Editorial Board

Dear Editor,

Enclosed is a revised version of our manuscript. It has been modified to address the concerns raised by the reviewers. We included additional data in figures 1 and 4, have added a figure 5. Alterations and additions to the manuscript text are highlighted in red. We are also including responses to the comments from the two reviewers. We are grateful for the helpful suggestions and hope our manuscript will now be considered suitable for publication in BMC Cancer.

Sincerely,

Bradley W. McIntyre, Ph.D.

Reviewer Jorge Filmus:

Response to General Comments:

We are pleased that Reviewer Jorge Filmus finds the work presented "interesting and potentially important" and that the experiments were "well performed and support the conclusions". We also agree that detailed "mechanistic insight" beyond our initial observations is not addressed in this manuscript. The manuscript does not focus on the differences between anoikis resistant and anoikis sensitive human osteosarcoma cells, but focuses on the fact that despite the induction of anoikis resistance, chemotherapy sensitivity is not altered. Therefore, elucidating the mechanisms involved in the acquisition and maintenance of anoikis resistance in osteosarcoma cells is beyond the scope of this paper.

Nevertheless, we acknowledge the importance of understanding anoikis resistance, and we have completed a mechanistic study in which anoikis resistance of human osteosarcoma cells is associated with the Src-dependent activation of the PI3-K/Akt pathway in a manner independent of FAK activation. A manuscript with these results has now been submitted and is currently being reviewed for publication.

Major Compulsory Revisions:

1. The focus of the manuscript is not on the molecular differences between anoikis resistant and sensitive cells but on the relationship of anoikis and drug sensitivity. Other investigators have found an association of anoikis resistance and drug resistance in their model systems (presented in the Discussion section of our manuscript), our goal was to present evidence supporting the concept that the acquisition of anoikis resistance does not necessarily lead to chemo-resistance in our model system, human osteosarcoma. Nevertheless, with regards to the molecular mechanisms that have been already associated with anoikis, our laboratory has previously reported that anoikis in human osteosarcoma cells involves the activation of
caspase 3 and the release of cytochrome c from the mitochondria (Marco RAW, et al. J Cell Biochem 2003, 1038-47). Furthermore, we have not seen a difference in integrin expression between anoikis resistant and sensitive populations.

2. Reviewer Filmus is correct about SAOS-2 cells having heterogeneous anoikis sensitive and resistant phenotypes, and that culturing these cells in suspension will result in the selection of the anoikis resistant ones. However, in our work published last year (Diaz-Montero CM and McIntyre BW. Eur J Can 2003, 2395-2402) we addressed the heterogeneity issue by generating clonal populations, and indeed we found different degrees of anoikis sensitivity among the clones tested. Importantly, culture of anoikis sensitive clones under cycles of adhered and suspended conditions could convert sensitive clones into anoikis resistant. This suggests that the resulting resistant phenotype was not due to selection of pre-existing resistant cells but that de-adhesion acts as a driving force towards anoikis resistance.

Based on the evidence from our previous study that directly addressed this issue, we believe that the process of deadhesion can convert an anoikis sensitive cell to resistant.

Minor Essential Revisions:
1. The statement regarding reference 37 (former reference 33) has been changed to " Furthermore, in breast cancer cell lines SKBR-3 and MDA-MB-453, anoikis resistance can be restored by induction of ILK, independently of Akt activity[37]".

2. The statement "our work suggests that in human osteosarcoma cells" has been changed to: "our work suggests that at least in two human osteosarcoma cell lines" (page 10).

Reviewer Janusz Rak

Response to General Comments:
We believe the comments (i - iv) by Dr. Rak clearly demonstrate that the research areas of cell survival and death are still controversial and in need of much more research. While issues related to senescence programs, adhesion as a survival factor in other unrelated tumor types (hematopoietic malignancies), phenotypes of chronically chemotherapy-treated cells, and separation of adhesion signaling, apoptotic mechanisms, and genotoxic insults are very intriguing, these observations in these disparate systems may not be related to each other or to the specific case of osteosarcoma. Partly in response to this uncertainty we performed a very straightforward study focusing on one issue - does the acquisition of anoikis resistance in osteosarcoma cells also result in a general resistance to chemotherapy?

Major Compulsory Revisions
1. We agree that in vivo studies would be useful in further confirming our findings. Unfortunately, SAOS-2 cells placed in mice do not grow well (after I.V. injection it can 6 months to get one lesion in the lung). However, it has been possible to create SAOS-2 sublines after multiple in vivo passages after I.V. injection that will yield microscopic lesion in the lungs in 6 weeks. The extent of differences between these sublines and the parental SAOS-2 cells are still being determined. Even if the I.V. model was more efficient, we are not convinced it is appropriate for these studies. Since, the cells are introduced I.V., they could quickly reach the lung and attach. In other words, they could reach the lung long before the induction of anoikis. Therefore, this model does not test the involvement of anoikis resistant cells during important events in tumor progression, such as detachment from the primary lesion, survival while in suspension and colonization of secondary organs. We also believe the I.V. model is not appropriate to test the in vivo relationship between anoikis and chemotherapy resistance. Intratibial injection of cells, followed by formation of primary lesions and metastasis, represents a model that more closely mimics the natural history of osteosarcoma. Unfortunately, SAOS-2 and TE-85 cells are not able to grow after intratibial implantation in the mouse.

2. Reviewer Janusz Rak is correct on the fact that human osteosarcoma cells have different degrees of anoikis sensitivity. Our lab has tested 4 different human osteosarcoma cell lines, SAOS-2, MG-63, TE-85 and KРИB; and found that indeed the levels of anoikis resistance vary. Both SAOS-2 and TE-85 cells show sensitivity to anoikis. In contrast, MG-63 and KРИB cells are anoikis resistant. Anoikis sensitive and resistant TE-85 clonal populations were generated following the same method of culture cycles under adhered and suspended conditions previously reported for SAOS-2 cells (Diaz-Montero CM and McIntyre BW. Eur J Can 2003, 2395-2402). Both anoikis sensitive (TE-85p) and anoikis resistant (TE-85ar) cells were assayed for sensitivity to the same chemotherapeutic agents reported in our manuscript. The findings corroborated our initial observation that anoikis resistance does not necessarily confer chemotherapy resistance in osteosarcoma cells. The data has been added to the manuscript.

3. Our study focuses on the relation between resistance to anoikis and response to chemotherapy. The data
showed that acquisition of anoikis resistance does not confer resistance to chemotherapy-induced apoptosis. As mentioned in the manuscript, 1 in vitro LD50 of chemotherapeutic agent was used. Although the experiments presented in the manuscript were performed with this only dose, it represents the middle point of a dose response curve that was performed for each agent in order to determine the in vitro LD50. The authors agree on the importance of elucidating the molecular mechanisms involved in anoikis. We have previously reported that anoikis of SAOS-2 cells involves the activation of caspase 3 and the release of cytochrome c from the mitochondria (Marco RAW, et al. J Cell Biochem 2003, 1038-47). Furthermore, a manuscript describing the involvement of Src-mediated activation of the PI3-K/Akt pathway is currently under editorial review.

In the revised manuscript, data from another apoptosis assay has been included as suggested by the reviewer. We have included apoptosis analyses using AnnexinV-FITC/PI staining. The results correlate with the analyses using PI staining followed by cell cycle analyses that were used in the original manuscript submission.

Minor Essential Revisions
1. It is correct that the assay described on page 7 does not specifically measure apoptosis. However, it represents a useful colorimetric method for the initial screening of chemotherapy-induced cell damage that certainly needs to be corroborated with more specific apoptosis assays such as the ones used in our manuscript. Interestingly, in our SAOS-2 model the data obtained with the live-dead assay is consistent with both apoptosis assays (Fig 1A, 1B, and 1C).

2. It is true that chemotherapy-induced cell death can involve other processes besides apoptosis. However, since anoikis resistance is a form of apoptosis resistance the discussion focused on resistance to apoptosis. A paragraph including other physiological processes involved in drug-induced death such as senescence and mitotic catastrophe has been added to the discussion (second paragraph).

Discretionary Revisions:
The references suggested were added to the manuscript.