Final Report:
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Mechanobiology of the neurovascular unit in health and disease

Metastatic complications are primordial cause of death in most cases of solid malignancies. Despite of the strong defence line of the central nervous system, detected neoplasms are predominantly of metastatic origin. Suppressing extravasation might be a promising tool of prevention and therapy. Unfortunately, our understanding of the mechanisms and driving factors of dissemination is still limited. One bottleneck of successful colonization is the interaction of the blood circulating tumor cells with the neurovascular unit. Mechanical aspects which are crucial for the interaction between the circulating tumor cells and the neurovascular unit are largely unexplored. The neurovascular unit plays crucial role in the maintenance of the proper homeostasis of the central nervous system. Endothelial cells and pericytes are the most exposed to mechanical stresses, therefore their mechanobiology is of primordial importance. Nanomechanical parameters of endothelial cells and brain vascular pericytes were recorded and compared in quasi-physiological and pathological conditions. Quasi- physiological conditions were provided as keeping the cells at 37°C degree in culture medium during measurements. In order to induce pathological conditions like e.g. inflammation, tumor necrosis factor alpha, lipopolysaccharides and interferon gamma were added to culture media. Nanomechanical parameters of the cells were followed by recording sequential elastic maps prior and after the administration of the above mentioned inflammatory agents. Part of these results were presented as a selected flash talk and poster presentation at the **AFMBioMed Conference** held in Munster. The scientific committee rewarded the poster with the third “Best Poster” offered by the organizers.

Intercellular interactions are based on intermolecular connections; therefore, direct measurement of single molecule interactions are of great interest. Not closely connected to the main stream of present research plan, two manuscripts were accepted for publication. The first investigates the direct connection between two bio-macromolecules predicting their connection establishment prior to the occurred event. This paper has appeared in the journal of **ACS OMEGA, DOI:10.1021/acsomega.8b02993**, a **Q1** ranked journal with an **IF: 2.584**. The second study presented the results of investigating the elastic properties of human retinal pigment epithelial cells in the presence of hyperosmotic and oxidative stress. Enhancement of neovascularization processes and protective effect on elastic properties of above mentioned cells in the presence of pituitary adenylate cyclase activating polypeptide was shown. This paper has appeared in the **ANNALS of the NEW YORK Academy of Sciences**, **DOI:10.1111/nyas.14189**, a **Q1** ranked journal with an **IF: 4,277**. The applied methods from these studies were used in the later phases of the investigations of the present project.

Investigations targeting the structure of single tumor cells of different origin using label free chemical imaging based on confocal Raman spectroscopy were also planned in the present project. Our goal was to build a hyperspectral database to identify cell type-specific spectral fingerprints. We planned to simultaneously test several clustering and analyzing methods for the optimal

discrimination of the obtained high resolution spectra. The amount and complexity of recorded spectra required introduction of special data analysing method. Testing and validation of several discrimination algorithms was performed in order to optimize and enhance data processing. This has proved to be more challenging task and more time consuming as the initial presumptions. Therefore, a custom built analysing algorhytm was introduced to deal with the large amount and variety of data. The obtained results are promising but more time is required to overcome all technical and computational demands in order to draw doubtless conclusions.

Cell-released extracellular vesicles are important mediators of intercellular communication. They serve as unique carriers of bioactive molecules and constitute pathway(s) of extracellular exit of molecules into the intercellular space, biofluids, and blood. These vesicles might be implicated as mediators of homeostasis and repair, while in cancer they may act as regulators of cell growth, clonogenicity, angiogenesis, thrombosis, and reciprocal tumor-stromal interactions. It has been shown that they might play key role in pre-metastatic niche formation and are involved in metastatic organotropism of different tumor types. Therefore, we have investigated the effect of tumor cell-derived exosomes on de-adhesion parameters of source cells exosome pre-treated brain endothelial layer. First step was to extract and isolate breast adenocarcinoma cells-derived exosomes. After validation of our isolates by their size and protein content, we have tested if their presence induces any alteration in de-adhesion pattern of adenocarcinoma cells directly contacting brain endothelial layer. Direct de-adhesion measurements were performed in order to compare adhesive parameters of the very same adenocarcinoma cell (MDA-MB-231) to exosome-pretreated endothelial layers. Our results indicated, that presence of exosomes lowers the adhesiveness of the endothelium when facing direct contact with breast adenocarcinoma cells. Size distribution of observed individual ruptures is close to those observed in case of cadherins and membrane related connections, nanotube tethers. This first contact appeared to be weaker in case when endothelial cells were pre-treated with tumor cell derived exosomes. These results underline that first contact of blood circulating tumor cells might not be decisive in the process of metastasis formation, although pre-metastatic niches might play important role in final colonization. The results of these investigations were presented in the **Q1** ranked journal of **Colloids and Surfaces B: Biointerfaces** (**DOI:10.1016/j.colsurfb.2021.111810**), having an impact factor of $\mathbf{IF}_{2020}$**=5,268**.

Successful brain colonization involves active participation of all cellular components at the tumor microenvironment. Breast adenocarcinoma is among the leading “sources” of brain metastases. In addition to immune cells, cancer associated fibroblasts and endothelial cells, pericytes are key components of the newly established tumour microenvironment, whose role in distant colonization has only recently been addressed. Hence, pericytes can be regarded as a second active defense line of the central nervous system against different solute and cellular elements circulating in the blood stream. Contractile proteins in cerebral pericytes may play important role in mechanical regulation of the endothelium and the blood flow. However, a detailed nanomechanical characterization of pericytes is still lacking. Our experiments targeted the nanomechanical comparison of pericytes and endothelial cells, with the aim to identify differences which might be important factors during brain targeted metastatic processes.

Intercellular adhesion measurements are affected inherently by variation of the tester and tested cells as well. In order to decrease this, we have used a method, which is able to test the adhesive properties of the very same tester cell to several surfaces or cell types. This method can increase the accuracy of the measurements substantially.

Besides endothelial cells the adhesive parameters of pericytes were tested to parental and brain seeking breast adenocarcinoma cells (MDA-MB-TGL and MDA-MB-BrM2). Upon measuring the adhesion properties of the very same tumor cell sequentially to collagen, endothelial cells and pericytes, the differences in adhesive properties can be characterized with higher accuracy. Our results indicate, that the endothelial cells exhibit lower affinity towards tumor cells, which might support their protective role as a firs defence line of the neurovascular unit. This protective ability might rely considerably on the surface proteins and proteoglycans of the endothelium, but their exact contribution and roles are not well described yet. Proteoglycans act as important mediators in several cell mechanics related regulation pathways. Syndecan-4 is a ubiquitous glycocalyx component proteoglycan, which plays important role in several signaling pathways. Syndecan-4 knockdown cells exhibited considerable mechanical alterations which manifested in overall higher apparent stiffness, as revealed by our high resolution nanomechanical mapping. Results on mechanical properties of syndecan-4 lacking myoblasts were published in the journal of **Cellular and Molecular Life Sciences, DOI: 10.1007/s00018-021-04121-0.**

Unfortunately, the timeframe of the present project was completely covering the recent global pandemic situation due to the SARS-CoV2 outbreak. As a direct consequence, practically all social events, conferences and on site meetings were cancelled, or postponed. These lockdowns were affecting roughly half of the project's timeframe, eliminating the possibilities to attend scientific meetings and conferences. After the release of severe regulations due to the pandemic, the opportunities for scientific conferences offered the organization of the **Regional Biophysics Congress** held at the marvellous city of Pécs at the end of August 2022. Within the frame of a lecture, our latest results in the field of tumor cell adhesion to the elements of the neurovascular unit were presented, gaining a warm and supporting attitude from the audience. One manuscript is under preparation which summarises the above mentioned data, but unfortunately could not be finalized yet. We truly hope we will be able to submit before the end of the present year.

As a summary, we can state that nanomechanical properties of invading tumor cells might have important aspects during colonization processes. These characteristics might be decisive for the outcome of metastatic processes. As a consequence, valuable information can be gathered upon the investigation of direct contact between tumor cells and components of the neurovascular unit which might serve not only better understanding of brain targeted colonization but in adequate circumstances might even serve as markers or complementary information in diagnostic tests.