to untreated control, whereas the relative cell cisplatin sensitivity remained unaltered". To further address this point, we examined the effects of Trametinib-carboplatin combination treatment in cell death (TUNEL) and proliferation (Ki67) in the model of OVCAR4 xenografts *in vivo*. Trametinib in combination with carboplatin did not induce more apoptosis or significantly reduce proliferation than carboplatin treatment alone.

These results have now been included in **Appendix Fig S3G-J** and described in the revised manuscript Results on p. 10. Altogether, this data suggests that the inhibition of broadly acting, proliferation-driving MEK-ERK1/2 pathway has a different mode of action that may not be directly relevant and related to the specific inhibition of the RSK-EphA2-GPRC5A axis affected by combinatorial treatment with RSKi and platinum. To address this consideration, we have now revised this aspect in Discussion in p. 17.

3. Were all the cell lines used validated as HGSC origin?

Response: This is an important question, since recent studies have brought into light striking findings on the use of cells that do not recapitulate the mutational landscape of specific OC subtypes, such is the case of HGSC: up to 90% of published studies using "HGSC" cell lines are actually based on cells that are TP53 wild-type and instead have characteristic mutations of other OC subtypes (3).

In this study we used TP53 mutant OVCAR3, OVCAR4, OVCAR8, TYK-nu and TYK-nu.R cell lines, from which OVCAR3, OVCAR4, TYK-nu and TYK-nu.R have been ranked based on the genetic alterations as highly likely HGSC, while OVCAR8 cell line has been described as likely HGSC (4). This reference and information have now been included in the cell line description in Materials and Methods on p. 19 of the revised manuscript. The HGSC patient-derived passaged cells OCKI_p01 - OCKI_p11 used in the experiments have also been analyzed by TP53 sequencing and tested for nutlin insensitivity as an indication of TP53 mutation status as shown in Appendix Fig S2C. The description of patient-derived cells has also been revised in Materials and Methods on p. 19-20 to clarify the presentation.

Referee #2 (Remarks for Author):

In this article, Moyano-Galceran L et al. investigated the effect of targeting RSK to overcome resistance to platinum-based therapy in high-grade serous ovarian cancers (HG-SOC). The current work provides new insight indicating that chemotherapy-induced RSK-EphA2-GPRC5A signaling switch is associated to resistance to the platinum and that pharmacological RSK inhibition prevented platinum-induced oncogenic EphA2-S897 phosphorylation and EphA2-GPRC5A co-regulation, and in combination with platinum can counteract HG-SOC drug resistance. Further studies should offer more definite insight into the specific mechanisms through which RSK inhibition might represent a possible novel therapeutic strategy for HG-SOC patients. These findings need to be strengthened by additional experiments.

Response: We thank Reviewer #2 for the interest in our manuscript and for the constructively critical comments that helped us to improve our manuscript. To better define the specific mechanism and strengthen the manuscript, we have performed various additional experiments during the revision, included the results and addressed the points as described below.

1. The hypothesis that platinum treatment leads to EphA2 upregulation and EphA2-pS897 phosphorylation in patient-derived HG-SOC cells ex vivo and in HG-SOC cell lines is intriguing, but the data are insufficient to offer more definite insight into the specific mechanism that regulate oncogenic EphA2 phosphorylation switch by platinum chemotherapy in HG-SOC cells.

Response: We thank the Reviewer for this constructive criticism, which motivated us to conduct several new experiments and allowed important improvements to the manuscript in terms of the specific mechanism underlying the oncogenic EphA2 phosphorylation switch linked to chemo-resistance in HGSC cells.

The following specific insights on these mechanisms have now been included in the revised version of our manuscript:

A) Clarified presentation of our original results showing that platinum induces ERK1/2-RSK pathway activation, which correlates with the EphA2-S897 phosphorylation (Figure 4H-I in the original and revised manuscript).

B) New results of siRNA experiments demonstrating that the depletion of specific activity of RSK2 in OVCAR8/4, as well as of RSK1 or RSK2 in TYK-nu and TYK-nu.R, will lead to the tumor suppressive EphA2 serine-to-tyrosine reversal (new **Fig 6D-F; new Fig EV3A-D** and described in the revised manuscript Results on p. 11-12). Silencing RSK2 specifically restored EphA2-pY588 in OVCAR4 and OVCAR8. This result indicates the

essential functions of these two RSKs in the platinum-induced oncogenic EphA2 switch.

C) New results of RSK1 and RSK2 siRNA experiments revealing an essential RSK1 function in the platinum-induced GPRC5A regulation in both the resistant OVCAR8 and TYK-nu.R, in association with apoptosis evasion, as indicated by PARP cleavage as well as reduced survival after RSK1 knockdown (new **Fig 6E-F**; new **Fig EV3**).

D) New results highlighting exclusive GPRC5A induction in HGSC cells, but not in corresponding tumor stroma or CAFs (new **Fig 8A-B; Appendix Fig S7A**).

The results C) and D) combined, i.e. the specific function of RSK1 in GPRC5A regulation coupled to cancer-specific GPRC5A induction, will help to explain the specific sensitization of the malignant cells to platinum-induced apoptosis, while stroma remains protected.

Platinum chemotherapy is known to induce oxidative stress/ROS-related ERK1/2 activation in different types of malignant and non-malignant cells (5). This notion has now also been included in the revised Discussion. In the originally submitted manuscript, we showed that both ERK1/2 and RSK were activated upon platinum treatment in our OC cell models (Figure 4H and I). Further, we showed that the EphA2-pS897 phosphorylation was repressed by pharmacological inhibition of either MEK-ERK1/2 axis or its downstream target RSK (RSK1-3 members/proteins inhibited by both the inhibitors; Figure 4E-I).

To clarify specifically the mechanism of the broadly acting ERK1/2-RSK axis in the EphA2 phosphorylation switch, we have now additionally assessed the effects of MEKi in OC platinum responses (new **Appendix Fig 3E-J**, see also response to comment 2 of Reviewer #1) and RSK expression of RSK1-4 in the OC cells used in the experiments (new **Fig EV3A**).

2. In order to evaluate the effect of platinum in HG-SOC cells, cell vitality experiments should be corroborated by apoptosis analysis (Annexin V, Cleaved caspase, etc). Similarly, in order to evaluate whether combination treatment of platinum with RSKi could affect key mechanisms that are important for HG-SOC cell survival, the authors should analyze apoptosis.

Response: We thank the reviewer for this excellent suggestion.

To evaluate the effect of platinum in HGSC cells, we assessed apoptosis (cleaved caspase-3; clCasp3) in patient-derived cancer cells cultured in 3D collagen. In this setting, cisplatin treatment significantly increased apoptosis (p = 0.022; See new **Fig EV1D-E**).

Further, to assess the effects of platinum alone or combined with RSKi on apoptosis in another highly relevant cell culture model, we stained for clCasp3 3D collagen OVCAR8-RFP spheroids treated with cisplatin alone or in combination with LJH685. When used alone, cisplatin treatment

induced apoptosis, while LJH685 alone had no significant effect on apoptosis. When used in combination, LJH685-cisplatin significantly induced apoptosis/clCasp3 (p = 0.002). Likewise, in a 3D co-culture model of patient-derived HGSC cells and CAFs, cisplatin- LJH685 combination further enhanced platinum-induced apoptosis. These results have now been included in new **Fig 7A-D, Appendix Fig S6A-B** and described in the revised manuscript Results p. 12.

To directly associate the RSK1/2 activities and EphA2-GPRC5A co-regulation to apoptosis and proliferation, we further detected cleaved PARP as a marker of apoptosis along with the proliferation marker PCNA by immunoblotting in RSK1/2 siRNA transfected TYK-nu.R. In the platinum-treated cells, siRNA-mediated RSK1 depletion led to GPRC5A suppression coincident with increased cleaved PARP (**Fig 6E**). Instead, RSK2 depletion increased cleaved PARP in the absence of cisplatin, whereas the proliferation marker PCNA was generally less affected by RSK1/2 knockdown, and even increased after cisplatin treatment in the resistant cells (**Fig 6E**).

Altogether, these results indicate that both platinum and RSKi-platinum combination treatments primarily decrease cell viability via increased apoptosis.

3. The authors should provide a better understanding of the supportive role of RSK-EphA2-GPRC5A in vivo. How does this work? Is this via a decrease in apoptosis (TUNEL) or an increase in proliferation rate (Ki67)?

Response: This is a valid point. To further address the role of RSK-EphA2-GPRC5A *in vivo*, we analyzed the OVCAR4 xenograft tumors presented in the originally submitted manuscript for EphA2, EphA2-pS897, GPRC5A, cleaved caspase-3 (clCasp3), TUNEL and Ki67. Carboplatin treatment increased EphA2, EphA2-pS897, clCasp3 and TUNEL, but did not alter Ki67. Notably, EphA2-pS897 and clCasp3 localized to different tumor cells and areas in the carboplatin treated tumors. Therefore, we conclude that the treatment-escaping HGSC cells activated oncogenic EphA2 signaling to evade apoptosis in response to platinum chemotherapy *in vivo*. These results have now been included in **Fig 3E-I, Fig EV2B-D** and described in Results p. 8.

Further, in two independent *in vivo* experiments, cisplatin treatment likewise induced apoptosis (detected by TUNEL) but had no major effect on proliferation (assessed by Ki67). In one of these experiments, BI-D1870 was used in combination with platinum for 48 h, significantly increasing TUNEL/apoptosis (2.5 ± 1.8 fold, p = 0.029) but not affecting Ki67/proliferation. These results (presented in **Fig 7E-H, Appendix Fig 3G-J**) suggest that both platinum and RSKi-platinum combination treatments decreased OC cell viability via increased apoptosis.

4. The inhibitor studies by using the pharmacological RSK inhibition to demonstrate the relevance of RSK-EphA2-GPRC5A signaling, should be complemented by the use of RNAi approaches (knock-down of EphA2 or GPCR5A or RSK).

Response: We thank the reviewer for this relevant suggestion. To further elucidate the RSK-EphA2-GPRC5A axis, we silenced RSK1 and/or RSK2 (the cancer-associated RSK proteins highly expressed in OC cells; see new Fig EV3A) in OC cells. As mentioned above, RSK1 and/or RSK2 depletion prevented the platinum-induced EphA2 phosphorylation switch in all OC cells. In OVCAR4 and OVCAR8, RSK2 silencing specifically restored EphA2-pY588, whereas RSK1 knockdown in the platinum resistant TYK-nu.R and OVCAR8 inhibited the increase of GPRC5A 46 KDa form and increased apoptosis as reflected by cleaved PARP (**Fig 6E**). These results have now been included in **Fig 6D-E**, the new **Fig EV3A-B** and described in the revised manuscript Results on p. 11-12.

In the originally submitted manuscript we had performed siRNA-mediated silencing of EphA2 and GPRC5A (see Appendix Figure S3C-D and S5A-C). We showed minor effect of siEphA2 on cell viability, which was consistent with our conclusion "Rather than blocking the entire signaling duality by EphA2 knockdown, the specific RSK-EphA2-pS897 inhibition and reversal to tumor-suppressive EphA2-pY588 correlated with the effective OC cell sensitization to platinum". We also noted an induction of GPRC5A upon siEphA2 as well as induction of EphA2 (total and pS897) upon siGPRC5A in TYK-nu cells. We have now clarified these results in manuscript p. 11.

5. To demonstrate that targeting RSK could improve platinum therapy response, the authors should analyze the effect of RSKi in vivo, in HG-SOC xenografts and PDX, providing indication whether combination with platinum can counteract HG-SOC drug resistance. Similarly, as suggested in point 2-3, the combination of RSKi and platinum should be evaluated in terms of apoptosis.

Response: To address this question, we conducted pilot experiments to test BI-D1870 in combination with carboplatin in two experimental set-ups with the same dosing (25 mg/kg i.p.): every two days for two weeks, or daily for 2 days. However, the longer dosing scheme of BI-D1870 in combination
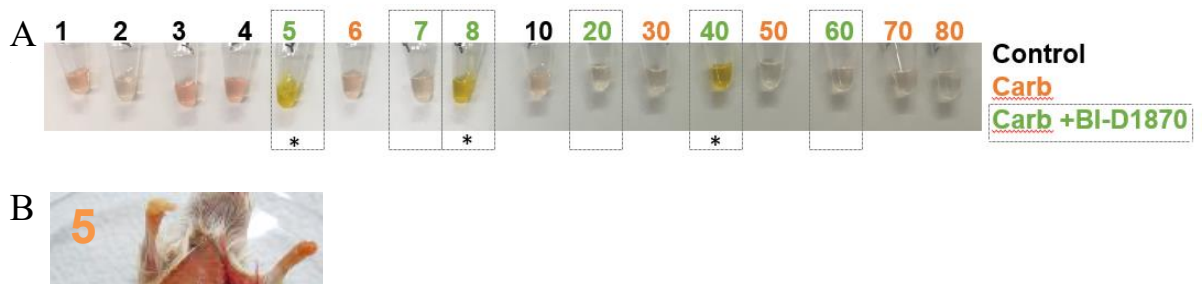
Figure 2 for Review. Manifestation of severe liver toxicity with BI-D1870 in combination with carboplatin. *A) Plasma samples of female SCID mice 14 days after the treatment start. Controls (black), carboplatin treated (orange), and carboplatin + 25 mg/kg BI-D1870 for seven times every two days (green, outlined). Note the yellowish shade of plasma (*) as a result of liver failure in 3/6 mice in this group. B) Icterus (#) of a mouse in the carboplatin + BI-D1870 group at sacrifice.*

with carboplatin resulted in severe liver toxicity, icterus, weight loss and increased red blood cell sedimentation rate in 50% of the treated mice (see below in Figure 2 for Review) forcing us to quit the dosing. From the shorter dosing scheme, we analyzed apoptosis by TUNEL staining, which revealed a significant induction of apoptosis compared to the control group (p = 0.029). We think that these results serve as convincing proof-of-principle for the mode of action by apoptosis induction. They have now been included in **Fig 7E-H** and described in the revised manuscript Results on p. 13.

To be successful in the *in vivo* tumor models, the inhibitor molecules need to entail well-optimized pharmacokinetic properties. To best of our knowledge, no good RSKi with favorable pharmaco-kinetic/-dynamic (PK/PD) characteristics *in vivo* has yet been developed. The commonly used *in vitro* inhibitors of RSK, LJH685 and BI-D1870, have both been preliminarily tested in PK/PD studies, showing poor drug stability, high clearance and short plasma half-life (6-9). Although the poor PK of RSKi could be overcome via comprehensive compound optimization, it is far beyond the scope of this study. However, as mentioned above, we conducted small RSKi pilot experiments in our orthotopic model of metastasized ovarian cancer in female SCID mice. The number of mice in these experimental groups was limited to 4-6, as we firmly think that the ethical 3R (replace, reduce, refine) principle governing all animal work should be the principal guideline when working with compounds with known suboptimal PK.

This 3R principle also guided our decision to not generate the PDX models suggested by the reviewer for treatment with these compounds. Moreover, the extensive efforts and time frames required for a PDX study would be out of the scope for the revision of this study and will, in our opinion, require another independent study.

6. Moreover what happens in the experiment where "sensitive" HG-SOC cells are used and EphA2 or GPCR5A or RSK are overexpressed? Would these GPCR5A overexpressing cells now be less sensitive to platinum? This experiment might add further support for targeting this signaling pathway in HG-SOC.

Response: To address this relevant point, we overexpressed RSK1/2, GPRC5A or EphA2 in cisplatin sensitive OVCAR4 and also in treatment-resistant OVCAR8 (only RSK1/2). Upon cisplatin treatment, the viability of the platinum sensitive OVCAR4 cells overexpressing RSK1/2 increased (5 µM cisplatin: RSK1_OE 106.0 ± 2.3 % and RSK2_OE 107.6 ± 6.1 % vs control 89.9 ± 4.9 %, p < 0.048). This increase in viability upon treatment was also seen in the more resistant OVCAR8 overexpressing RSK1. In OVCAR4 overexpressing GPRC5A or EphA2, viability was also increased when compared to treated control (GPRC5A OE at 10 µM cisplatin: 41.3 ±

0.3 % vs 2.5 ± 24.7 % in control, p < 0.002; increased viability after EphA2 OE at 5 µM cisplatin: 100.7 ± 0.3 % vs 84.6 ± 10.4 % in control, p < 0.029). These results have now been included in the new **Fig EV3C-F** and in Results p. 12.

7. The acquisition of drug resistance can be explained in part by intrinsic properties of cancer cells, but could be dependent also by the tumor microenvironment (TME). In order to demonstrate that targeting RSK can disable critical signaling in HGSOC cells, but not alter cell vitality in cancer-associated fibroblasts (CAF), the authors should evaluate the heterogeneous expression of EphA2 or GPCR5A or RSK on tumor cells and CAF. Moreover, it should be interesting to evaluate host component effects in HG-SOC treated with RSKi in mono and combination therapy with platinum co-cultured in the absence and in the presence of CAF.

Response: To address this interesting suggestion by the reviewer, we have now assessed the expression of RSK, EphA2 and GPRC5A in HGSC frozen tissue sections by immunofluorescence and in patient-derived HGSC cells and CAFs by immunoblotting. Immunofluorescence showed that RSK and EphA2 expression was higher in the cancer cells than in the stroma and that GPRC5A localized exclusively in the areas with cancer cells. Immunobloting for RSK, EphA2 and GPRC5A also showed that despite variable expression of these proteins in different patient-derived cancer cells, they were notably more expressed in cancer cells than in CAFs. These results have now been included in **Fig 8A-B, Appendix Fig S7A** and described in the revised manuscript Results on p. 13.

To assess the host component effect in HGSC cells apoptosis, we generated mono- and co-culture spheroids of OVCAR8-RFP/OCKI_p13 cancer cells and patient-derived CAFs and embedded them in 3D collagen. Please see the answer to Reviewer #1, question 1 for detailed description of these results.

We think that these results altogether have markedly improved the revised manuscript. Firstly, the cancer-specific GPRC5A induction, coupled with the direct link between RSK function and platinum induced GPRC5A regulation identified during revision when performing the siRNA experiments (**Fig 6, Fig EV3B**), provide a more specific mechanistic insight into the revised study. Secondly, our OC-CAF co-culture results highlight that even in a culture where CAFs seem to further inhibit OVCAR8 platinum response, the RSKi-cisplatin combination can induce apoptosis (new **Fig 7**).

8. To better define GPRC5A as a predictive marker the authors should provide more details using larger cohort of platinum-sensitive and resistant HG-SOC patients. Moreover the authors should evaluate the expression of RSK-EphA2-GPRC5A axis as predictor signature of poor platinum-based therapy responses and shorter survival in HG-SOC patients.

Response: We have now clarified the definition of platinum sensitive and resistant HGSC patients (N=136) according to clinical standards regarding the treatment (one should not consider platinum as single agent when defining patient groups, but rather group together patients with platinum single and double treatments). We have accordingly modified the Appendix Materials and Methods section in p. 17, **Fig 9E** and **Appendix Table S7** as well as the Results in p. 14.

Moreover, we have analyzed two independent HGSC cohorts (TCGA dataset for OC with N = 578, http://cancergenome.nih.gov/; GSE4997 dataset with N = 204, (10)) to validate the power of the RSK-EphA2-GPRC5A signaling axis as predictor signature. Survival analysis of the TCGA dataset validated our findings on GPRC5A association with worse overall survival in this case at the mRNA level. Moreover, survival analysis of the GSE49997 cohort further uncovered the potential of the combination of EphA2+GPRC5A mRNA expression as an approach to predict progression-free survival of the patients (p = 0.020 for EphA2+GPRC5A with and without RSK1 and RSK2). These results have now been included in **Fig 9F-H, Fig EV5** and described in the revised manuscript Results on p. 14-15.

References

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2nd Editorial Decision   30th January 2020

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the reviewer who was asked to re-assess it. As you will see the reviewer is now overall supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following amendments:

***** Reviewer's comments *****

Referee #2 (Remarks for Author):

The authors have presented a novel approach with mechanistic underpinnings of how to improve currently available platinum-based therapy for high-grade serous ovarian cancers. The authors have responded to all of the reviewers' comments including providing considerable additional data. I have no further concerns.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dr. Kaisa Lehti	
Journal Submitted to: EMBO molecular medicine	
Manuscript Number: EMM-2019-11177	

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Minimum number of technical and biological replicates was set to 3 based on researchers experience. For animal studies, the number of mice per treatment group was limited to 4-6 to minimize the number of animals used yet provide adequate statistical power. For patient studies, sample size was dependent on availability and increased during the revision to improve accuracy.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, the number of mice per treatment group was limited to 4-6 to minimize the number of animals used yet provide adequate statistical power.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For the first animal study, additional RSK inhibitor-treated group (N = 6) were excluded from the final analysis due to extensive liver toxicity. Mice were sacrificed on week 7. For the second animal study, spontaneous death or reaching humane endpoint during the treatment course was used as criteria to exclude individual mice. Analysis and data presented in the paper includes only the individuals surviving until the day of sacrifice.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice were randomly assigned into groups while simultaneously ensuring equal distribution of tumor burden (based on bioluminescent signal) within each group.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly assigned into groups while simultaneously ensuring equal distribution of tumor burden (based on bioluminescent signal) within each group.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Separate or partially separate group of researchers conduted the collection of the patient samples / preparation of TMA /animal studies to those researchers analyzing the results obtained from the experiments. Additionally, see the answers above.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No specific blinding was done (see statement about randomization).
5. For every figure, are statistical tests justified as appropriate?	Yes, for all statistics normal distribution was determined before choosing the appropriate method to determine statistical power. For numerical values, two-sided Student's t-test and Mann-Whitney U test were used. For survival analysis Log Rank test and Cox Regression were used. For correlation analyses Pearson Chi-square, gamma and kappa tests were used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Kolmogorov-Smirnov and Shapiro-Wilk normality tests together with histogram analysis were used for determining the distribution of data. When data did not meet the assumption of normal distribution, appropriate statistical test was used (specific method used for each assay is indicated in corresponding figure legend).
Is there an estimate of variation within each group of data?	Yes, SD was used.
Is the variance similar between the groups that are being statistically compared?	Yes in most cases, when not, unequal variance test was performed.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The antibodies used were as follows: primary antibodies against cleaved caspase-3 (Asp175 Alexa Fluor® 488 Conjugate, immunofluorescence (IF) 1:50), E-cadherin (24E10, #3195S, immunoblotting (WB) 1:1000), EphA2_pS897 (#6347, WB 1:750, IF 1:100), EphA2_pY588 (#12677, WB 1:750), ERK1/2 (#9107S, WB 1:500), ERK1/2_pT202/Y204 (#9101, WB 1:500), PARP (46D11, #9532, WB 1:1000), PCNA (D3H8P, #13110, WB 1:1000), RSK1 (D6D5, #8408, WB 1:1500), RSK2 (D21B2 XP, #5528, WB 1:1500), RSK1/2/3 (#9355, WB 1:750, IF 1:25) and RSK_pT359/S363 (#8753, WB 1:750) all from Cell Signaling Technologies. Primary antibodies from Santa Cruz Biotechnology against EphA2 (C-3, # sc-398832, WB 1:750), p53 (DO-1, # sc-126, IF 1:200), PDGFRβ (D6, # sc-374573, WB 1:500), Vimentin (V-9, # sc-6260, WB 1:2000, IF 1:100), β-actin (C-4, # sc-47778, WB 1:2000) and β-tubulin (D-10, # sc-5274, WB 1:500). Primary antibodies against CK7 (OV-TL 12/30, Invitrogen, MA5 11986, WB 1:1000, IF 1:100), EphA2 (ECD, R&D Systems, #AF3035, IF 1:100), FSP1 (AT1C3, LifeSpan Biosciences, LS-C755562, WB 1:750), GAPDH (Sigma, Atlas Antibodies, # G8795, WB 1:15000), GPRC5A (Sigma, Atlas Antibodies HPA007928, WB 1:1000, IF and immunohistochemistry (IHC) 1:250), Ki67 (Leica, Biosystems, #ACK02, IHC 1:200), N-cadherin (BD Transduction Laboratories, # 610920, WB 1:1000) and PAX8 (Proteintech, # 10336-1-AP, WB 1:2500, IF 1:100).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NIH:OVCAR3, OVCAR4 and OVCAR8 (National Cancer Institute, USA); TYK-nu and TYK-nu.R (Japanese Collection of Research Bioresources Cell Bank; Japan); ARN8 and CCL-137 (American Type Culture Collection; USA). Cells were routinely checked using MycoAlertPlus™ Mycoplasma Detection Kit (Lonza).

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mouse ICR-SCID, C.B-17,female, 6-8 weeks. Finland: IcrHan®Hsd-Prkdcscid, Envigo. Japan: Icricl-Prkdcscid, Japan CLEA. Housing in IVC (individually ventilated cages), 4-5 mice per EU-standard sized cage, nest boxes and material provided as enrichments, temperature 20-24C, RH 45-65%, 12-h light rhytm, aspen-bedding. Welfare checked daily by the animal facility personel and/or the study-conducting researchers.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Tumor xenograft experiments in mice were performed in compliance with ethical regulations for animal experiments and wellfare, and the studies were approved by the National Animal Experiment Board in Finland (ESAVI/8983/04.10.07/2015) and the Animal Experiment Board in Osaka City University in Japan (19001).
10. We recommend consulting the ARRIVE guidelines (see list list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Both the collection of fresh patient material and the use of tissue mircroarray were approved by The Swedish Ethical Review Agency (Etikprövningsmyndigheten; 2016/1197-31/1, 2016/2060-32, 2012/539-31/1).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Abdominal ascites fluid and omental tumors were collected from consented patients at the Karolinska University Hospital.The study was conducted according to the criteria set by the Declaration of Helsinki and the Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	No patient photos were included in this publication.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	All human patient data and samples were received and used as described in the ethical approval and written informed consent.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	No clinical trial data were included in this publication.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	No clinical trial data were included in this publication.
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.	This is to confirm that guidelines are being followed.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No data was generated in this study to be deposited in a public database .
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	This study will not fall under dual use research restrictions.