Author's response to reviews

Title: Role of LPAR3, PKC and EGFR in LPA-induced cell migration in oral squamous carcinoma cells

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Author’s response to reviews: see over
Dear editor and reviewers!

Thank you for reviewing our manuscript and for giving valuable comments. We have now done additional laboratory, graphic and written work answering to all the referees’ comments.

Below is an itemized list of all the changes in the manuscript and figures. In the manuscript, additions are highlighted in yellow, and text to be removed is highlighted in red.

Reviewer 1’s (Hiroaki Niiro) comments:

Major compulsory revisions:

1. “Importance of LPAR3 in LPA-mediated cell migration would be a novel aspect in this paper. The conclusions were, however, mainly drawn from the results using the chemical inhibitor (i.e. Ki16425). Given the data showing that this inhibitor also partially blocks EGF effects (Fig. 4), another technique (e.g. silencing of LPAR3) should be employed to show more direct evidence for this conclusion.”

   Answer: First of all, we want to emphasize that our conclusion did not solely rely on the use of this inhibitor. In addition, we used OMPT, an LPAR3 agonist. The combined effect of the antagonist, the agonist and the protein expression of LPAR3 but not LPAR1 in the E10 cells made us draw the conclusion.

   We have now additionally performed siRNA silencing of LPAR3 in the E10 cells. Surprisingly, this did not give the same inhibition of LPA-induced migration as did the inhibitor. We therefore checked mRNA levels of LPAR1-3 with RT-qPCR. The mRNA level of LPAR3 was downregulated as expected upon siRNA silencing. However, the level of LPAR1 and 2 were at the same time dose-dependently upregulated. In addition, the LPAR1/3 antagonist was still able to reduce the LPA-induced migration in the LPAR3-silenced cells. These results may suggest that RNA silencing with a three days incubation time alters the natural relationship between the receptors, a condition that does not occur when an inhibitor is given short-time along with the stimulating agent. This fact might affect the outcome, in this case leading to the possibility of LPAR1-induced migration. The LPAR2-receptor agonist LP-105 could still not induce any migration, not even when given in combination with LPAR3 siRNA silencing and LPAR1/3 inhibitor.

   We have added the following paragraph to the Results section, highlighted in yellow in the
manuscript:

“To further examine the effect of LPAR3 on LPA-stimulated cell migration, we pretreated the cells with siRNA against LPAR3 (Figure 6). The LPAR3 siRNA did not block LPA-induced cell migration, but the migration was still inhibited by the use of LRAR1/3-inhibitor Ki16425 (Figure 6B). We then examined the expression of LPAR1 and LPAR2 upon the use of LPAR3 siRNA, and found that LPAR1 and 2 mRNA were upregulated while LPAR3 mRNA was (partly) downregulated (Figure 6A). This suggests that LPAR3 was not sufficiently suppressed and/or that LPAR1 may take over as an inducer of cell migration when LPAR3 has been downregulated. The effect of LPAR2 was examined using the specific agonist LP-105. Although we saw an upregulation of LPAR2 mRNA after LPAR3 silencing, this agonist did not induce migration, neither in the absence nor the presence of the concomitant use of LPAR1/3 inhibitor.”

In addition, this is added to the Discussion:

“However, the results could suggest that upon downregulation of LPAR3 with siRNA in the E10 cells, LPAR1 may substitute for LPAR3, but we have insufficient evidence for this.”

The results are shown in a new figure, named Figure 6.

2. “Although the authors previously identified critical signaling pathways of EGF/HGF-mediated migration of these cells, this issue remains elusive in the context of LPA-mediated migration. Which MAP kinase or PI3 kinase is more crucial? The finding that blockade of EGFR transactivation almost completely abrogates cell migration in E10 and SCC-9 cells would be a bit disappointing. Molecular dissection of EGFR-dependent and –independent pathways would strengthen the paper.”

Answer: To answer the question, we stimulated the E10 cells with LPA after treating with either MEK/ERK inhibitor, p38 inhibitor or PI3K inhibitor in the same manner as in our previous paper (Brusevold et al. 2012). For LPA-induced cell migration, all three pathways where crucial, as either would inhibit cell migration. Even though the p38 inhibitor inhibited less than the other two, the inhibition was still considerable. These results are added to Figure 7 (previous Figure 6), and a new paragraph is added on page 16, highlighted in yellow: “The importance of downstream kinase pathways in cell migration was studied. Blocking of the MEK/ERK kinase, the p38 kinase and the PI3 kinase with PD98059, SB203580, and LY294002 respectively, all inhibited the LPA-induced cell migration in the E10 cells (Figure 7B).”

3. “Statistical analysis should be carried out to help interpret the results.”

Answer: We here present the results of t-tests of some key experiments, with p-values in the figures and/or in the figure legends and manuscripts. The following paragraph is inserted to the Materials and methods section in the manuscript:

“Statistical analysis
Statistical analysis for the migration studies was performed using Sigmaplot 11.2 (Systat software, Inc., San Jose, CA, USA). Mean percent wound closure of groups was compared using t-test for normally distributed data and Mann-Whitney rank-sum test when data were not normally distributed. A difference was considered to be statistically significant where the corresponding p-value was ≤ 0.05. Exact p-values are given in figure legends. Densitrometric analyses of immunoblots were obtained with ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Data were expressed as mean ± SEM from independent
experiments and visualized with GraphPad Software. The statistical significance of differences was analysed by unpaired t-test using GraphPad Software.”

Minor essential revisions:

1. “Explanation of the discrepancy of LPAR expression at mRNA and protein levels would be addressed: levels of LPAR2 protein are much higher in E10 cells than SCC-9 cells (Fig. 2).” Our answer to that is that there is not necessarily a consistency between mRNA and protein levels. Studies have shown that there are more often no correlation than a positive correlation between mRNA and protein level. As for the expression levels of LPAR2 in our cells, the receptor does not seem to contribute to cell migration neither at high nor at low protein and mRNA levels.

2. “Previous papers showed that in addition to EGFR, HER2 is strongly tyrosine phosphorylated upon LPA stimulation in SCC-6 cells and gastric cancer cells (Cancer Res, 2002; BBRC, 2005). Is this not the case in this study?”
   Answer: We don’t think this happens in the SCC-9 cells, as there is no tyrosine phosphorylation at all in these cells after LPA stimulation. For comparison, we show western blots with EGF stimulation where tyrosine is strongly phosphorylated corresponding to pY1173EGFR. This is added to Figure 8, and the figure legends correspondingly. In addition, a sentence is changed in the results section, page 17, with changes highlighted in yellow.

3. “The detection of production of endogenous EGF-like proteins (e.g. amphiregulin, HB-EGF, TGFβ) might help confirm the requirement of EGFR transactivation in SCC-9 cells.”
   Answer: We measured the levels of amphiregulin, HB-EGF and TGF-alpha in the supernatants of LPA-stimulated SCC-9 cells. Detection with the ELISA method revealed that there was a low basal level of amphiregulin in these cells, which was not increased at 3 and 5 min, but slightly increased by 10 and 30 min stimulation with LPA as shown in the figure below. HB-EGF and TGF-alpha was hardly detected. The failure of these cells to respond with increased production of EGF-like ligands corresponds to the lack of EGFR transactivation. We will use
these results, along with further examination in a future study.

**SCC-9 cells**

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Control 3 min</th>
<th>LPA 3 min</th>
<th>LPA 5 min</th>
<th>LPA 10 min</th>
<th>LPA 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphiregulin</td>
<td></td>
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<td></td>
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<tr>
<td>HB-EGF</td>
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<tr>
<td>TGF-alpha</td>
<td></td>
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</tr>
</tbody>
</table>

Discretionary revisions:

1. "In Fig.1A, the data of E10 cells at 23 h are shown. The first paragraph in Results sections, however, describes the optimal time point of 24 h. Which time point is correct?"
   **Answer:** The correct time point is 24h. Figure 1 is corrected. 23h was erroneously stated in the figure since preliminary experiments were conducted with 23h observation time.

2. "GF109203X blocks activation of both conventional and novel PKCs (Fig. 6). More selective PKC inhibitor would help uncover a critical pathway for cell migration."
   **Answer:** We have not applied any PKC inhibitors in these experiments. It would of course be interesting to reveal more of the signaling pathways for LPA-induced migration, but this was not possible for us to do within this time frame.

3. "Akt is apparently constitutively phosphorylated in D2 cells compared with E10 and SCC-9 cells (Fig. 7 & 8). Is this a part of reason for spontaneous migration of D2 cells?"
   **Answer:** The constitutive Akt phosphorylation in the D2 cells might very well be an explanation for the spontaneous migration in these cells. Clearly other mechanisms also contribute, e.g. Met receptor is also constitutively activated. This might be studied further in later projects in our group. See also “question 2, discretionary revisions” of reviewer 2.

**Reviewer 2’s (Bryan Allen) comments:**

Major compulsory revisions:

1. "Provide E10 pAKT data after exposure to VPC."
   **Answer:** E10 pAKT data after exposure to VPC is added to figure 3A.
2. “Every Western series needs loading controls. For example, faint bands appear visible in the SCC-9 pEGFR gels outlining a possible bubble in the gel. The loading control will help clarify this.”

   **Answer:** Loading controls are inserted whenever missing. In addition, we apply Ponceau staining of every western gel, and gels with bubbles are excluded. The faint staining in the gel in the SCC-9 p-EGFR must be some kind of background smear, and not antibody binding. This is confirmed in several blots.

3. “Quantify Westerns using ImageJ or another program with supporting statistical analysis in order to make statements about the extent of Akt, ERK, p38 and EGFR phosphorylation inhibition.”

   **Answer:** Quantification and statistics are added, see answer to “comment 3 in Major compulsory revisions” from reviewer 1.

Minor essential revisions:

1. “Figure 4, Figure labels differ from figure legend. Stimulated with LPA for 0 or 1 min.”

   **Answer:** The differences between figure labels and legends in Figure 4 are corrected. Changes in the figure legends are highlighted in yellow.

2. “Specify how GF2, 5uM differs from GF2 either in the text or figure legend.”

   **Answer:** We’re sorry about the confusing labeling of this. The labels are corrected and specifications in the figure legends are made.

Discretionary revisions:

1. “Expose SCC-9 and D2 cells to varying concentrations of LPA”

   **Answer:** We have not done proper dose-curves, but found 10 µM of LPA to be working. Higher doses were toxic to the SCC-9 cells.

2. “Repeat SCC-9, E10, and D2 EGFR inhibition Western blot studies with cetuximab as described Figure 7. Will likely provide interesting clarification on D2 cells EGFR insight into the extracellular ligand binding site vs the intracellular kinase site.”

   **Answer:** We have added western blots with cetuximab for the D2 cells as they showed discrepant results with these inhibitors in the migration assays. The results show that Akt phosphorylation is not inhibited by cetuximab in these cells. The following sentence is added to the manuscript, highlighted in yellow: “Interestingly, cetuximab did not inhibit Akt in the D2 cells, which may reflect properties of the EGFR system in these cells and corresponds to the failure of cetuximab to inhibit migration in D2 cells.”

Sincerely,

Ingvild J. Brusevold