INSTITUTO SÍRIO-LIBANÊS
DE ENSINO E PESQUISA

São Paulo, 22th Oct 2019.

Rebuttal document JoVE60531
Dr. Bing Wu
Review Editor *JoVE*

Re: Revisions required for your JoVE submission JoVE60531 - [EMID:4fc1e164d71dfd01]

Dear Dr. Wu,

Thank you for your message informing us that our manuscript entitled "Quantification of tumor cell adhesion to lymph node cryosections" (JoVE60531), has been editorially and peer-reviewed for JoVE. We have followed your recommendations for video production and formatting, and we are also very grateful for the reviewers' comments and thoughtful suggestions.

Based on these, we have carried out additional experiments and revised the manuscript accordingly. Please, find attached a point-by-point response to reviewers' concerns. We revised the manuscript using the tracked changes function of Word to help the reviewers identify our changes.

We believe we have produced an improved version of our manuscript, and we hope the reviewers now deem it acceptable for publication in JoVE.

Sincerely,

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Reply to the Editor's comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

R: Thank you, Dr. Wu, for your comments on the language. We now have proofread the manuscript by a professional native speaker.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e., "This figure has been modified from [citation]."

R: We have modified Figure 1 accordingly, and now all figures from the revised manuscript are original.

3. Please only highlight the protocol steps for filming. Please do not highlight any text in Introduction, Representative Results or Discussion.

R: These instructions were followed as recommended.

4. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations.

R: Thank you for the suggestions. We updated the discussion session accordingly.

5. Please ensure that the references appear as the following. 6. Please do not abbreviate journal titles for references. 7. Please sort the items in alphabetical order according to the name of material/equipment. 8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). Any text that cannot be written in the imperative tense may be added as a “Note.” 9. Please add a one-line space between each of your protocol steps. 10. Please use h, min, s for time units.

R: Thank you for all these points. In the revised manuscript, we have adjusted these specific issues that you highlighted. Thank you very much for the time and energy you spent on our behalf.

Reply to Reviewer #1

Manuscript Summary: This manuscript describes a method to examine tumor derived factors that promote adhesion of tumor cells to molecules, cells and structures present in lymph nodes. This method has advantages relative to in vitro tumor adhesion assays and also limitations as described by the authors. The authors were transparent when discussing the advantages and disadvantages of the research tool that they developed. The methods were easy to follow and a very relevant example of how to apply their assay was provided.
R: Thank you very much for your kind words about our paper.

Minor Concerns:
The manuscript does require a good amount of copy-editing.
R: We have made careful modifications to the original manuscript. The manuscript was proofread by a professional to minimize spelling and grammar errors.

Reply to Reviewer #2

Manuscript Summary: This technical paper is based on a recent publication by the same authors in which NDRG4 promoter hypermethylation was identified as a biomarker of metastatic breast cancer (Jandrey et al NPJ Breast Cancer 2019). In that paper, the authors have shown that MCF-7 cells in which NDRG4 was silenced using shRNA had an increased adhesion to LN cryostat sections. Increased β1 integrin clustering at the leading edge of tumor cells resulting in increased cell adhesion and migration towards vitronectin was identified as the underlying mechanism.

Here, the authors provide a step-by-step detailed description of the adhesion assay used, supplementing previously published data. The assay described is a further optimization of a previously published technique (i.e. Brodt et al). They propose that it can be used as a low-cost, rapid, sensitive and environmentally friendly protocol with clinical relevance to lymph node metastasis in breast cancer patients.

Critique:

The description of the assay is very clear. This adhesion assay could be useful to other investigators who wish to establish a functional in vitro correlate of lymphatic metastasis as part of a broader profiling of metastatic cancers.

R: We appreciate your time, comments, and insights. We are delighted to hear that you think our method is relevant, clearly described and contextualized by our previously published data.

Major Concerns:

1. For their studies, the authors selected human breast cancer MCF-7 cells that are known to be non-metastatic in xenotransplanted mice. Moreover, they provide no evidence that NDRG4 silencing in these cells actually alters their lymph-node metastasizing potential (e.g. in an animal model). Thus, while differences in adhesion of the two cell lines were demonstrated, the correlation to LN metastasis is indirect, as it relies on other data showing NDRG4 promoter methylation in clinical specimens. To strengthen the impact of this work, the authors should use cancer cell line pairs for which a difference in lymph node metastasizing potential that correlates with differences in adhesion to the cryostat LN sections can be demonstrated.

R: You are correct that the correlation between our method and LN metastasis is indirect and should be complemented with additional experiments. Lymphatic dissemination of tumor cells requires a series of complex events, like remodeling of cell-cell interactions, local invasion, chemotaxis, cell migration, etc. In my experience, even the use of "pairwise" adhesion assays, using a couple of different cell lineages, will hardly indicate direct correlation with LN-metastatic potential *in vivo*. For example, as you well noticed, even a non-metastatic breast tumor cell line, like MCF-7 (see manuscript) or the T47D tumor cells (data not shown), strongly adhere to LN sections *in vitro*, at similar levels to that observed for the highly metastatic MDA-MB-231 tumor cells (data not shown from our lab). However, it is well known that orthotopic MCF-7 xenograft tumors cannot reach sentinel LNs, while MDA-MB-231 tumors spontaneously metastasize to LNs (Pathak et al., *Cancer Res*, 2006). The main bottlenecks for MCF-7 LN-metastasis formation occur in steps in the metastatic cascade before reaching and adhering to LNs, like the inability of MCF-7 cells to efficiently escape from the primary tumors. This paper aims to carefully describe

a simple method to quantify cell-matrix adhesion properties in a more realistic ECM *in vitro*. The purpose of this assay is not to establish direct correlations with potential LN metastasis but is to allow a rapid and low-cost functional analysis of specific adhesiveness of tumor cells to ECM, cells, and structures present in LN. Nonetheless, to address the review's comment, we included a paragraph in the discussion section highlighting this limitation of the assay.

2. Because the lymph nodes used are derived from rats, the authors should consider using rat breast (or other) carcinoma lines or human lymph nodes, if available. At the very least, potential differences in the stromal components of human and rat lymph nodes should be discussed.

R: We agree with the reviewer that potential differences in the stromal components of human and rat lymph node could impact the performance of the assay. However, we believe that the impact would be minimal as the composition of rat, mouse or human LNs includes the same structural and functional proteins that are part of native mammalian ECM (e.g. collagens, fibronectins, laminins, etc), all preserving similar binding sites that are necessary for cell adhesion (Badylak SF Transpl Immunol 2004). Nevertheless, to address the review's comment, we included a paragraph in the discussion section highlighting potential differences in stromal components and their potential impact in our assay.

Minor Concerns: As a control, the authors used BSA-coated dishes. As an additional and more relevant control, the authors should consider using dishes coated with ECM proteins such as fibronectin, vitronectin and collagen. This will inform on whether the differential adhesion to LN sections is merely a result of differential adhesion to specific ECM proteins, or the LN stroma has additional complexity and constituents (e.g. cells) that could contribute to the differential adhesion.

R: Thanks for raising this critical point about controls. Dishes with purified ECM compounds, or decellularized LNs, certainly could be used, but the results would be cell-type specific, as different cell lines possess different adhesive affinities towards different LN components. The purpose of this paper is to describe a general protocol to evaluate cell-matrix adhesion in frozen LNs. To evaluate whether the differential adhesion to LN sections is a result of binding to

collagen, vitronectin, or other structures present in LN sections, is beyond the scope of this particular article. To address the reviewer's comments these points are now addressed in the revised version of the manuscript.

Reply to Reviewer #3

Major Concerns: Although the method is well described and complete, I suggest to authors to include photographs showing the identification and isolation of lymph nodes, instead of drawings. This is the most challenging step in this methodology and deserves a proper representation.

R: We agree with the reviewer's suggestion. These photographs were incorporated in the revised version of the manuscript (see new Figure 1). Thank you for this relevant suggestion to better illustrates our method.

Minor Concerns:
- Page 1, line 31 - in the second sentence of the abstract, replace carcinomas for cancer.
R: We agree and have changed the sentence accordingly.

Reply to Reviewer #4

Major Concerns: To better represent figure 1, the authors should provide annotations for the location of lymph nodes. (…)
R: Thank you for this valuable comment that Reviewer 3 also pointed out. Instead of drawings, we now included photographs showing the identification and isolation of rat LNs in the revised version of the manuscript (see the new Figure 1).

(…) The discussion should also outline the advantages in utilizing this methods in a clinical setting i.e studies that would benefit in utilizing this method. Authors should also mention if similar methods can be used for different organs and cell types i.e spleen, WBC.
R: The reviewer raised an interesting perspective to use this method in the clinical setting. Metastatic cells exhibit organotropism and measurements of adhesive strength in different organs could be a useful means to predict organ-specific cancer metastasis. We now elaborate on this point in the discussion

section of the revised version of the manuscript. Nevertheless, we are hesitant to take a strong position on this point (like utilizing it in a clinical setting), because metastatic growth requires a series of more complex events, than a simple tissue-specific adhesion.

Minor Concerns: The manuscript requires linguistic revision. i.e line 104-105, line 123, line 158 etc. The protocol section needs revision for imperative statements.

R: Thank you for your recommendations on the language. The manuscript was proofread the manuscript by a native speaker.