Author's response to reviews

Title: Gonadotropin-Releasing Hormone Receptor Activates GTPase RhoA and Inhibits Cell Invasion in the Breast Cancer Cell Line MDA-MB-231

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“Human Gonadotropin-releasing hormone receptor activates the GTPase RhoA and inhibits cell invasion in the breast cancer cell MDA-MB-231”

Answer to Referee 1

Major compulsory revisions:

1. As underlined by the authors, different breast cancer-derived cell lines express specific binding sites for GnRH and respond to GnRH agonists with reduced proliferation and metastatic behavior (references n. 20, 21). An antimetastatic effect of GnRH agonists has been shown to reduce metastasis formation by MDA-MB-231 breast cancer cells in vivo (reference n. 21). The results reported in this paper demonstrate that the GnRH agonist Buserelin inhibits the invasive potential of MDA-MB-231 breast cancer cells only when they are artificially forced to overexpress either the wild-type or the mutant GnRHR. In MDA-MB-231 cancer cells transfected with the empty vector, the GnRH agonist does not apparently affect the invasive behavior of the cells. These results should be confirmed by experiments performed in breast cancer cells expressing high levels of GnRHR in basal conditions (without the need to artificially overexpress these receptors). These data would improve the relevance of the paper.

Answer:
Low levels of GnRHR expression in breast cancer cell lines have been previously documented in many cell lines (eg. Morgan K et al. BMC Cancer. 2011 Nov 3;11:476); this particular feature has hampered the analysis of the GnRHR-mediated signaling pathways involved in cell proliferation and invasiveness. Further it is not known whether the phenotype of breast cancer tissues changes with density of the GnRHR. Thus overexpression via transfection has become a useful tool to overcome this problem. Over-expressed receptors will then mimic what actually might potentially occur in breast tumor tissues naturally expressing high GnRHR levels (Everest HM et al. Endocrinology 2001, 142:4663-4672). Our data showed lower levels of functional endogenous receptor in MDA cells and higher levels of functional receptor in MDA cells transfected with either WT GnRHR or GnRHR-DesK191. Only by employing this procedure, we were able to measure the effect of Buserelin-stimulation on cytoskeleton regulation, antimigratory behavior, and cell adhesion in this highly invasive cell line. Several lines discussing this particular issue were added to the “Discussion” section of the revised ms (PAGE 19; LINES 10 TO 19.

2. Abstract, Conclusions, third sentence: ‘These observations offer new insights into the molecular mechanisms whereby this malignant cell line expresses its invasion potential’. According to the data reported in the paper, the GnRH agonist does not efficiently affect the invasive behavior of MDA-MB-231 cells in basal conditions (i.e., when transfected with an empty vector). These malignant cells
need to be transfected with the GnRHR in order to respond to the GnRH agonist with a decreased invasive behavior. Thus, in line with comment 1), this sentence does not seem to be supported by the results here described.

Answer:

In the section “Abstract/Conclusion”, the sentence “These observations offer new insights into the molecular mechanisms whereby this malignant cell line expresses its invasion potential” was replaced with “These observations offer new insights into the molecular mechanisms whereby activation of overexpressed GnRHRs affects cell invasion potential of this malignant cell line, and provide opportunities for designing mechanism-based adjuvant therapies for breast cancer” (PAGE 4, lines 5-9).

3. Which is the effect of the GnRH agonist on the proliferation of MDA-MB-231 breast cancer cells, transfected with either the empty vector or with the wild-type/mutant GnRH receptors?

Answer:

The primary aim of the present study was to determine the mechanisms subserving the effect of GnRHR activation on cell migration, rather than on cell proliferation, given that a number of previous reports have already analyzed in some detail this latter issue. In fact, some of these studies have shown that GnRH decrease cell proliferation. We are currently assessing the effects of several GnRH analogs on both cell invasion and proliferation using our overexpression model of the GnRHR DesK191 and hope to have new data on this interesting issue in the near future.

4. Radioligand binding assays: which is the value of the binding affinity constant for the overexpressed GnRH receptors?

Answer:

The binding affinity data (Ki) were added to the “Results” section in the revised version of the manuscript (PAGE 14; LINES 14 TO 18).

5. In MDA-MB-231 breast cancer cells overexpressing either the wild-type or the mutant GnRHR, Buserelin stimulates intracellular production of IP3. According to the literature, a Galphai protein also seems to be deeply involved in the direct antitumor activity of GnRH analogs (Limonta et al., Endocrinology 1999, 140:5250-5256; Imai and Tamaya, Vitam Horm 2000, 59:1-33; Grundker et al., Endocrinology 2001, 142:2369-2380). This point should be addressed in the ‘Discussion’.
Answer:

This is an interesting point. Previous reports (Limonta P et al. Endocrinology 1999, 140(11):5250-5256; Maudsley S et al. Cancer Res 2004, 64(20):7533-7544; Grundker C et al. Endocrinology 2001, 142(6):2369-2380) have shown that, the GnRHR is coupled to Gαi in different reproductive tumors, but not in breast neoplastic tissue. In fact it has been shown that in several extrapituitary tissues, including neoplastic tissue, this receptor is coupled to Gq/11 (Everest HM et al. Endocrinology 2001, 142:4663-4672). Nevertheless, we can not rule out the possibility that the GnRHR might also couple to other G proteins, depending mainly on the cell context and the particular GnRH analog employed. In our hands, high doses of GnRHa did not modify basal levels of cAMP as a surrogate marker for Gαi coupling. A paragraph discussing this issue has been added to the “Discussion” section of the revised manuscript (PAGE 19; LINES 21 TO 25 and PAGE 20; LINES 1 TO 7).

Minor points:

1) Methods, Constructions, second sentence: 'As previously shown [60]'.
Reference n. 60 does not exist in the 'References' section.

In the “Methods/Constructions” section, reference 60 was checked and added as reference number 31 (PAGE 8; LINE 7)

The authors thank this reviewer for her important observations.
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Answers to Referee 2

Although these engineered cell lines provide a consistent story, it is still unclear how readily non-engineered (ie non-GnRHR transfected) models with lower levels of GnRHR receptor might be modulated by this GnRHR activation. It could be argued though that this model might reflect those breast cancers with the highest levels of GnRHR expression.

Answer:

This is an important point.

Low levels of GnRHR expression in breast cancer cell lines have been previously documented in many cell lines (eg. Morgan K et al. BMC Cancer. 2011 Nov 3;11:476); this particular feature has hampered the analysis of the GnRHR-mediated signaling pathways involved in cell proliferation and invasiveness. Further it is not known whether the phenotype of breast cancer tissues changes with density of the GnRHR. Thus overexpression via transfection has become a useful tool to overcome this problem. Over-expressed receptors will then mimic what actually might potentially occur in breast tumor tissues naturally expressing high GnRHR levels or changing phenotypes during invasion. Only by employing this procedure, we were able to measure the effect of Buserelin-stimulation on cytoskeleton regulation, antimigratory behavior, and cell adhesion in this highly invasive cell line. Several lines discussing this particular issue were added to the “Discussion” section of the revised ms (PAGE 19; LINES 10 TO 19. The final statement of the Abstract (Conclusions) was also changed, accordingly.

Minor essential revisions:

-Methods: Page 8: Constructions: Reference 60? The authors state “As previously shown [60], the GnRHR-DesK191 is expressed at higher levels compared to the WT receptor.” Could the authors please correct this reference (there is no reference 60) and provide within this manuscript some data to indicate the relative levels of receptor.

The reference was modified and added to the manuscript as reference number 31. PAGE 8; LINE 7. In the “Results” section, PAGE 14; LINES 11-21, lines were added indicating the Ki values for [125I]-Buserelin of the endogenous and transfected receptors. Relative levels were only measured by radioligand binding assays.
- The source of buserelin should be indicated.

The source of Buserelin is now indicated in the reviewed version. PAGE 9, LINE 15.

- "inhibits" rather than "inhibit" in the title

“Inhibit” was replaced with “inhibits” in the title.

- Page 18. Line 1 “finding “ should be “a finding”

“finding” was replaced by “a finding” PAGE 18, LINE 7.


“parallely” was changed by “paralleled”, PAGE 20, LINE 20

The authors thank this reviewer for his interesting observations.