Author's response to reviews

Title: Ionizing radiation induces tumor cell lysyl oxidase secretion

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Author's response to reviews: see over
Dear Prof. Pajonk,
Associate Editor of *BMC Cancer*

We would like to thank you and the reviewers for the valuable comments concerning the content of our manuscript entitled “Ionizing radiation induces tumor cell lysyl oxidase secretion”. Enclosed you will find the revised manuscript. We carefully considered the reviewers comments, responded to them as outlined and have uploaded a revised version of the manuscript.

The major changes in the revised version include (see detailed comments to reviewers below):

**Suppl. Fig. 2:** Analysis of LOX secretion in response to tumor cell irradiation in multiple tumor cell lines and at multiple time points.

**Suppl. Fig. 3/4:** Analysis of LOX transcription in response to tumor cell irradiation in multiple tumor cell lines and at multiple time points.

**Suppl. Fig. 5:** Quantification of LOX-immunostaining.

Besides smaller textual changes, we also extended the discussion section with the points requested by the reviewers and exchanged the order of the authors (agreed by all authors). We believe that the revised manuscript now fully addresses the concerns of the reviewers and that our manuscript is now acceptable for publication in BMC Cancer.

Sincerely Yours,

Martin Pruschi, PhD
(Corresponding author)
COMMENTS TO REVIEWERS:

Reviewer 1 (Franz Rödel):

We would like to thank this reviewer for his detailed input. Overall, this reviewer regards our manuscript as an interesting investigation in the field of molecular radiobiology but requests additional corrections:

Major Compulsory Revisions:

Point 1;2: The reviewer indicates that comparative results in an alternative cell line and time kinetics of both protein and mRNA expression of LOX would strengthen our data.

Answer to Point 1;2: We agree with this reviewer that additional data would further strengthen our already existing set of data. We therefore performed additional experiments and included these data into the revised version:

The revised manuscript now includes additional results on

a) IR-induced LOX secretion analyzed in additional cell lines (SW620 and A431) (Suppl. Fig. 2).

b) Time kinetics of LOX secretion in 3 cell lines (A549, SW620, A431) (Suppl. Fig. 2).

c) LOX mRNA expression in an additional cell line (SW620) and at multiple time points (Suppl. Fig. 3; Suppl. Fig. 4). Similar to our previous data in A549 cells, we observed that LOX mRNA-transcription was also not enhanced in this cell line – in response to IR. As positive/technical control, we determined IR-induced CDKN1A-expression.

Point 3: The reviewer asks to a) include the number of animals/group and the number of repeated experiments into the figure legend and b) to quantify histochemical staining.

Answer to Point 3: As requested by this reviewer we included a) the missing information and b) quantified the histochemical staining of LOX (Suppl. Figure 5).

Point 4: The reviewer asks to include a mock-control into our experimental set-up.

Answer to Point 4: Based on our own experience and also in comparison with other published siRNA-based experiments, we strongly believe that a siRNA-control sample directed against luciferase serves as a better control (mock-control) than just treatment with the transfection reagent alone. Luciferase is not expressed in the target cells and the mixture of “unspecific siRNA plus transfection reagent” is more equivalent to the mixture of “LOX-directed siRNA plus transfection reagent” than the “transfection reagent” alone.

Minor Essential Revisions:

Point 5/Answer Point 5: We agree with the reviewer and added the missing information on these cell lines to the method section in the revised manuscript.
**Point 6/Answer Point 6:** We extended the legends of the figures (or referred to the methods section) with the respective information on the statistical evaluation.

**Point 7:** The reviewer asks for more detailed verification of the statistical evaluation of data presented in Fig. 2.

**Answer to Point 7:** Fig. 2 shows absolute LOX levels secreted from the multiple cell lines investigated. Absolute levels of secreted LOX from both D341 and DAOY cells are very low, but are significantly upregulated in response to IR in these cells. The outcome of the statistical test depends on fold changes rather than absolute levels. Even though changes of absolute levels are difficult to be detected in this form (linear scaling of the y-axis), we nevertheless prefer to show absolute levels of secreted LOX – at least for Fig. 2. We thereby can also demonstrate that each tumor cell type has different basal levels of LOX secretion and may respond differently to varying doses of IR.

**Point 8/Answer to Point 8:** We thank the reviewer for this note and briefly discussed this concept in the discussion section.

**Point 9/Answer to Point 9:** We agree with the reviewer and a) removed this term and b) extended the section on LOX-immunostaining. We also quantified the histochemical staining of LOX as requested, see above (Suppl. Fig. 5).

**Point 10/Answer to Point 10:** We agree with the reviewer and added the missing information.

**Reviewer Nr. 2 (Peter Huber):**

We also would like to thank this reviewer for his input. Overall, this reviewer regards the topic of our manuscript as an interesting and important subject and describes it as a small and compact preclinical proof of concept paper. This reviewer does not ask for specific mandatory additional experiments or changes, but requests some additional explanations/change of wording to be integrated into the revised version.

We also agree with this reviewer that additional experiments probing the impact of LOX on clonogenic survival etc. would be of great interest. However, additional detailed experiments on these endpoints outreach the scope of this manuscript. However and as already mentioned in the original manuscript, we could not observe a correlation between IR-induced LOX levels and radiosensitivity in the cell lines investigated. In the revised manuscript, we rather consolidated our already obtained results on additional cell lines and at additional time points as requested by reviewer Nr. 1, at this stage of the project. The new set of data strongly corroborates our previous findings.

**Major changes in the revised manuscript related to Reviewer Nr. 2:**

**Point 1:** The reviewer questions the interpretation of our results as “new escape mechanism” after irradiation (as mentioned in the abstract and in the last section of the discussion part).

**Answer to Point 1:** We agree with the reviewer that “new escape-mechanism” might be an overly strong statement. But at the same time irradiation induces a
multi-fold stress response that includes the secretion of different factors that might promote tumor progression. LOX is known to play a dominant role in hypoxia-dependent cancer cell dissemination. As such, our novel data on IR-induced LOX-secretion intriguingly suggest that IR-induced LOX-secretion from tumor cells might promote an “escape-mechanism” on sublethally-irradiated cells. However, we agree with the reviewer that we did not perform sufficient mechanistic experiment to proof this statement. We therefore do not use the term “escape-mechanism” in the revised version. IR-induced secretion of LOX from tumor cells may contribute towards an IR-induced migratory phenotype and tumor progression, as e.g. previously identified for IR-induced matrix metalloproteinase activity.

**Point 2:** The reviewer asks to discuss our results in the light of the previously identified role of LOX and fibrosis.

**Answer to Point 2:** We have shortly addressed this aspect already in the original manuscript. We have now integrated additional comments on this topic into the discussion section of the revised manuscript. Overall, Cox et al. (Cancer Research, 2013, 73, 1721ff) previously demonstrated that (IR-induced) LOX-dependent collagen crosslinking creates a growth-permissive fibrotic microenvironment supporting metastatic growth. However, those results did not include experiments demonstrating IR-induced LOX-secretion from the tumor but from the normal tissue. Our new results now demonstrate that IR also induces LOX secretion from tumor cells and from the tumor (detectable in the murine blood). We agree with the reviewer that IR-induced LOX secretion from tumor cells and the tumor is part of an early response and might not affect distant fibrogenesis. Of note, we here did not observe an increase of murine LOX in the serum of mice not carrying tumor xenografts but still locoregionally irradiated (data not shown). We will further investigate these aspects as part of our planned in vivo studies using a disseminating tumor model to be irradiated with stereotactic body irradiation.

We believe that the revised manuscript now fully addresses the concerns of the reviewers and that our manuscript is now acceptable for publication in BMC Cancer.