Reviewer's report

Title: Cystatin E/M suppresses legumain activity and invasion of human melanoma

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Reviewer: Christoph Borner

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Major Compulsory Revisions

Unfortunately, the revised MS by Briggs et al. has not really improved. Although parts of the text were changed to make some of the statements clearer, there are no additional data presented which would support a causal relationship between cystatin E/M secretion and the metastatic potential of melanoma cells. The whole study remains descriptive. While the authors clearly show that overexpression of cystatin E/M in two cystatin E/M non-expressing metastatic melanoma cells (A375 and MCC11) results in the inhibition of legumain activity (Fig. 3C/D) and a consequent decrease in the invasion capacity of the cells in the Matrigel gel assay (Fig. 4), the authors do not even attempt to demonstrate that the metastatic melanoma cell lines secreting massive cystatin E/M endogenously (MCC57 and MCC72) have a lower invasive capacity than the cell lines where no secreted cystatin E/M was detectable (MCC11, MCC35, MCC52, MCC69B). The authors argue that the Matrigel assay is too expensive to be performed for all cell lines. In addition they state that measuring the invasive capacity in the whole panel of cell lines will not necessarily reflect the protease/inhibitor in question, but rather the total levels of proteases and inhibitors in the different cell lines. I do not think it would not be too costly to just compare two high and low cystatin E/M-producing metastatic cell lines, Moreover, even though other protease/inhibitor pairs may contribute to the metastatic potential, the secretion of massive amounts of cystatin E/M (as it is the case for MCC57 and MCC72) should clearly diminish this potential because it blocks legumain, a crucial metastasis promoting protease.

Additional points:

1) The positive correlation between high cystatin E/M secretion and low residual legumain activity in the conditioned media assay does not hold true for all cell lines. For example, MA11 cells show high cystatin E/M levels but almost no legumain inhibitory activity (Fig. 1A/B). Conversely, the media of MCF10A, 92.1 and 624.38 cells exerts relatively good legumain inhibition, but no cystatin E/M secretion. The legumain activity data do not even correlate with cystatin C (which could replace cystatin E/M) because 92.1 cells do not secrete this protein while the other two cell lines do (MCF10A and 624.38). Moreover, the metastatic cell line MCC52 and MCC57 display similar legumain inhibitory activity, but only the latter cell line really expressed cystatin E/M.
2) The authors promised to show some human melanocytes as control lines (apart from HDF and HaCaT) but none are really presented in the revised MS (see author’s comments to point#2).

3) I do not buy the argument of the authors that the difference of the ELISA (Table 1) and Western blot data (Fig. 1A) is due to the possibility that in the ELISA assay part of the cystatin E/M is bound to the legumain protease. Then you should be able to show that legumain and/or cathepsin B or L are secreted. At least for legumain this does not seem to be the case (as stated in the last sentence on page 18 (cathepsin B and L activities were not shown). Thus, it remains peculiar that cystatin E/M can be detected by ELISA in the MCC70, but not MCC13, 57 and 72 cell lines although all cells have a low expression level of legumain, cathepsin B and L (Fig. 2).

4) It is very interesting that most of the analyzed cell lines which secrete lots of cystatin E/M (with the exception of HCT-116) also exhibit low levels of processed, active legumain, cathepsin B and L. The authors interpret this correlation as an inhibition of the protease processing/activities by cystatin E/M. However as the inactive proforms of these proteases are also diminished (Fig. 2), an alternative explanation could be that cystatin E/M and cathepsin expression levels are counterregulated by some unknown mechanism. In this case the lower metastatic potential of the cystatin E/M expressing cells might also be due to lower total expression levels of legumain, cathepsin B and L. This possibility should be envisaged and discussed. This is why the authors have to perform the Matrigel assay on the metastatic cell lines. If cystatin E/M expressing cells migrate faster, overexpression of legumain or cathepsin B/L and underexpression of cystatin E/M by shRNA will distinguish between the importance of the protease expression as compared to inhibitor levels.

5) There is another discrepancy between the cellular legumain activity (shown in the referees comments part) and the Western blot data of the processed, active protease form. While the amount of the active 36 kD form of legumain is comparable between the DU145, MCF10A, MCC13, MCC70, MCC57 and MCC72 cell lines, the legumain activities tremendously vary. Why? I do not understand this, especially because legumain activity/protein does not seem to be lost from the cell (secreted).

6) The data obtained from legumain, cathepsin B and L activity measurements using cleaved peptide substrates should be shown in the MS (not only mentioned on in the last paragraph on page 18).

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:

I declare that I have no competing interests