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**The role of podoplanin in glioblastoma stem cell-like spheroid cultures**

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Glioblastoma multiforme is the most common primary malignancy of the human brain and one of the most malign cancers. Within the last one and a half decades, the concept of tumor stem cells has emerged as a potential explanation as to why malignant diseases can recur even many years after an initially successful treatment. It postulates a hierarchical model of tumor initiation and development in which only a small, phenotypically distinct subpopulation of tumor cells is responsible for sustained tumor growth, progression or initiation, while the rest of the cells lack this ability. Like for many other cancers, the existence of tumor stem cells has been shown for glioblastoma, and when cultured in stem cell medium *in vitro*, glioblastoma stem cells notably grow as free-floating spheroids. However, to this day neither a single marker nor a suitable combination of markers has been found that could reliably identify glioblastoma stem cells. Podoplanin is a transmembrane glycoprotein upregulated in a number of human cancers including glioblastoma. Multiple lines of both *in vitro* and *in vivo* evidence indicated that podoplanin might have a distinct role in glioblastoma stem cells or could possibly serve as a marker. Based on these, the aim of this study was to illuminate the role of podoplanin in glioblastoma stem cells and to determine whether podoplanin might be suitable as a glioblastoma stem cell marker, thereby ultimately paving the way for future therapeutic targeting of the elusive population of glioblastoma stem cells.

A preliminary investigation in adherent glioblastoma cell lines revealed an association between podoplanin expression and the capability to form spheroids, which is considered a parameter related to stemness. Flow cytometry analyses of podoplanin expression in primary glioblastoma stem-cell like spheroid cultures showed that the number of podoplanin-expressing cells varies heavily between different cultures. Since it has been postulated that glioblastoma stem cells are enriched at the surface of spheroids rather than its core, immunofluorescence stainings of cryosections were conducted to determine the localization of podoplanin-expressing cells. Podoplanin expression was found to be homogeneous throughout the spheroid, indicating that it might be expressed not only in glioblastoma stem cells but also in other cell populations within the spheroid, such as transit-amplifying cells.

An important hallmark of tumor stem cells is the ability to create a cellular hierarchy. To investigate if podoplanin-expressing cells are capable of this, spheroid cultures were fluorescence activated cell-sorted into podoplanin-positive and -negative subpopulations. Both podoplanin-positive and -negative subpopulations yielded progeny that was heterogeneous with respect to podoplanin expression, and hence no hierarchy. Moreover, podoplanin was found to be quickly re-expressed in podoplanin-negative populations, and the extent of re-expression was dependent on the original percentage of podoplanin-

expressing cells in the respective culture, hinting at a potential intrinsic balance. Global gene expression profiling of podoplanin-positive and -negative cells and subsequent analysis of differentially expressed genes revealed a preponderance of genes associated with proliferation in the podoplanin-positive group. The analysis also revealed that podoplanin-positive cells harbor a mesenchymal gene expression signature, while their negative counterparts harbor a proneural signature.

Possible co-expression of podoplanin with other, already described potential glioblastoma stem cell markers was also subject of the study. In order to identify suitable glioblastoma stem cell marker candidates for co-expression studies with podoplanin, the expression of several candidates (CD133, Nestin, Sox2, CD44, CD29, CD49f, CD15, Bmi1 and Msi1) was analyzed in the primary glioblastoma spheroid cultures using complementary methods such as gene expression profiling, real-time quantitative polymerase chain reaction and flow cytometry. Subsequently, only CD44 and CD24 were found to have a substantial positive or negative correlation with podoplanin expression. To determine if a combination of these markers defines a glioblastoma stem cell population, cells from the spheroid cultures were single cell-sorted into all eight possible subpopulations according to their CD44, CD24 and podoplanin expression status and compared in terms of spheroid formation capacity and the ability to re-establish the original heterogeneity of the tumor. No significant differences were observed, suggesting that the combination CD44/CD24/PDPN does not specify a distinct population of glioblastoma stem cells.

To the best of knowledge, this is the first study providing an in-depth characterization of podoplanin expression in primary glioblastoma stem cell-like spheroid cultures as well as the first study to demonstrate a spheroid formation-promoting effect of podoplanin in glioblastoma cells. The investigation yielded novel findings on the role of podoplanin in glioblastoma stem cell-like spheroid cultures and contributes to the debate on the suitability of podoplanin as a glioblastoma stem cell marker. Taken together, the results presented here indicate that podoplanin is not suitable as a marker for glioblastoma stem cells, neither alone nor in combination with the other investigated marker candidates. The data suggest that in glioblastoma stem cell-like spheroid cultures, podoplanin expression is associated with a specific functional state of the cells promoting their adhesion and assembly into spheroids rather than a distinct cell type.