Author’s response to reviews

Title: Angiotensin II type 2 receptor signaling significantly attenuates growth of murine pancreatic carcinoma grafts in syngeneic mice

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Version: 2 Date: 1 September 2009

Author’s response to reviews: see over
August 31, 2009

Andrea Bucceri, Ph.D.
Scientific Editor
BMC-series Journals
BioMed Central
Floor 6, 236 Gray's Inn Road
London, WC1X 8HL

RE: Manuscript # 8449177322741726
Title: Angiotensin II type 2 receptor signaling significantly attenuates growth of murine pancreatic carcinoma grafts in syngeneic mice

Dear Dr. Bucceri,

Thank you very much for your kind comments on the above-referenced manuscript. We are very pleased that you and the reviewers have acknowledged the significance and novelty of our study and given us a chance to revise our manuscript.

We understand the concerns pointed out by the reviewers and agree with most of the reviewers’ comments. Therefore, we have modified the manuscript accordingly. Essentially, our modifications clarify the data presentations, insert additional data indicating significantly higher vessel density in AT2-KO than wild type mouse tumors, and correct a number of typographical and presentation errors. In addition, we have removed data relating to ERK ½ and associated statements throughout the manuscript in response to the comments by reviewer 1, 2 and 3. Although these modifications have further clarified the involvement of the AT2 receptor signaling in pancreatic cancer growth, our overall conclusions have not changed. A list of modifications and item-by-item responses is included in the bottom of the cover letter.

I hope this letter will clarify the modifications and that the revised manuscript will be approved for publication in BMC Cancer.

Thank you very much for your time and consideration.

Sincerely,

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Reviewer-1 report

Title: Angiotensin II type 2 receptor signaling significantly attenuates growth of murine pancreatic carcinoma grafts in syngeneic mice

Version: 1 Date: 31 May 2009

Reviewer: Diego F Calvisi

Reviewer's report:

Pancreatic cancer is one of the most aggressive and lethal human malignancies, characterized by a high rate of recurrence and resistance to conventional chemotherapeutic treatments. Thus, investigation of the molecular mechanisms leading to pancreatic tumor development and progression is required. In the present manuscript, Doi et al. assessed the effect of host angiotensin II (Ang II) type 2 receptor (AT2) expression on the growth of pancreatic carcinoma. For this purpose, the Authors have investigated the growth of mouse pancreatic ductal carcinoma grafts in syngeneic wild type and AT2 receptor–deficient (AT2-KO) mice. In particular, the Authors determined the role of AT2 receptor signaling in stromal cells on the growth of murine pancreatic carcinoma cells (PAN02) via in vitro and in vivo approaches. The results show that the growth of subcutaneously transplanted syngeneic xenografts of PAN02 mouse pancreatic ductal carcinoma cells was significantly faster in AT2-KO mice than in control wild-type mice. This was associated with higher cell immunoreactivity for Ki-67 and lower apoptosis in xenografts grown in AT2–KO mice than in wild-type mice. Furthermore, the growth of PAN02 cells was significantly decreased when grown with AT2 receptor gene-transfected wild-type and AT2-KO mouse-derived fibroblasts. The Authors conclude that Ang II might play a role in the growth of pancreatic carcinoma cells through modulating functions of host stromal cells. Furthermore, the present data indicate that Ang II AT2 receptor signaling is a negative regulator of pancreatic carcinoma cell growth.

The paper by Doi et al. is novel and provides new insights on the pathogenesis of human pancreatic cancer. The techniques used and statistical methods applied are appropriate. The main strength of the paper is the demonstration of a role of the AT2 receptor in pancreatic carcinogenesis and, consequently, its possible implication as a target for pancreatic prevention and treatment. However, the work appears incomplete and not conducted in depth.

Major Compulsory Revisions:

The paper investigates only superficially the molecular mechanisms responsible for pancreatic cancer cell growth following suppression of AT2 receptor expression. Although the Authors investigated the involvement of the ERK proteins, further work is certainly
required. For instance, the Authors should address whether the suppression of the AT2 receptor leads to the activation of the AT1 receptor (with an opposite, pro-oncogenic role) in their model of pancreatic cancer. This is a crucial question that requires to be answered. If the AT1 receptor is overexpressed following inactivation of AT2 receptor, the Authors should investigate whether AT1 overexpression results in activation of protooncogenes such as c-fos, c-jun, and c-myc, which are cited in the Discussion section but not investigated. Furthermore, the study of activation of the Ras cascade is limited to ERK1/2 in the present manuscript, whereas the Authors should investigate whether other effectors of Ras involved in cell proliferation, apoptosis, and/or angiogenesis (JNK, PI3K, p38MAPK, HIF-1alpha, VEGF-A) are also triggered by AT2 receptor downregulation in pancreatic cancer.

Response: We thank the reviewer for comments acknowledging the novelty of our study. We essentially agree with the reviewer’s critique regarding superficial exploration of the molecular mechanism by which AT2 expression regulates pancreatic cancer cell growth. We have considered possible receptor cross-talk and examined apoptosis and cell proliferation more than we have presented in the manuscript. However, since PAN02 cells do not express angiotensin II receptors (Table 1 and page 11, lines 4-3 from the bottom), PAN02 growth regulation by angiotensin II receptors appears to be stroma-dependent. This indicates that most suggested signaling components can be modified by angiotensin II receptor expression in stromal cells, but not in PAN02 cells. Indeed, other MAPK signaling components and apoptosis-related enzymes did not show any significant change in PAN02 cells in co-culture with fibroblasts (data not shown). As presented in the original figure 6, we were able to detect a mild change in Erk1/2 phosphorylation, but not other MAPK. Due to the statistically insignificant result, we have removed Figure 6 and statements relating to Erk1/2 phosphorylation throughout the manuscript. While we acknowledge that further studies are needed, our in vivo findings are interesting and we were able to confirm them in cell culture studies. Therefore, we would like to publish these findings and obtain some feedback from the readers at this stage.

Specific, additional concerns:

1.) The quality of Figures 2 (A,B), 3 (A,B), and 4 (A,B) is not appropriate and needs to be significantly improved. Higher magnification of the aforementioned Figures is recommended.

Response: As suggested, we have incorporated better quality figures in Figures 2-4 in the revised manuscript.

2.) Evaluation of microvasculature in tumors from AT2-wild type and AT2-KO mice should be performed using a more appropriate and objective (quantitative) method than the simple H&E staining. Authors should perform immunohistochemistry with an endothelial cell marker (such as CD34, for example) and count the microvessel density in AT2-wild type and AT2-KO tumors.

Response: As instructed, we have added tumor vessel densities of wild type and AT2-KO mouse tumors as determined by immunohistochemical analysis using a vascular endothelial
cell-specific anti-von Willebrand factor antibody (figure 4, page 12, last paragraph).

3.) In Figure 6, the difference in activated (phosphorylated) ERK1/2 levels between untreated and treated PAN02 cell is very small and cannot support the Authors’ conclusions.

Response: As pointed out, the ERK1/2 activation is mild. Therefore, we have removed this result and related statements throughout the manuscript.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests
Reviewer-2 report

Title: Angiotensin II type 2 receptor signaling significantly attenuates growth of murine pancreatic carcinoma grafts in syngeneic mice

Version: 1 Date: 4 June 2009

Reviewer: Anson Lowe

Reviewer's report:

Manuscript by Doi et al.

The manuscript by Doi et al. proposes that the angiotensin II receptor suppresses the growth of the pancreatic adenocarcinoma cell line, PAN02. The authors conclude that the effects on PAN02 are due to stromal derived AT2. Overall the effects on tumor growth and ERK phosphorylation are modest and not that convincing. One caveat, however, is that I was not able to interpret figure 5 at all, which is most likely due to a formatting error. Thus the data will need to be reexamined in the context of a corrected figure 5.

Major compulsory revisions:

1. The authors have previously published work on AT2 with respect to models of lung and colon cancer with apparent disparate results. The do not comment why AT2 appears to suppress tumor growth for pancreatic cancer cells and not for the previous two models where the opposite effect was found.

Response: Our previous two publications evaluated the effect of AT2 expression on chemical carcinogen-induced tumorigenesis in the colon and lung. Our conclusion in these studies was that AT2 expression stimulates tumorigenesis, which includes carcinogen metabolism and cell transformation in these models. The current study was to evaluate the effect of AT2 expression on the growth of pancreatic cancer cell xenografts; the research was focused only on the growth regulation of cancer cells. Our observations suggest that the AT2 expression attenuates the growth of pancreatic cancer cells. This conclusion was indirectly supported by other reports (described in page 16, line 2-4) and by our unpublished other studies). Therefore, our current study results and conclusion are not contradictory to our previous studies. To avoid confusion, we have added a brief statement regarding this issue in the revised manuscript (page 6, lines 4-8).

2. Figure 1 – How was the endpoint determined? Why is it that only the last time point shows a significant difference in size? The figure legend should note what is represented by the error bars. From the data, a significant difference was noted only for size and not weight at the same time point.

Response: Our end point determination was decided by tumor size. There is a strict Animal Care & Use regulation as to the maximal tumor diameter which may be reached before the experiment must be ended. Since wild type mouse tumor size was approaching the maximal size allowed, we ended tumor measurement. We agree with the reviewer that there is not
much difference between wild type and KO-mouse tumor size in the early stages of tumor growth; however, tumor size differences became obvious at the end point. This is a quite common growth pattern for transplanted tumors. Initially, the tumor cells rebuild an appropriate tumor microenvironment and tumor growth is generally slow; once tumor cells have generated appropriate environments, they show rapid growth. In the revised manuscript we have added information about the error bars in the Figures 1-6 legends.

3 Figure 3 – changes in the apoptotic index were not statistically significant. Thus the authors should not refer to this result as a positive result throughout the paper.

Response: We understand the reviewer’s comment. Although the results are not statistically significant due to the large variations, we think it will be worthwhile to retain the results in the manuscript. However, we have carefully avoided strong citations throughout the revised manuscript.

4 Figure 4 – It is difficult to assess vascular density with the images provided. The use of an objective criteria, such as immunocytochemistry with an endothelial marker would be preferred. This would also permit some form of quantitation.

Response: As suggested, we have analyzed tumor vascular density by counting vessels identified by immunostaining of vascular endothelial cells using an anti- von Willebrand factor antibody. A new figure (Fig. 4C and 4D) and accompanying statements (page 12, last paragraph) have been added in the revised manuscript.

5 Figure 5 – this is an important figure that was very difficult for me understand. I believe that there may be a major formatting error in the copy I received. The figure legend states that the bars are colored in the following order, black, grey, white, and dark grey; whereas I see black, grey, striped light pink, dark grey, and dark pink striped. To my eye, the following pairs of bars are colored the same and received the same treatment but have profoundly different OD (2&3, 6&7, 10&11, 14&15). It does not make sense. I cannot see the point the authors are trying to make. In addition, it is not clear how one distinguishes the cell growth due to the fibroblasts from the cancer cells? Although it is stated earlier in figure 1 in data not shown that PAN02 cells do not express AT2, I think it would be worth showing the data for both PAN02 and the fibroblasts.

Response: We apologize for poor presentation of these data. In the revised manuscript we have presented the figure very clearly. In addition, we have added a clear statement regarding AT2 expression in the pancreatic cancer cells and the fibroblasts we used (Table 1; page 11, lines 4-3 from the bottom).

6 The effects on ERK1/2 phosphorylation are modest. Are we to assume that the increase in ERK1/2 phosphorylation is derived from the PAN02 cells. Why can’t increase ERK phosphorylation be from the fibroblast where AT2 is presumably located?

Response: Although our Western blot samples were from PAN02 cells co-cultured with fibroblasts (PAN02:fibroblasts = 5:1), the majority of Western blot signals were derived from PAN02 cells. The amount of the proteins recovered from fibroblast alone culture (the
same number of cells were cultured in parallel) did not provide any readable Western blot bands. Therefore, the ERK1/2 phosphorylation detected in this experiment reflects changes in PAN02 cells. However, as pointed out, the ERK1/2 activation is mild and the difference between two groups is not statistically significant. Therefore, we have removed this result and related statements throughout manuscript.

**Minor Essential Revisions**

Figure 2 – It is difficult to evaluate the immunocytochemistry. I would suggest enlarging the image so that Ki-67 positive cells can more easily be detected by the reader.

**Response:** As suggested, we have added a better magnified picture in the revised manuscript; this will make detection of Ki-67 positive cells easier.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests.
Reviewer-3 report

**Title:** Angiotensin II type 2 receptor signaling significantly attenuates growth of murine pancreatic carcinoma grafts in syngeneic mice

**Version:** 1  **Date:** 5 June 2009

**Reviewer:** Marina Pasca di Magliano

**Reviewer's report:**

In the manuscript entitled “Angiotensin II type 2 receptor significantly attenuates growth of murine pancreatic carcinoma grafts in syngeneic mice” the authors explore the potential role of Angiotensin II type 2 receptor (hereby AT2) in the stroma in regulating the growth of subcutaneously transplanted pancreatic cancer cells. They use a pancreatic cancer line derived from C57/B16 mice, and inject it subcutaneously in syngenic mice that are either wild-type or knock-out for AT2; in addition, they conduct some in vitro experiments using the same cancer line and mouse skin fibroblasts.

The concept of tumor-associated fibroblasts promoting tumor growth is well established. We have, however, a limited understanding of how fibroblasts promote tumor growth, and therefore studies aimed at understanding the mechanism that mediate the tumor-promoting ability of components of the tumor stroma are interesting and have potential therapeutic implications.

**Major Compulsory revisions:**

I have some major concerns regarding the manuscript:

1) The relevance of AT2 to pancreatic cancer is hard to explain unless expression of this gene is shown in the disease. Moreover, expression of the gene should be demonstrated in the fibroblasts infiltrating the grafted tumor cells in the wild type mice. Failing that, it is hard to attribute the difference in tumor growth to knocking-out a gene that might not be expressed in the first place. This is particularly relevant since the authors mention in the introduction that the gene is expressed during embryogenesis but not in adult tissues.

**Response:** First we thank reviewer for acknowledging the significance of our research. Although we have stated that AT2 expression in PAN02 cells is undetectable by real time PCR in the original manuscript (page 11, lines 4-3 from the bottom), we have mistakenly omitted information regarding AT2 expression in the skin fibroblasts. Data on AT2 expression in these cells are now included in the revised manuscript (Table 1, page 11, lines 4-3 from the bottom). Although AT2 expression is shown to be very low in adult tissue in general, this receptor is inducible in several pathological conditions such as wounds (Steckelings *et al.* Br J Dermatol, 153:887-93, 2005) and cardiac hypertrophy (Lorrell *et al.* Am J Cardiol, 83:48H-52H, 1999). AT2 expression is also increased in some dietary conditions (Tamura *et al.* Can J Physiol Pharmacol, 78:548-56, 2000). Furthermore, AT2
expression is reported in rodent pancreatic ductal epithelium (Leung et al. J Endocrinol, 153:269-74, 1997) and is inducible under pathological conditions (Ulamov et al. A J Physiol, 296:G284-94, 2009). However, there is no report describing a change of AT2 expression in human pancreatic cancer. Based on this background information, we have conducted the present study. We have added this information in the introduction more clearly (page 6, line 4-8).

2) The system used to study tumor-stroma interaction is not ideal, as the tumor cells are transplanted subcutaneously. Subcutaneous fibroblasts are very different from pancreatic ones; moreover, in a subcutaneous location pancreatic cancer cells fail to elicit a desmoplastic reaction resembling primary tumors. A more relevant approach would be constituted by orthotopic transplantation of tumor cells. A second possibility would be to transplant pancreatic fibroblasts together with the tumor cells. In any case, as per point 1, the expression of AT2 should be evaluated in the different cell populations.

**Response:** We understand and agree with the reviewer’s comment that subcutaneous model is not the ideal model for studying pancreatic cancer. However, our primary aim was to find a potential relationship between AT2 receptor signaling and the growth of pancreatic cancer. Since PAN02 cells do not express AT2 but primary cultured skin fibroblasts do express AT2 (this statement is now in revised manuscript (page 11, lines 4-3 from the bottom), this subcutaneous model appears to be a good approach to evaluate the role of the AT2 in mouse studies. Since this first study with subcutaneous tumor model and skin fibroblasts provided us with promising results, we are going to use an orthotopic pancreatic cancer model using not only murine cells but also human pancreatic cancer cells in a follow up study, pending IACUC approval.

Similarly, the use of mouse skin fibroblasts is not likely to be relevant to pancreatic cancer; a more advisable approach would be conducting those experiments with pancreatic fibroblasts.

**Response:** Again we agree with the reviewer’s comment. However, as mentioned above, the primary aim of this study was to clarify the relationship between AT2 and pancreatic cancer. Since primary cultured skin fibroblasts do express AT2, and we used a subcutaneous model, we believe skin fibroblasts seem to be a natural choice for an interacting stroma.

3) The authors conclude that AT2 constitutes a promising new target in pancreatic cancer. However, AT2 appears to act as a suppressor of tumor growth: the authors should explain how they would use it as a therapeutic target. Moreover, given that the findings apply to a single pancreatic cancer cell line, and that the tumor histology in the manuscript do not resemble human pancreatic cancer, I think the authors should explain the limitations of their system.

**Response:** We agree with the reviewer’s comments regarding the limitations of our experiments and the resultant interpretation. Therefore, we have added a brief explanation regarding this issue (page 18, last paragraph in the discussion). In terms of a potential therapeutic translation, we are planning to use over-expression of AT2 for either treatment
or prevention of pancreatic cancer. Since AT2 over-expression in vasculatures do not cause phenotypical changes, AT2 over-expression by gene therapy appears to be a viable therapeutic choice.

Below are specific comments on the results:
1) The changes in tumor growth reported in figure 1A are only statistically significant at the last time-point. This data set is obtained by caliper measurements that are by their own nature not very accurate. The data in figure 1B, tumor weight at dissection, reflect only a marginal difference in tumor growth that might not be statistically relevant. Therefore, I am not convinced that loss of AT2 has an effect on tumor growth.

Response: Our end point determination was decided by tumor size and condition. There is a strict Animal Care & Use regulation as to the maximal tumor diameter which may be reached before an experiment must be ended (1.5 cm diameter or ulcerative tumors). Since wild type mouse tumor size was approaching the maximal size allowed, we ended tumor measurements. We agree with the reviewer that there is not much difference between wild type and KO-mouse tumor size in the early stages of the tumor growth; however, tumor size differences became obvious at the end point. This is a very common growth pattern for transplanted tumors. Initially, while the tumor cells rebuild an appropriate tumor microenvironment, tumor growth is generally slow; once tumor cells have generated appropriate environments, they show rapid growth. In Fig. 1B, tumor weight at dissection reflects approximately a 60% increase in tumor size in the AT2-KO mice. Because tumors sometimes fuse to skin, inaccuracies in tumor weights can occur. Although the differences in tumor weights here are not statistically significant due to large variation, the trend for larger tumors in AT2-KO mice corroborates the caliper data. We believe that these data taken together indicate that the loss of AT2 has an effect on tumor growth.

2) The data in figure 2 cannot be interpreted: the increase in proliferation cannot be attributed to a specific cell compartment in absence of a counterstaining: epithelial and fibroblast markers should be used to determine which cell population is affected.

Response: Generally, pancreatic tumors contain characteristically small amounts of stroma in the tumor tissue. PAN02 tumors follow this pattern: the majority of tumor tissue consists of tumor cells. Our analysis of Ki-67 positive cells, along with their morphology, indicates that all Ki -67 positive cells are tumor cells. Therefore, elimination of the possibility of fibroblasts or vascular endothelial cells appears to be unnecessary. However, for the sake of clarity of this issue, we have replaced pictures in Figs. 2 and 3 and added a brief explanation (page 12, first and second paragraphs).

3) The data in figure 3 is non statistically significant.

Response: Due to a large variation, the two groups are not significantly different. Accordingly, we have clearly stated in the revised manuscript that apoptosis is likely not involved in AT2-KO-induced faster tumor growth (page 13, second paragraph). Although these are negative data, we would like to leave it as it is.
4) In order to establish vascular density of the tumors in the different animal cohorts, the authors should either use in vivo imaging systems or quantify by FACS the endothelial cells in a representative and statistically adequate number of samples. The data in figure 4 does not support the conclusion that a different vascular density is observed in the different cohorts.

**Response:** Since we recognized a clear difference in the vasculature density between wild-type and KO-mouse tumors, we did not perform the immunohistochemical quantification initially. However, as suggested we have added data indicating vascular density differences (immunohistochemically stained vascular endothelial cells in tumors from wild type and AT2-KO mice) in the revised manuscript (page 12, last paragraph and Fig. 4).

5) The experimental design for this data set should be clarified, and the figure labeling should be improved to allow the reader to interpret the data. I was not able to interpret the data presented in the experiment. Are the authors showing that angiotensin 2 has a tumor promoting ability? And that AT2 is tumor-inhibiting? What is the effect of AT2 antagonist? In any case, cell proliferation is reported for both fibroblasts and tumor cells together; it might be more useful to sort the tumor cells or culture them in separate trans-wells, to be sure that the differences in proliferation can be attributed to the tumor cells.

**Response:** As suggested, we have clarified the data in the Figure 5 legend of the revised manuscript.

6) The authors hypothesize that AT2 influences tumor growth by affecting the level of phospho-Erk in the tumor cells. However, the data presented does not appear to reach statistical significance, and, in the western blot, the levels of total ERK appear to change as well as phospho-Erk; therefore, it is my impression that the conclusions are not adequately supported by the data.

**Response:** We agree with the reviewer that the changes in ERK1/2 phosphorylation are modest and the difference between two groups is very small. Therefore, we have removed this result and related statements throughout manuscript.

**Minor Essential Revisions:**

1) Abstract: The background section should explain why the authors hypothesize that AT2 might play a role in pancreatic cancer (is it expressed in the disease? In which cell types? Is it expressed in normal pancreas?)

The methods section should be written such that can be interpreted even without reading the whole manuscript – reference to specific staining performed could be removed, and replaced with a description of the two experimental settings used: a syngenic transplantation model, where pancreatic cancer cells are injected subcutaneously; and an in vitro co-culture of tumor cells with fibroblasts. The conclusions section of the abstract states that AT2 would constitute a therapeutic target; this should be explained, especially since the receptor appears to be a negative regulator of tumor growth.

**Response:** We have completed the necessary changes in the revised manuscript, as
suggested by the reviewer.

2) The background should be more specific, so that people not in the renin-angiotensin field can understand the relevance of the study. Page 4, end of the page: “AT2, (…), is primarily expressed in the mesenchyme of fetus limited in adult tissues” – this sentence is incomplete. Page 5 “Our previous study revealed… deficiency”: the study should be cited or explained – which organ system? Which carcinogen? How is it affected by AT2? More or less tumor growth?

Page 5, second paragraph: This needs to be explained better: which inhibitors have a role in cancer, how do they affect tumor growth – is AT2 a positive or negative regulator of cancer growth? The sentence “This suggests… role in cancer” does not logically follow the previous statements; this paragraph should be rewritten so that non-specialists can follow the logic behind it.

Response: We have modified the above points in the revised manuscript as suggested by the reviewer.

3) In the Results: the use of past tenses in the paragraph titles is somewhat unusual – in general, the paper would benefit from some text editing.

Response: We have edited the revised manuscript as instructed.

4) Each panel of each figure should be cited in the text – several are missing. The figures would benefit from more extensive labeling, figure 5 in particular.

Response: Necessary corrections have been incorporated in the revised manuscript.

Level of interest: An article of limited interest

Quality of written English: Not suitable for publication unless extensively edited

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests
Reviewer-4 report

Title: Angiotensin II type 2 receptor signaling significantly attenuates growth of murine pancreatic carcinoma grafts in syngeneic mice

Version: 1 Date: 8 June 2009

Reviewer: Martin Fernandez-Zapico

Reviewer's report:

The study by Doi et al evaluates the role angiotensin II type 2 receptor (AT2R) signaling on the growth of pancreatic tumors in a murine AT2R knockout (AT2R KO) syngeneic model. The authors found an increase growth rate in pancreatic tumor containing fibroblast from AT2R KO as compared to the wild type ones. The proliferation index was higher in the AT2RKO tumors and apoptosis levels were reduced in this group. Similarly, co-culture experiments using AT2R KO fibroblast show increased pancreatic cancer cell growth as compared to the groups cultured with wild type fibroblast. Together, these results led the authors to conclude that AT2R plays a role in pancreatic carcinogenesis by regulating the stromal compartment and it is a potentially important target for chemotherapy for this tumor.

This is a very interesting and novel study testing the role AT2R in pancreatic cancer stromal compartment. However, there are few small aspects of the manuscript that are still premature for publication. I would like the authors to address the comments posted below before considering the manuscript for publication. Previous reports have shown increase VEGF production by the ATR2 signaling activation and suggested an oncogenic function for this pathway. The authors should determine the vessel density by immunohistochemical methods and expression VEGF in the different experimental groups. A paper by Anandanadesan R et al suggested the activation of MAPK pathway by ATR2 in pancreatic cancer. Comments about these findings as well as the potential role as tumor suppressor or oncogene in these tumors should be added to the discussion. Finally, the effect of AT2R deficiency on the fibroblast function should be further characterized. For instance, what is the effect on fibroblasts proliferation? The H/E staining showed increased fibrosis. The authors should determine the fibrotic index by immunohistochemistry and discuss potential implications for pancreatic tumorigenesis.

Response: First, we would like to thank the reviewer for appreciating the novelty of our work. As suggested, we have studied vessel densities in tumors grown in wild type and AT2-KO mice and found significantly higher vessel densities in tumors from AT2-KO mice than in wild type mice. This result has been added in the revised manuscript (page 13, last paragraph and Fig. 4). In addition, we have added comments concerning the above mentioned paper as suggested by the reviewer (page 16, lines 2-4) although the paper by Anandanadesan et al. focuses on the AT1 receptor more than on the AT2 receptor. In terms of the function of tumor stromal fibroblasts, we would like to address this issue in the future.
However, since AT2 receptor expression has been shown in various tissue fibroblasts including skin, cardiac and vascular tissues, but not in the pancreatic cancer cells used here (Table 1; page 11, lines 4-3 from the bottom), it is reasonable to speculate that the AT2 signaling in stromal cells plays a role in the growth of pancreatic cancer in vivo. A brief discussion of this issue has been added in the revised manuscript (page 14, first paragraph in Discussion).

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests