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Opponent's review of the PhD thesis

The role of the tumour microenvironment in melanoma cell invasiveness
Role nádorového mikroprostředí v invazivitě buněk melanomů

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The presented Ph.D. thesis was carried out at the Department of Cell Biology, Faculty of Science at Charles University in the Laboratory of cancer cell invasion. The thesis is based on two original research articles published in journals with an IF (2.78 a 3.98), the candidate is the first and second author, respectively, in these articles. In addition, the candidate is the first author of a review article that was published in Clinical Cancer Drugs.

The Ph.D. thesis focuses on the molecular mechanisms that drive the invasiveness of cancer cells, with a special focus on the invasion of melanoma cells. The topic is of high importance for both basic and translational research as these mechanisms are largely unknown and significantly contribute to cancer related morbidity and mortality.
In the first part, the candidate participated on a project evaluating the role of the proteoglycan NG2 in cancer cell invasion. Using activated Rho pulldown and gelatin degradation assays she could show that NG2 activates Rho signaling with subsequent mesenchymal to amoeboid transition, which is accompanied by decreased ECM degradation and increased invasiveness.
In the second part, the interaction between melanoma cells and cancer associated fibroblasts (CAFs) was examined to determine its effect on melanoma invasion. Using a 3D invasion assay with conditioned media with or without neutralizing antibodies, the candidate reveals a complex bidirectional interaction between cancer cells and CAFs, and identifies Il6 and Il8 as important mediators of the pro-invasive effect of CAFs.
The results also include a short summary of the published review article and unpublished results from an ongoing project. In this part, the candidate highlights the advantages of advanced invasion models for drug testing, and using the 3D invasion assay demonstrates the pro-invasive effect of conditioned media from a co-culture system of fibroblasts and keratinocytes. Preliminary data on the anti-invasive effect of curcumin in the 3D invasion assay is also shown.

From my point of view, the presented thesis nicely demonstrates that both the cancer cell autonomous mechanisms and clues from the tumor microenvironment are critical in driving the invasiveness of cancer cells. It also provides an example of using model systems such as 3D invasion assays, which more closely mimic the *in vivo* conditions, in preclinical research. The results obtained in these systems can differ from the results in less complex 2D assays and seem to be more appropriate for the preclinical testing of anticancer therapies. Last but not least, I

value the efforts of the candidate to propose potential future cancer treatments based on her research.
The work of the opponent is made easier as the majority of the results in this Ph.D. thesis are part of peer reviewed original articles that were published in respected international scientific journals. This proves that the candidate mastered advanced methods in biochemistry and cellular biology and is able to design experiments and interpret and summarize their results.

The presented Ph.D. thesis is written in English, has 93 pages, 15 figures, cites 173 mostly recent references and is standardly structured. The articles, on which the thesis is based, are included as supplements. The introduction provides a short overview of cancer with the focus on cancer cell invasion and metastasis. Adequate information is provided on the main areas covered in the studies. Nevertheless, the organization of the topics is slightly disarranged, possibly due to the somewhat heterogeneous nature of the aims of the work. The results are in large part taken from the attached original manuscripts and are divided into 4 areas; the incorporation of a short summary of the review article would in my opinion be more suitable for the introduction or discussion section. In the discussion section, the main findings are summarized and the results are critically analyzed.

I have the following critical comments regarding the submitted thesis:
The aims and objectives of the work (p 36)
- surprisingly, the objectives are more precisely formulated in the Summary of the Ph.D. thesis (on p6) than in the main text. Unfortunately, the data for one of the objectives stated in the Summary of the Ph.D. thesis (the optimization of the 3D spheroid invasion assay) are not shown. The link between the two aims is somewhat hard to identify at the first sight and would deserve to be introduced in a better way.
- It is in my opinion very unfortunate that the first aim is formulated as "to study the mechanisms in which NG2 activates Rho/ROCK signaling." As later stated by the candidate (p47) this aim was not further pursued because a competing team published the results in 2013 (Biname et al 2013). The aims of the thesis should be compatible with the presented data.
- The results regarding curcumin are very loosely if at all connected with the stated aims and objectives.

Minor comments:
1) In some of the graphs, the statistical significance of the observed differences is not stated (e.g. Figure 15, p66, Figure 16, p68, but also figures from the original manuscript on p 53, 56, 57 etc.), making these figures hard to interpret. Were the experiments depicted in figure 6 (p51) and 9 (p56) and 10 (p57) run in parallel? Strictly speaking only the experiment depicted in figure 12 (p59) seems to test the effect of conditioned medium vs. conditioned medium with the anti Il6 and anti Il8 antibodies.

2) Experiments with curcumin (4.4.2)
In the Discussion section, these results are discussed in relation to the role of cancer associated fibroblasts (5.2.6., p80), but the link is unclear to me. I would also disagree with the generalization that "natural compounds usually show decreased levels of toxicity compared to synthetic products" (p 80).

3) As far as I can judge, the thesis is written in relatively good English. Nevertheless, typos and unusual formulations can be found and in some parts of the text these are disturbing. Some

examples are listed below in an appendix of this report. They are intended for the author's information and I do not consider it necessary to discuss them during the thesis defense.

4) Figure 15 (p75) is part of the discussion, but as it illustrates the lay-out of the 3D invasion assay, it would be more appropriate in the Material and Methods section.

5) The bibliography is not provided in a uniform format, the numbering of alphabetically ordered references is rather unusual.

Reading of the thesis further evoked the following questions:
1) In the discussion (p 71) the candidate states that *"NG2 levels do not differ between primary and metastatic lesion during early stage cancers, however NG2 levels are significantly greater in metastatic lesions during later stages (Burg et al 1998). This suggests that NG2 plays a role in the migratory potential of cancer cells and poor prognosis."* A similar statement in the introduction (p22) refers to Huggins et al 2014.
Please clarify this statement (the presence of overt metastases is somewhat incompatible with "early stage cancers") and explain how would this particular finding support the conclusion that NG2 contributes to the metastatic spreading?

2) The presented data show that silencing of NG2 promotes the extracellular matrix degrading activity in cancer cells and would result in lower ROCK activity. ROCK inhibition (alone or in combination with other factors) can reprogram several cell types and in some cases induce a more stem-like phenotype in cancer cells.
Could you speculate more on the proposed potential of therapeutic targeting of NG2 in cancer treatment (p71) in light of these facts?

3) What was the ratio between melanoma cells and fibroblast during the preparation of conditioned media and how many cells were used for the preparation of conditioned media by melanoma cells? For the co-culture, the cells were seeded at 1:10, but this could have significantly changed during the two days of cultivation prior to media conditioning. This could substantially influence the interpretation of the contribution of individual populations to cytokine production (p53, Figure 8). Why are the cytokine concentrations expressed in pg/mol?

4) A more general question- the dormancy of melanoma cells in the target tissue (in addition to their invasion from the original site and subsequently from the vasculature) is likely an important determinant of overt metastatic spread in melanoma. Is something known about the microenvironmental factors that regulate the dormancy of micrometastases in melanoma?

Conclusion:
In spite of the above-mentioned objections the thesis demonstrates the preparedness of Njainday Pulo Jobe for the scientific career. **I therefore recommend the thesis for defense and acceptance as part of the requirements for awarding the Ph.D. degree.**

Prague 20.8.2016

Petr Bušek, M.D., Ph.D.

Appendix to the opponent's review

The title of the Summary of the Ph.D. thesis contains a typo (*The Role of the Tumour Microenvironment on Melanoma…*)

p 2 typo *BIBIOLOGRAPHY*

p10 *"Concomitantly, there is subsequent increase…"* sounds strange.

p 11 typo *"…in other to colonise distant sites in metastasis*."

p12 The sentence *"The migration of amoeboid cells may be inhibited due to structural limitations as cells that contain a nucleus larger than the collagen pores cannot be negotiated."* sounds strange.

p22 *"NG2 binds to a number of ECM components such as collagen II, …, laminin, and its popular, highly characterized interaction with collagen VI."* sounds strange.

p23 *"transfection of NG2 and cell subsequent cell spreading…"*

p34 *…"may have significant implications for a number or targeted therapies, such as MAPK, as it can result…."* Inhibitors of the MAPK pathway" or similar should be used instead of "MAPK"

p41 "*Primary antibodies diluted according to manufacturer's instructions and incubated overnight…"* a verb is missing

p52 *"Basal amounts of Il-6 by BLM…"*, a verb is missing.

p65 *"In addition, we are also analyzing the effect of conventional treatment on keratinocytes, and how this affects melanoma cell invasion."* It is not clear what effects and which "conventional" treatments are being tested.

p71 The sentence *"Although a better understanding of the role NG2 in malignancies will further aid the development of targeted therapeutic agents specifically and directly against NG2, in order to avoid limitations."* sounds strange.

p77 *"5.2.4 Il-6 and Il-8 in combination is more effective in inhibiting CAF-dependent increase in invasion"* This part refers to the inhibitory effects of the neutralizing antibodies against the two cytokines, as also correctly stated in the heading of section 4.2.3. *"Blocking Il-8 and Il-6 inhibits CAF mediated increase in invasion"*

p78 *"…further highlighting the significant of the extended tumour microenvironment in cancer progression."* sounds strange

p78 *"in order to promote an objective response and disease maintenance."* remission?

p80 *"As demonstrated by the Nobel Peace Prize winner in 2015 that discovered a natural compound to treat popular parasitic diseases (website:nobelprize.org)".* Not a specialist in this field, but "The Nobel Peace Prize 2015 was awarded to National Dialogue Quartet "for its decisive contribution to the building of a pluralistic democracy in Tunisia in the wake of the Jasmine Revolution of 2011".(source: *nobelprize.org)*