Title: Glioma-associated mesenchymal stem cells indicate new avenues for brain tumor therapy.

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Abstract: Throughout the last years researchers recognized the pathological and therapeutic relevance of the brain-tumor microenvironment. Our research-group showed that factors released from endogenous neural stem and precursor cells (NPCs) can be adopted for glioma therapy. Our new data indicate that another glioma-associated cell-type, mesenchymal stem cells (MSCs) has even a broader role in primary brain tumors: Depending on the environmental conditions MSCs can release tumor-suppressive or tumor supportive factors. In a first part of our project we will explore the molecular factors that are responsible for the tumor supporting role of MSCs - these MSC-derived molecules will constitute new targets for adjuvans treatments of malignant gliomas. In a second part of our project we will establish the MSC-released tumor suppressive molecules - this will enable us to translate these natural, endogenous anti-tumor factors into new glioma-therapeutics. In a collaborative approach with researchers from Heidelberg (Christel Herold-Mende) and Hamburg (Katrin Lamszus) we have the unique opportunity to uncover different aspects of MSC-mediated effects on glioma progression, including immune-modulation and metabolic support of tumor growth.

1. State of the art

1.1. Brain tumors and the brain-tumor microenvironment: Malignant brain tumors like glioblastomas (GBM) belong to the most aggressive forms of cancer. The current survival rates for patients with GBM are less than 12 months with debulking surgery alone, 12.1 months with the addition of radiotherapy, and 14.6 months after adding radio- and chemo-therapy (the standard of care is the DNA-alkylating agent temozolomide)[1]. A significant barrier for therapeutic treatment is built-up by the tumor-host interface[2], which supports invasion, angiogenesis and resistance to anti-angiogenesis. In order to effectively treat this disease, we need to fully understand the basic biology behind its development and progression, including the effects of other cells in the tumor microenvironment (see Figure-1, below).

1.2. Mesenchymal stem cells are part of the GBM microenvironment. Mesenchymal stem cells (MSCs) are tissue stem cells which retain a potential to differentiate into osteocytes, chondrocytes or adipocytes[3]. MSCs have initially been purified and characterized from the bone marrow and today MSCs are known to be preserved in all organs except from the peripheral blood[3]. The MSCs are recognized for their ability to migrate to zones of tissue injury and are recruited into tumors[4]. The tropism of exogenously cultivated and genetically modified MSCs is e.g. exploited to deliver therapeutic substances to GBM[4]. Recently, endogenous MSCs could be purified from GBM[5]. These GBM-derived MSCs do not form tumors upon transplantation showing that MSCs are not a transdifferentiated tumor cell-type, but indicate that MSCs are part of the natural GBM microenvironment. A range of pro- or anti-tumorigenic effects of MSCs has been reported, which comprise pro- or anti-angiogenic effects[6, 7]. Furthermore, MSCs were found to mediate direct effects

against GBM cells, which include inhibition of tumor cell migration[8] or induction of GBM cell-death[9, 10]. In summary, this shows that MSCs are a potentially important component of the brain tumor parenchyma. However, the signaling pathways which control the pro- or anti-angiogenic effects of MSCs are unknown. Likewise, the molecular nature of MSC-derived tumor suppressive components has not been identified. As previously shown by our laboratory (see below), parenchymal components can be a target of GBM adjuvans therapy and natural tumor suppressors (released from parenchymal cells) can be adopted for therapeutic use. In the current project we will explore, if MSCs are a relevant targets for anti-angiogensis and we seek to identify the MSC-derived GBM-suppressing factor(s).

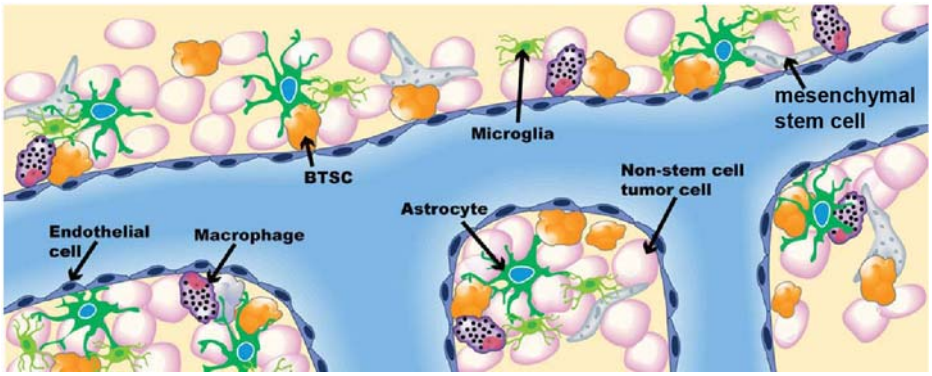

Figure-1. The glioblastoma microenvironment. The microenvironment of glioblastoma is composed of several stromal cell types which are believed to make distinct contributions to tumor progression and invasion. These cells include but are not limited to mesenchymal stem cells, astrocytes, macrophages, pericytes, and endothelial cells; modified from[11].

2. Own contributions. Previously, we showed that different glioma-associated parenchymal cells have very profound effects on glioma progression[11, 12]. For example, our data indicated that gliomas manipulate the immune-phenotype of brain specific macrophages (termed microglial cells) to propel tumor cell invasion[13-17]. We observed that GBM actively induce the accumulation of brain-specific macrophages (termed microglial cells) by releasing the chemoattractant GDNF[18]. Hence, GBM consist to a large part, up to 30%, of microglia. Tumor-associated microglia initiates a metalloprotease-cascade, which mediates extracellular matrix degradation and in turn facilitates the diffuse infiltration of GBM cells into the brain parenchyma[14]. This is pathologically important since the enormous invasion of GBM prevents complete surgical resection or efficient radiotherapy of these tumors. Furthermore, we have shown that some cells of the tumor microenvironment can have efficient anti-tumorigenic effects. We reported that neural precursor cells (NPCs), which e.g. generate all neurons, astrocytes and oligodendrocytes during development and which persist in the adult CNS, can release different anti-tumorigenic substances (these are GBM cell-death inducing lipids named vanilloids[19] and bone morphogenetic protein-7; BMP7[12]). The anti-tumor response of NPCs is restricted to the young brain, but exogenous application of NPC-derived tumor suppressors (e.g. of the vanilloids) also has therapeutic effects in the aged brain[19]. Altogether, our data showed that brain tumor parenchymal cells can indicate new tumor therapies (as shown with NPC-derived anti-tumorigenic molecules) or can be a new target for adjuvans therapies against gliomas (as demonstrated for microglia). The role of the tumor micro-environment as a target for adjuvans therapies is just emerging and the use of natural tumor suppressors released from subsets of parenchymal cells is largely unexplored[2].

3. Pilot studies, pro and anti-tumor effects by MSCs. We found that mesenchymal stem cells (MSCs) can induce both pro- and anti-tumorigenic effects in human primary GBM cells in vitro, depending on simple changes in culture conditions. Primary cultures: Bone marrow aspirates are obtained at the University Clinics, Munich; filtered cells are purified by gradient-centrifugation, the mononuclear cells are collected, washed and cultured in DMEM 20%FCS - only early passages are used for experiments. Glioblastoma tissue is obtained from the Clinic for Neurosurgery, Munich; biopsies are homogenized filtered purified by gradient-centrifugation, the mononuclear cells are collected, washed and resuspended in Sorting Buffer for depletion of CD133+ cells. For labeling, CD133 Microbeads and (MicroBead Kit, Miltenyi) are used. The CD133-negative fraction (flow-through) has been cultured in DMEM 20%FCS. The isolated BM and gbMSC are analyzed for their marker expression according to the definition for minimal criteria of mesenchymal stromal cells[20]. Hence, the cells are stained with antibodies against CD73, CD14 (Miltenyi), CD90, CD105, CD45, CD31 (all AbD Serotec) and CD34 (BD) and the corresponding isotype controls. Cell fluorescence is evaluated by flow cytometry on a FACS Calibur instrument (Becton Dickinson) and the data are analyzed by using FlowJo software (Tree Star, Inc.). A total of at least 20000 events are acquired to determine the expression of the different cell surface markers tested and PI staining is used to exclude dead cells.

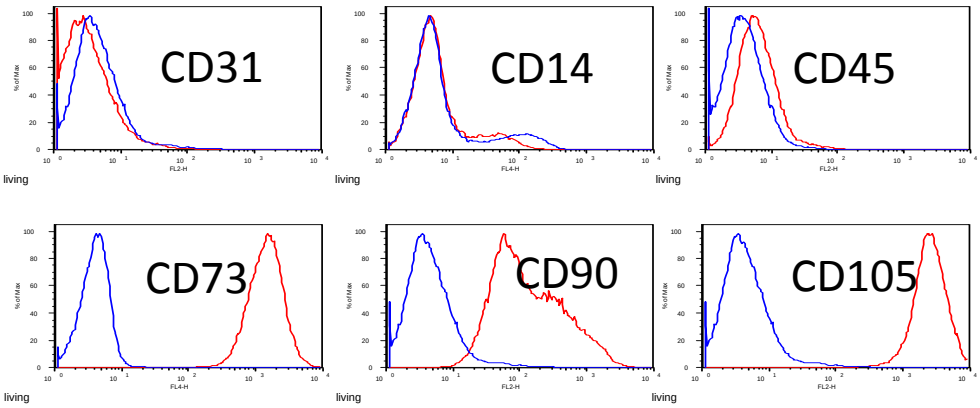

Figure-2. The marker profile of human mesenchymal stem cells, as determined by FACS analysis. Cells purified from a human glioblastoma sample were analysed by FACS and identified as MSCs, by strong positivity for CD73, CD90 and CD105 (> 95% of cells positive) and by very low expression of CD31, CD14 or CD45 (< 5% of cells positive).

Also MSCs are differentiated into the adipocytic-, chondrocytic- and osteocytic-lineage. All cultivated MSCs will be analyzed for chromosome aberrations by array-CGH. Additionally, we will determine the specific genomic aberration profile for each parental tumor. Thereby, we will determine that our bone-marrow and tumor derived MSCs did not acquire genomic defects during cell culture. Also, we will assure that GBM-derived MSCs do not harbor genomic lesions that are similar to the tumor biopsy (from which the MSCs originated).

MSCs are pathologically relevant as they accumulate in the tumor microenvironment and the tumor supporting effects of MSCs are a potential target for new (MSC-directed) adjuvans-therapies. The anti-tumorigenic factors released from MSCs are highly interesting as they hold the key for new GBM therapies: the MSC-derived molecules (or pharmacological compounds mimicking the anti-tumor action of MSC-derived factors) can be used to induce GBM cell-death.

We have now set-up a simple cell culture model, which can be used to switch MSCs from a pro- to an anti-tumorigenic state. We will use this model to uncover the molecular pathways controlling pro-

and anti-glioma effects of MSC. The pro-tumorigenic effects may be suppressed by new adjuvans therapies directed against GA-MSCs. The anti-tumor effects will be characterized molecularly and will serve as a blue-print to establish new glioma therapeutics.

We have established a range of MSC cultures that are derived from human bone marrow (e.g. BM30 and BM6; see Fig. 1) and from human GBM (e.g. gb863; see Fig. 1), which have a bona fide MSC marker profile (as determined by FACS measurements) and which are genetically intact (i.e. GBM-derived cells are not of tumor-origin; as determined by array-comparative genomic hybridization; array-CGH). MSCs are cultivated under standard conditions (i.e. in medium containing 20% fetal calf serum; FCS) and then primary human glioblastoma cells (e.g. GBM20; see Fig. 1) are exposed to supernatants from such cultures (MSC-conditioned media). The viability of the GBM cells decreases after three days of stimulation with MSC-conditioned media (containing 20%FCS; see Fig. 1A). However, when MSCs are maintained for short periods of time (3 days in our experiments) under starvation conditions (0% FCS) and if GBM20 cells are the challenged with MSC-conditioned media we observe that the viability of GBM cells increases under these very restrictive conditions (see Fig. 1B). Hence, MSCs release anti-tumor factors under standard conditions, but can strongly support the viability of GBM under stressful (nutrient restriction in 0% FCS medium) conditions. These data were retrieved in four independent experiments and were backed up by additional studies investigating the GBM cell number after exposure to MSC-conditioned media (under both culture-conditions).

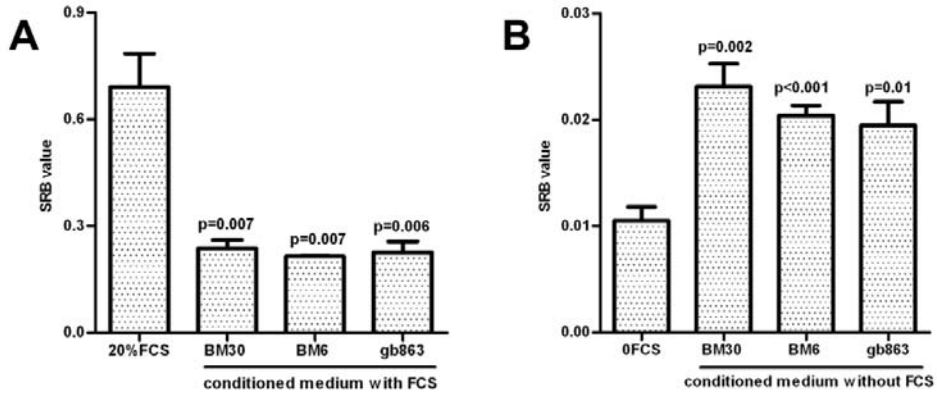

Fig. 3. MSCs have profound impact on GBM viability. (**A**)Cell-viability measurements (indicated as SRB-values) showed the growth of a glioblastoma primary culture was suppressed by MSC-conditioned media (BM30, BM6, gb863) with 20% FCS, i.e. the SRB values in MSC-exposed GBM cells are lower than under control conditions (20% FCS). (**B**) Cell-viability of GBM cells is increased by MSC-conditioned media containing 0% FCS, i.e. the SRB values in MSC-exposed GBM cells are higher than under control conditions.

Importantly, we found that the MSC-mediated anti-tumor effect is molecularly different from previously described tumor suppressive mechanisms by tumor stromal cells[19]. We found that the MSC-derived molecules, which rapidly modulate glioma cell numbers, are not vanilloids or cannabinoids (i.e. selective antagonists for vanilloid or cannabinoid receptors could not blunt the anti-glioma effects of MSC-released molecules; data not shown). Hence, the MSC mediated tumor suppression is pharmacologically different from the NPC-derived tumor suppressors (vanilloids - and the pharmacologically related cannabinoids) that were previously described by our group[19].

4. Aims of the proposal

We have determined previously, that MSCs which are kept in serum-containing medium **(A)** exert anti-tumor effects against primary human GBM cells and against human glioma cell lines (see pilot studies). **(B)** Interestingly, a shift in the culture conditions (exposing MSCs to serum-free medium) converted the tumor-suppressive functions into pro-tumorigenic actions. Now we will use this simple experimental set-up to uncover those anti-tumor factors that are released from MSCs and to uncover new targets for stroma-directed adjuvans therapies in gliomas. Currently, it is unclear if peripheral MSCs (e.g. bone-marrow derived MSCs, which exert a strong tropism towards gliomas) and glioma-associated MSCs are molecularly or biologically distinct. Our pilot-studies suggest that both cell-entities mediate very similar effects on gliomas in vitro (when applying serum-rich or serum-free cultivation conditions). In our current project we will investigate if this also applies to a larger number of MSC samples from both the bone-marrow and from glioma.

(A) We have previously shown that endogenous stem and precursor cell-derived factors (from NPCs) can be used for glioma therapy. Our new data exhibited that MSCs also mediate an anti-tumor effect against glioma cells, which can be exploited for therapeutic use. Importantly, we have previously determined that the MSC-mediated tumor suppression is molecularly distinct from the NPC-mediated cell-death induction in gliomas. Hence, we can discover a new tumor-suppressive factor, which is released from endogenous cells in humans and which should therefore be tolerated by glioma patients after exogenous application.

(B) Serum-free culture conditions induce a tumor supporting phenotype in glioma cells, which may indicate that glioma cells living under conditions of poor nutrition or in stressful environments benefit from the support of neighboring MSCs. Glioma cells may encounter a stressful/ nutritionally poor environment in therapeutically/ pathologically relevant areas like e.g. hypoxic regions and in the invasive zone or during therapy. We will explore the molecular factors that are responsible for the tumor supporting role of MSCs in an environment that may otherwise be unfavorable for glioma cells. These MSC-specific factors may have prognostic power and can also constitute a target for adjuvans treatments, which are suited to reduce invasion or therapeutic resistance in gliomas.

5. Work plan.

We will perform **(5.1.)** genetic and **(5.2.)** proteomic assays to explore the MSC-derived molecular factors that are responsible for mediating the pro- and anti-tumor effects on glioma cells. **(5.3.)** We will use molecular modeling, genetic and pharmacological approaches to validate candidate MSC-derived factors that can suppress or support gliomas. **(5.4.)** We will determine the power of the newly discovered MSC-specific molecules as prognostic markers for glioma patients.

5.1. Work-package-1; Genetic assays to uncover the MSCs-derived factors mediating pro- and anti-tumorigenic effects in glioma cells.

Cell culture: Bone marrow- and glioma-derived MSC cultures are established in our laboratory. The live-span of these cultures is limited in vitro and only early passages will be used for our experiments. Therefore, new MSCs will be obtained by a standardized protocol. Briefly, bone marrow aspirates are obtained from the Stiftung Aktion Knochenmarkspende Bayern from healthy donors and isolated as described [21]. Glioblastoma tissue will be obtained from the Departments of Neurosurgery in Munich (University Clinics Munich; Prof. J.C. Tonn) and in Berlin (Charité University Clinics; cooperation partner is Dr. M. Synowitz). GBM samples will be dissociated into single cell suspensions, mononuclear cells will be separated by gradient centrifugation, collected, washed and depleted of CD133+ cells by microbeads (Miltenyi). The CD133-negative fraction is expanded in vitro and will be

purified by FACS (using a BD Aria II cell sorter) with pre-established markers (see also pilot studies). Per definition MSCs are plastic-adherent cells expressing CD105, CD73, and CD90 (> 95% of cells must be positive for these markers) and must be devoid of CD45, CD34 or CD14 (< 5% of cells may be positive for these markers)[20]. Furthermore, all MSC cultures must have the potential to differentiate into the osteoblast-, chondroblast- or adipocyte-lineage. MSC differentiation will be determined in vitro after exposing MSCs to defined osteogenic, chondrogenic or adipogenic conditions and by using selective differentiation markers[20].

Microarrays: We will use expression analysis (by microarrays; Affymetrix Gene ST 2.0 arrays) to compare the gene-expression profile of human MSCs (from bone-marrow or from GBM under both cultivation conditions, i.e. with/without serum) and from GBM cells receiving the different MSC-conditioned media (as compared to non-conditioned control medium). We will analyze three different MSC lines (under each condition) and three primary human GBM cultures receiving conditioned medium (from one MSC-line cultivated with/without serum). Hence, we will prepare 18 samples for gene-expression profiling by microarray analysis.

Array-CGH: Subpopulations of highly aggressive and tumorigenic glioma cells (so-called glioma stem cells) have a potential to trans-differentiate into other cell-lineages like e.g. endothelia or pericytes. Pericytes (in many cases) are MSC-like cells and therefore, there is a possibility that glioma stem cells may also generate MSCs. Furthermore, normal glioma cells may (theoretically) exhibit an aberrant marker profile and differentiation potential that could qualify them as MSCs. In order to differentiate physiological MSCs from tumor derived MSC-like cells we will investigate the genomic make-up of our cultivated cells by comparative genomic hybridization on array-chips (array-CGH). This experiment will show, if our cells are euploid (genetically intact) or of tumor origin.

Intra-experimental controls: In one large part of our experiments we maintain MSCs under serum-free conditions. This is different from the standard cultivation technique for MSCs (which includes 20% of serum in cell culture media) and can potentially change some characteristic features of MSCs. In our pilot experiments we observed that for the timing of the experiments (3 days in serum-free medium) our MSCs retained their MSC-phenotype, nevertheless we will control the MSC-marker profile before and after all experiments.

5.2. Work-package-2; Proteomic assays to uncover the molecular factors from MSCs mediating pro- and anti-tumorigenic effects in glioma cells. In cooperation with the Charité Core Facility for Proteomics we will use a combination of size exclusion-, ion exchange- and reverse phase-chromatography to fractionate our MSC conditioned medium. Thereby we will generate fractions according to the molecular weight and polarity of the protein content of the media. Initially, we will separate a smaller range of fractions that are given to human GBM cultures, subsequently the GBM cell-viability will be read-out. If a fraction contains e.g. a cell-death inducing stimulus for GBM cells, we will subsequently separate that particular fraction further and again determine the cytotoxic responses in GBM cultures. The protein-content of a fraction of MSC conditioned medium that induces a strong physiological/pathological response (i.e. modulates GBM cell-viability) will then be identified by mass-spectrometry. For our collaborative project together with the group in Hamburg we will explore (by mass-spectrometry) if MSCs (under different in vitro conditions) release cytokines or metabolites which can support GBM cell growth.

5.3. Work-package-3; Molecular modeling and target-validation. Expression analysis and (in silico) molecular pathway modeling will indicate which signal transduction pathways control the pro- and anti-tumorigenic phenotype in MSCs. Genetic and proteomic marker profiles that are obtained in the

essays described above will be analysed in silico in cooperation with the respective core-facilities of the Charité. In the bioinformatics approach we will sequentially analyze the following data: Firstly, the genetic profiles of MSCs maintained under both cultivation conditions (i.e. in serum-containing or serum-free medium) will indicate those genetic changes that also control the anti- to a pro-tumorigenic phenotype. Hence, this first dataset harbors gene expression changes that indicate the release of tumor-suppressive or -supportive factors from MSCs. Secondly, the expression profile of glioma cells that are exposed to MSC-derived pro- or anti-tumor factors (as compared to controls) will indicate the signaling pathways that are activated in glioma cells by the MSC-derived molecules. Thirdly, we determined (by proteomics) all MSC-released factors in a biochemically separated fraction which induces e.g. the reduction in glioma cell viability. The combined data will allow us to narrow down the number of candidate factors (from MSCs) which are present e.g. in anti-tumorigenic MSCs (but which are absent in pro-tumorigenic MSCs), which are released from MSCs and which activate signaling in glioma cells. We will then interfere with the expression or the release of these candidate factors by genetic (siRNA) or pharmacological modulation of MSCs to determine the physiological role of the respective molecule. For our collaborative project together with the group in Heidelberg we will investigate in expression arrays if GBM induce gene expression changes in MSCs, which are indicative of a switch towards an immune-modulatory phenotype.

5.4. Work-package-4; MSC-specific prognostic markers. In work-package-3 we have determined those genes which are specifically expressed in MSCs cultivated as tumor suppressive or as tumor supporting cells. In collaboration with our co-applicant and collaboration partner Christel Herold-Mende (University of Heidelberg) we will determine those genes which can be specific for MSCs under pathological conditions, i.e. which are absent from the tumor-free brain, or from T-cells, microglia, macrophages and tumor cells proper. These data will be retrieved from the micro-array results and will subsequently be validated by immunohistochemical analysis in human and mouse brain tumor samples. Then we will determine which of these MSC-specific molecules has prognostic relevance for glioma patients, by interrogating a glioma database (containing gene-expression and survival data), like e.g. the TCGA (http://www.cbioportal.org/public-portal/) and the REMBRANDT (https://caintegrator.nci.nih.gov/ rembrandt/login.do) databases.

6. Time-course and milestones for the experiments in Munich

Timeline: months	*6*	*12*	*18*	*24*
Work-package-1; Genetic assays to uncover the MSCs-derived factors mediating pro- and anti-tumorigenic effects in glioma cells.				
Work-package-2; Proteomic assays to uncover MSC-released molecular factors mediating pro- and anti-tumorigenic effects.				
Work-package-3; Molecular modeling and target-validation.				
Work-package-4; MSC-specific prognostic markers.				

7. Milestones for the laboratory in Munich

Work-package-1; Genetic assays to uncover the MSCs-derived factors mediating pro- and anti-tumorigenic effects in glioma cells. In the first year of the project we will generate a micro-array dataset of experimentally treated MSCs and glioma cells. We will control for the physiological function of all our cells (with cell biological assays) and we will gain new tumor cells as well as tumor-derived or bone-marrow derived MSCs, which will be used to back-up findings by our microarray screening approach: All cells - previously established and new cells - under all experimental conditions - will also be analyzed by additional techniques (e.g. real-time PCR) for gene expression changes (as obtained by microarrays) of some defined genes (which are pathologically relevant as determined by e.g. by database interrogation).

Work-package-2; Proteomic assays to uncover MSC-released molecular factors mediating pro- and anti-tumorigenic effects. We will biochemically separate a fraction of proteins from MSC conditioned media, which mediates a defined biological activity in glioma cells (i.e. a pro- or anti-tumorigenic effect). Then we will use proteomics to identify all proteins in that fraction of medium.

Work-package-3; Molecular modeling and target-validation. We will model signal transduction pathways in glioma cells and MSCs to identify the mechanisms (and molecular nature) of the pro- or anti-tumorigenic effect mediated by MSCs. The functionality of these candidate-molecules will by validated by loss-of-function (siRNA or pharmacological blockade) or gain-of-function (e.g. forced expression) approaches.

Work-package-4; MSC-specific prognostic markers. The validated MSC-derived pro- or anti-tumorigenic molecules identified in work-packages 1 to 3 will be investigated for specificity in MSCs and for their pathological relevance. We aim to uncover MSC-specific prognostic markers.

Outlook: **(1.)** In the future, we will interfere pharmacologically with pro-tumorigenic effects of glioma-associated MSC to convert pro-tumorigenic mesenchymal host cells into tumor suppressive cells. This would open up a new avenue for glioma adjuvans therapies. **(2.)** We will determine if our newly discovered, MSC-specific prognostic markers can serve as predictive markers for MSC-directed adjuvans therapies. **(3.)** We will investigate if the MSC-derived molecules, which may be larger biomolecules that can be difficult to synthesize or may have limited distribution within the body, can be subsidized by small molecules that are therapeutically applicable.

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8. Budget, costs for the group in Munich:

Personnel:	1 postdoctoral scientist for 2 years (annual salary according to DFG listing is € 59.700,00)	
	Personnel (total costs for 2 years):	€ 119.400

Justification: This subproject is methodically challenging and work intensive. We request funding for a post-doc position as we would need a scientist that is well-trained and can immediately start the project.

Consumables:	
Media, supplements and plastic materials for cell-cultures	€15.000
Array-CGH	€15.000
Reagents for qPCR	€10.000
Cell based assays (proliferation, viability, cell-death)	€10.000
Reagents and antibodies for immunohistochemistry	€8.000
Reagents and antibodies for FACS	€8.000
Kits and Reagents for mRNA amplification and array hybridization	€6.000
Affymetrix ST 2.0 Gene expression arrays	€6.000
Total consumables:	€78.000
Total costs for 2 years:	€197.400