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The PhD thesis by Agata Marta Rybicka is entitled *"Characterisation of canine mammary cancer stem-like cells and their interaction with tumour-associated macrophages"* and has been submitted to the Faculty of Veterinary Medicine, Warsaw University of Life Sciences, (WULS) at Poland. As an external evaluator chosen to review this doctorate thesis, I have thoroughly read the text and evaluate it by focusing in four directions: the novelty and scientific merit, the aim and main tasks, the experimentation and the results obtained. To this end, my review follows such structure.

Novelty and scientific merit

The development of suitable models to understand interactions between tumor cells and their microenvironment is vital for cancer prognosis, diagnosis and pharmacotherapy. To this end, the use of canine mammary tumor in vitro models to uncover novel pharmacologically exploitable targets as well as to understand the molecular interactions of macrophages with cancer stem-like cells (CSLCs) within the tumor microenvironment is novel and represents a fundamental issue within the doctorate thesis.

Aim and main tasks

The work presented in the thesis document focuses in the broader era of cancer stem cells (CSCs) that, nowadays, represents a very interesting scientific topic in tumor pathophysiology and therapeutics. The way by which CSCs interact at the molecular level with other cells in the microenvironment contributes to tumor progression and aggressiveness, as well as to the therapy response and the final clinical outcome. To this end, the aim of the submitted thesis to identify molecular targets governing such interactions and validate them as predictive biomarkers is of crucial importance in modern pharmacology of new anticancer drug development.

Experimentation

To experimentally approach the aim and the main tasks of the doctorate thesis, Ms. Rybicka applied the following methodologies:

1. Use of three established canine mammary tumor (CMT) lines, to identify the canine mammary cancer stem-like cells (CMSLCs) through the use of the surface markers Sca1, CD44 and EpCAM. [Stem cells antigen 1 (Sca-1), Epithelial cell adhesion molecule (EpCAM), CD44 antigen]
2. Application of flow cytometry analysis to quantify the number of the CMSLCs within the entire CMT cell population.
3. Exploitation of microarrays to reveal miRNA molecular signatures based on the genetic expression profiles of canine miRNAs in CMSLCs compared to that in differentiated tumor cells.
4. Execution of microarray DNA experiments to assess CSLCs interactions with macrophages by developing cultures of CSLCs in vitro, as well as co-cultures of CSLCs with tumour-associated macrophages (TAMs).

Results

1. The identified of CSLCs population in the three CMTs as quantified by flow cytometry analysis has been shown to be within the range of 0.2% to 3.75% of the entire tumor cell population. The methodology used in the two published papers that are included in the submitted document, however, differs between the two applied detection and separation strategies for the isolation of CSLCs population.
2. Transforming growth factor-beta (TGF-β) is a well-known player in maintaining the stemness of embryonic stem cells. It is thus interesting, that through functional analysis of the identified molecular signatures in the expression profiles of the 24 down-regulated miRNAs in CMSLCs, compared to differentiated tumor cells, it has been revealed that the majority of targeted genes belong to the TGF-β signaling pathway. Such a result proposes that the identified miRNAs could be further pharmacologically exploited to uncover candidate molecules within them as potential miRNA therapeutics in CMTs. The potential clinical usefulness and the pharmacological benefit in targeting TGF-β signaling for mammary tumor therapy remains, however, to be further experimentally validated.
3. The co-culture of CSLCs with tumour-associated macrophages (TAMs) followed by DNA microarray analysis has permitted the identification of the macrophage attracting factor / monocyte chemo-attractant protein-1 (CCL2/MCP1) gene, as a contributing factor to enhance the infiltration of macrophages into the tumor mass. This role has been shown to exist by analyzing, through RT-PCR, samples of various grades of primary CMTs, where CCL2 gene overexpression has been well-correlated with tumor aggressiveness and metastatic potential. Moreover, the formation of 3D tubules in human umbilical venous endothelial cells (HUVECs) angiogenesis *in vitro* assay has been increased only by assessing CSLCs co-cultured with TAMs or by the addition of CCL2 in culture media. The latter,

is important since the survival rate in primary CMTs has also been correlated with the level of angiogenesis seen within the tumor mass. The existing complexity at the molecular level, however, within those major cancer cell decisive processes of proliferation, angiogenesis, and metastasis, further necessitates a more targeted in vivo models approach to again clinically validate the obtained results.

Evaluation

In conclusion, Ms. Rybicka has presented a solid and innovative work from its design to methodological approach and the execution of experiments, whereas she has produced a readable document at a very satisfactory level. The work is presented in two publications in a clear manner and the conclusions follow logically from the experimental observations. The third publication been included represents a review article that covers well the scientific era of the experimental work conducted and executed. It is interesting that Ms. Rybicka convincingly explains in the doctorate document some methodological discrepancies that exist within the two published research papers. Also, the exemplification of the terminology used, especially for cancer stem cells, tumor initiating cells and cancer stem-like cells, helps towards the better understanding of the work presented. Ms. Rybicka has worked extremely hard and she has gained considerable experience in a diverse range of molecular biology and genomics techniques. The subject matter of the thesis is particularly demanding in that it requires a broad appreciation of several source data handling and experimentation with a high level of understanding of specialist techniques from miRNA/DNA microarray analysis to cell studies and primary tumor samples usage. Finally, the thesis mirrors the candidate's development into an independent researcher.

Proposal

Following the evaluation presented above, I also confirm that the PhD thesis of Ms. Agata Rybicka meets all the criteria according to the proper Polish Law *(Ustawa z dnia 14 marca 2003r. o stopniach naukowych i tytule naukowym oraz o stopniach I tytule w zakresie sztuki; Dz. U. Nr 65, poz. 595, z pozn. zm.).* Therefore, I propose Ms. Agata Rybicka to go to the next step of the doctoral degree conferral procedure. Moreover, I propose to the Faculty Council of the Faculty of Veterinary Medicine to award this PhD thesis with a proper distinction.

Sincerely yours,

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