Author’s response to reviews

**Title:** Expression of integrin alpha3beta1 and cyclooxygenase-2 (COX2) are positively correlated in human breast cancer

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**Author’s response to reviews:** see over
May 15, 2014

Dear Editor,

Herein we submit our revised manuscript entitled “Expression of integrin α3β1 and cyclooxygenase-2 (COX2) are positively correlated in human breast cancer” (MS:2095327853117939; Anshu Aggarwal et al.). Included in this coverletter is our point-by-point response to the reviewers’ comments. We thank the reviewers for their excellent comments and suggestions. We have revised our manuscript in accordance with the concerns that were raised, which we think has strengthened the study. Revisions are underlined in the manuscript text. Please note that we have also added an additional author, Dr. Anupam Batra, who assisted in the completion of new experiments that were requested by Reviewer #2.

We thank you for your consideration, and we hope that you find our revised manuscript to be suitable for publication in BMC Cancer.

Yours sincerely,

C. Michael DiPersio, Ph.D.
Professor, Center for Cell Biology & Cancer Research
Response to Reviewer Concerns

Aggarwal et al., MS:2095327853117939

REVIEWER 1:

1. Reviewer Comment: “Recently, molecular mechanism of upregulation of alpha3 integrin and its possible involvement in EMT have been reported in FEBS J (2012 279,p4535) and Cancer Sci (2013 104,p1189), respectively. Thus, the author should discuss on them in “Discussion” section.”

Response: We thank the reviewer for pointing out these two studies, which are now cited and discussed in the revised manuscript.

REVIEWER 2:

1. Reviewer Comment: “The positive correlation between α3 and COX2 expression was found in both normal breast tissue and breast tumor tissues of different grade and cancer stage. Therefore the clinical relevance of the pro-tumorigenic and pro-metastatic function of α3β1-dependent regulation of cox2 gene expression previously found in a breast cancer cell line remains uncertain. This might be due to the broad heterogeneity of breast cancers and to the limited number of samples analysed in this study. However, one particularly striking function reported for α3β1 integrin in this context is its paracrine induction of angiogenesis. Therefore, to strengthen the potential physiological role of α3β1-dependent regulation of cox2 gene expression in promoting tumor growth, the authors should analyse potential correlation between α3 and/or COX2 expression and elevated vessel density in these clinical samples.”

Response: We agree that this is an important question to address with regard to linking the current findings to our previous studies, and we thank the reviewer for suggesting it. To address this question, we performed immunostaining of tumor samples with anti-Von Willebrand factor (anti-vWF) to stain blood vessels, then quantified and compared blood vessel density across samples of varying α3 expression. While we observed an overall trend of elevated blood vessel density with increased expression of α3, one-way ANOVA did not reveal a statistically significant difference in blood vessel density among the groups, possibly due to limited sample size of the groups (see new Fig. 3 of the revised manuscript).

2. Reviewer Comment: “The authors preface their study in the introduction by stating that a number of studies have shown that α3β1 promotes tumor growth, invasion and/or metastasis of breast cancer or other carcinoma cells. However, a number of recently published studies illustrating this statement have been omitted and should be added to reinforce this statement (for example Shirakihara et al., Cancer Sci. 2013; Zhou et al., Mol Cancer Res. 2013; Cagnet et al., Oncogene 2013). These studies should also be included into the discussion.”

Response: We thank the reviewer for pointing out these recent studies, which we now cite and discuss in the revised manuscript.

3. Reviewer Comment: “The authors describe a staining for α3 observed primarily in the cytoplasm of tumor cells. This observation does not fit with integrin α3β1 being a cell surface receptor. The authors should provide explanations for such a cellular localization of α3β1 in tumor cells.”

Response: We now provide a brief explanation for this staining pattern in the text. Although the α3β1 integrin functions as an obligate heterodimer from its position on the cell surface, detection of the α3 subunit is not restricted to the cell surface in epithelial and tumor cells, especially when its expression...
levels are high. Presumably, cytoplasmic staining includes the pool of α3 subunit that has not yet reached the cell surface, and may also include that which has been internalized (some integrins are known to be recycled). Importantly, the immunohistochemical methods that we used will detect all subcellular pools of α3. Consistently, we and others have observed similar cytoplasmic staining patterns for α3, or other integrin subunits, in sections of breast or other tumors (for examples, please see Shirakihara et al., Cancer Sci (2013) 104: 1189–1197; Moreno et al., Histol Histopathol (2002) 17: 347-352; Hashida et al., Int J Cancer (2002) 97: 518–525; Morodo et al., Oral Diseases (2004) 10: 277–282), as well as in epidermis (see figure provided for Reviewer 3, below).

**Reviewer 3:**

1. **Reviewer Comment:** “Given that α3β1 is a cell surface receptor, it was surprising that the staining for α3 was cytoplasmic and no evidence for membrane staining was obvious in the sections shown in Figs 1A and 2A. The authors should address this inconsistency. What is the evidence for the specificity of the α3 antibody that was used for staining? Evidence that the antibody is specific for the α3 subunit under the conditions used for fixation and staining of the tissue sections is necessary and would strengthen confidence in the conclusions of the manuscript.”

**Response:** Regarding the cytoplasmic staining, please see our above response to Reviewer 2, comment 3. The specificity of the antibody is an important point, especially since cross-reactivity of anti-α3 antibodies with the α6 integrin subunit was a possibility. Indeed, for these studies we used a rabbit polyclonal antibody that was raised against the α3 cytoplasmic domain, which shows some homology with the α6 cytoplasmic domain, and it is well known that some anti-α3 antibodies can cross-react with α6. The specificity of the anti-α3 serum that we used in our study has been demonstrated in previous studies from our group by western blot, immunoprecipitation, and immunohistochemistry of frozen tissue sections (i.e., DiPersio et al., J Cell Sci (2000) 113: 2909-2921; Mitchell et al., J Cell Sci (2009) 122: 1778-1787). Nevertheless, as the reviewer points out it was important to confirm that the anti-α3 antibody is specific under conditions of tissue fixation and staining that we used in the current study. Therefore, we prepared paraffin sections of neonatal epidermis from wildtype or α3-knockout mice and treated them with the same antigen-retrieval and staining protocols that were used in the current study. Below we have included a reviewer figure showing that the anti-α3 antibody stains basal keratinocytes of epidermis, as expected, while the corresponding preimmune serum produces only background staining. Importantly, identical treatment of α3-knockout epidermis with the anti-α3 serum produced only background staining, failing to detect α3 in the basal keratinocytes. Since α3-null epidermis expresses α6 integrin in the basal keratinocytes (DiPersio et al., J Cell Sci (2000) 113: 3051-3062), these results indicate that the α3 antibody does not detectably cross-react with α6 or other proteins. We have added a short statement to the methods section regarding confirmed specificity of the anti-α3 antibody.

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<tr>
<th>preimmune serum</th>
<th>anti-α3 serum</th>
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<td>wildtype</td>
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2. **Reviewer Comment:** “Combining the tissue array dataset and AMC pathology archived dataset would create a larger “n” for the study and provide more confidence in the correlations that are identified. This may also resolve the inconsistencies of the associations with different receptor subgroups (ER, PR, HER2).”
Response: We had originally analyzed our data in the manner suggested by the reviewer, as well. We found that Spearman’s rank correlation coefficient analysis of the combined datasets also revealed a statistically significant positive correlation of α3 and COX-2 (n=127; rₛ=0.36; p<0.0001). Thus, with regard to the relationship between α3 and COX-2, it did not matter whether we combined the datasets or treated them as distinct datasets.

However, with regard to the relationship of either α3 or COX2 with different receptor subgroups (ER, PR, HER2), there are differences when the datasets are analyzed individually versus combined. Where the association of either α3 or COX2 with ER and PR were significant in the AMC dataset but not in the TMA (shown in Table 1), these associations were significant in the combined dataset. On the other hand, where the association of either α3 or COX2 with HER2 was significant in the TMA but not the AMC dataset (shown in Table 1), this association was not significant in the combined dataset.

After discussing with our biostatistician how best to analyze and present these data, we decided that the AMC pathology and commercial TMA datasets should not be combined since they were obtained from distinct institutions and had been pre-scored for receptor status by different pathologists. Given the caveats of combining datasets that had been scored by different groups, we felt it was most conservative to present a separate analysis of each dataset. Importantly, while keeping the two datasets separate did reduce sample size, the data still supported our main conclusion that α3 and COX-2 are correlated, and also demonstrated that this correlation holds in two independent data sets.