Anti-Oncogenic Activities Exhibited By Secretomes Of Mesenchymal Stem Cells Are Mediated By Modulation Of *KITLG* and *DKK1* Genes In Glioma Stem Cells.

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Abstract

Background. Cancer stem cells (CSCs) use their stemness properties such as self renewal, toxicity, plasticity, and communication with the tumor microenvironment (TME) to perpetuate their lineage and survive chemotherapy. Learning how to interrupt the self renewal ability or modulate the interaction of CSCs with the TME signaling will dramatically improve therapeutic impact on patient's remission. Anti-tumor properties of mesenchymal stem cells (MSCs) are currently under investigations and different approaches have been applied to gain beneficial effects. However, different types of MSCs yielded different conflicting results. In order to investigate if different types of MSCs preconditioned in the same culture conditions can exert alike anti oncogenic effect on glioma stem cells, we planned this study.

Methods. GSCs were isolated from U87 cell line by FACS cell sorter, characterized and established as gliospheres. Condition media from MSCs of Wharton Jelly (WJ-MSCs) and bone marrow (BM-MSCs) were harvested and used as treatments on gliospheres (3D) to investigate the effect on proliferation, invasion and self renewal properties of GSCs. Microarray analysis was used to determine the effect at molecular level. Specific human CSC gene arrays were applied to validate the findings of the microarray explicitly the pluripotency of the GSCs.

Results. Our results from functional and molecular assays showed that condition media (CM) from both types of MSCs inhibited the metabolism by interrupting oxidative phosphorylation, arrested the cell cycle, induced cell differentiation, targeted the pluripotency and up-regulated the immune response in GSCs. Moreover, condition media from both types of MSCs significantly affected the same genes (*KITLG* and *DKK1*) causing a similar effect while using slightly different routes and signaling pathways signifying their individual effects.

Conclusion. We conclude that mesenchymal stem cells possess antitumor properties and paracrine factors of mesenchymal stem cells in combination with anti-immune modalities can provide novel therapeutic targets for glioma treatment.

1. Background

The modern theory of carcinogenesis focuses on the presence of malignant transformations in adult tissue stem cells (1). The theory of cancer stem cells (CSC) is old, as it was first described in 1973 (2). Later, in 1997, the existence of a heterogeneous tumor cell population was mentioned in leukemia for the first time (3). Analysis of this cell population revealed stem cell properties, such as self-renewal capacity, high proliferation rate and maintenance of tumor cell population (4,5). These properties form the basis of the modern accepted hypothesis of cancer stem cells (CSCs) (6, 7, 8).

The CSC hypothesis gained credibility because all main cancer-origin theories (genetic/epigenetic events, chemical-, infection-, virus-induced carcinogenesis) indicated that the tissue stem cell is involved in the generation of cancer (3,9,10). Moreover, recent studies also suggest that CSCs are a major driving force for tumorigenesis, metastasis, aggressiveness and resistance to treatment (11). The presence of CSCs

has been reported in both hematologic malignancies and solid tumors (*i.e.*, breast cancer, brain tumors, malignant melanoma, or prostate cancer) (12, 13).

Glioblastoma multiforme (GBM) is the most malignant type of brain tumor and is still incurable, with the overall survival rate being less than 15 months (14, 15, 16). The highly infiltrative growth of GBM and its resistance to chemo/radiotherapies, prevents complete elimination of tumor cells, despite improvements made in surgical techniques and therapeutic protocols (18). The highly tumorigenic subpopulation of glioblastoma CSC (GSCs) is thought to be one of the reasons for a high GBM recurrence rate (19). The resistance to radiation and chemotherapy of GSCs suggests that new therapeutic approaches are needed to focus on specific targeting of GSCs to improve the survival of GBM patients. Many current therapies, such as the drugs that target different signaling pathways, tumor transcription factors, cells in the tumor microenvironment, and the use of tumor-inhibiting miRNA (20) are presently being investigated in the development of novel therapeutic targets.

However, targeting GSCs can be tricky as GSCs would normally be quiescent and enter the cell cycle only after exposure to external stimuli such as growth factors (21). Therefore, GSCs would only be vulnerable to treatments while they are in an actively growing state.

Cellular therapy for cancer is being revisited in view of using mesenchymal stem cells (MSCs) for cancer treatment. MSCs are multipotent stem cells most often isolated from perinatal sources such as umbilical cord Wharton's jelly (WJ-MSC), placenta (PL-MSCs) and adult tissues such as bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) (22). They have been shown to have wide therapeutic potential because of their immuno-modulatory ability, wound- and neoplasm-directed homing, and tissue repair ability (23–24). Additionally MSCs have been shown to cross the blood-brain barrier (BBB), a characteristic that represents an important aspect when considering MSCs as a therapeutic option for brain tumors (25). Recently, pre-clinical studies demonstrated that MSCs might suppress the growth of GBM cells, though conflicting studies have also found the opposite effect (26). However, the mechanisms by which MSCs may suppress GBM growth have not been illustrated yet.

There has been active research to enhance the anticancer effects of mesenchymal stem cells, such as overexpressing anticancer genes (27) or using engineered delivery systems with an anticancer drug (28). However, these methods can reduce the viability of mesenchymal stem cells, modify endogenous genes, or exert toxicity on normal cells. Such negative effects limit the clinical application of cell-based therapy. Moreover, the issue of whether the MSCs of the tumor microenvironment and their molecular crosstalk with other cells results in tumor-suppressive effects or, favors tumor promotion is still unclear (29).

It has been reported that paracrine factors of MSCs showed enhanced beneficial effects on recovery from injury or disease in some experimental models (30–35). MSC harvested from numerous anatomical locations display similar immunophenotypic profiles. However, the secretome of MSCs appears to vary significantly, depending on the age of the host and diverse stimuli present in the niches where the cells reside. (36). In order to improve the therapeutic ability of MSCs, the composition of the MSCs' secretome can be modulated by preconditioning the MSCs during *in vitro* culture (37). Preconditioning of MSCs by

hypoxia, inflammatory stimulus, and other factors/conditions prior to their use in therapy is a new strategy currently being investigated (38).

Based on this knowledge, we planned to investigate the anticancer effect of the secretome from MSCs preconditioned with GSC growth factors to explore the biological mechanisms and signaling pathways that are associated with anti or pro-tumorigenic effects. In doing so, we evaluated the effects of factors secreted (condition media, CM), under same culture conditions, from two different populations of MSCs (bone marrow-derived (BM-MSC) and Wharton's jelly-derived MSC (WJ-MSC) on GSCs survival, invasion and self-renewal capability. Further, the genetic changes at signaling pathway levels were explored by microarray analysis followed by further validation of the effect on the pluripotency of GSCs with human CSC arrays.

2. Methods

2.1. Isolation of glioma stem cells (GSC) from GBM cell line (U87- ATCC)

2.1.1. Monolayer generation of cell line

The GBM cell line U87 (U87MG ATCC-HTB14™) was expanded according to the protocol described by ATCC with a slight modification. Briefly, the cell line was expanded in standard culture media,{DMEM-F121:1, L-Glutamine 200 mM,10,000 U/ml penicillin/streptomycin, 25 µg/mL amphotericin B and 10% heat-inactivated Fetal Bovine Serum(GIBCO/Invitrogen Corporation,USA)}. The cells were seeded at 10^4 cells/cm² in T75 cultures flasks and maintained at 37°C and 5% CO₂. The culture medium was exchanged every 2-3 days.

2.1.2. FACS Sorting and enrichment of glioma stem cells

Cell line expanded at different passages (P3-P9) was investigated for the percentage of CD133 positive population which was sorted by using FACS JAZZ cell sorter (BD, Biosciences, USA) according to manufacturer's instruction. Briefly, upon reaching the 80-90% confluence, U87 cancer cells were harvested using 0.25% trypsin EDTA (Invitrogen, USA), washed twice with cold PBS (Invitrogen, USA) and centrifuged at 300xg for 5 minutes. Cell pellet was resuspended in 100 ul of BD FACS staining buffer (BD Biosciences, USA) and 10ul of CD133 antibody (eBioscience, USA) were added. Mixed well and put at 4°C for about 30 minutes in dark. Next 500 ul of cold PBS were added to wash the cells and centrifuged at 300 xg for 5 minutes and pellet was resuspended in 500 ul PBS for sorting. Sorted populations were divided into positive and negative fractions and were evaluated for the enrichment of CD133 positive cells using FACS Canto ii and analysed by FACS DIVA software Version 7 (BD, Bioscience, USA).

2.2 Characterization of sorted populations.

2.2.1. First Gliosphere generation.

Sorted tumor cells from both positive and negative populations were directly seeded in cancer stem cell media later termed here as gliosphere media (GSM).{Dulbecco's Modified Eagle Medium/F12 (Gibco, USA),supplemented with N2 (Gibco, USA), EGF (20 ng/mL, Invitrogen), bFGF (20 ng/mL, Gibco, USA), leukemia inhibitory factor 10 ng/ μ L (LIF; Chemicon, Germany), and B27 (1:50; Life Technologies, USA)}, in 6-well ultralow attachment plates (Corning, USA) to enrich for glioma stem cells (gliospheres) and were maintained at 37⁰C in a humidified atmosphere of 5% CO₂/95% air. The morphology of gliospheres from positive and negative populations was observed under a phase contrast microscope (Zeiss, Germany) for 10 days.

2.2.2 Glio-subsphere generation.

CD133⁺ and CD133⁻ selected cell populations, derived from first gliospheres, were dissociated using StemProAccutase Cell Dissociation Reagent (GIBCO, USA) according to manufacturer's instructions. Cells were subsequently re-suspended in GSM and seeded in 24-well ultralow plates (Corning, USA) at 10³cells/well. Formation of free floating sub-spheres was observed by phase-contrast microscopy (Zeiss, Germany) and the experiment was conducted till passage 12 (gliosphere, G12). Cells in each passage were kept in culture between 7-10 days.

2.2.3. Monolayer generation

Similarly small fractions of sorted cells from positive and negative populations were cultured in standard culture media (DM-F12+10%FBS, GIBCO, USA) in order to investigate their expansion ability adherently after sorting. The morphological changes were observed under phase contrast microscope (Zeiss, Germany). The experiment was conducted for 6 passages and one passage time was kept between 5-7days.

2.2.4.Immunofluorescent assay of CD133 positive population

A panel of GSC markers, neuronl lineage markers and late differentiated neuronal markers, was selected (Table.1) to characterize the sorted CD133⁺ population by immunocytochemistry. Briefly, subspheres at G13 were harvested by treatment with StemPro Accutase Cell Dissociation Reagent (GIBCO, USA), washed with PBS (pH = 7.4 GIBCO,USA) followed by centrifugation. After resuspension, cells were seeded on polyL-Lysine coated cover slips (sigma-Aldrich, USA) at the seeding density of 20⁴cell/ cover slip and were immersed in GSM with 2% FBS (GIBCO, USA). After two days the cells were fixed with 4% formaldehyde for 20 minutes followed by washing. Cover slips were then permeabilized with 0.3% Triton-X-100 for 15 minutes at room temperature. Blocking was performed with 10% normal goat serum (GIBCO,USA) for 1 hour at room temperature. Cells were stained with the primary antibodies in 1% blocking buffer overnight at 4⁰C with shaking followed by wahing with 0.1% Triton-X-100 in PBS three times for 5 minutes each. Next, cells were stained with secondary antibodies (Table.1) in 1% blocking buffer for 1hour at room temperature in the dark. Cells were washed with 0.1% Triton-X-100 in PBS three times for 5 minutes each. Nuclei were stained with DAPI (Thermo Fisher Scientific, USA) for 5 minutes,

coverslips mounted onto slides. Cell imaging was performed on inverted phase contrast fluorescence microscope (Zeiss Axio Observer Z1, Germany). The description of the primary and secondary antibodies used for the assay is given in the Table 1.

2.3. Functional Assays

2.3.1. Preparation of MSCs condition media (CM) under Gliosphere Media Conditions

Mesenchymal stem cell lines of bone marrow (BM-MSCs) and Wharton's Jelly (WJ-MSCs) were acquired from Cell Therapy Center, University of Jordan which had been expanded and characterized as described previously (39). Three biological samples of WJ-MSCs (WJ1, WJ2, WJ3) and three from BM-MSCs (BM1, BM2, BM3) at passage 3 were used to generate condition media. All six cell lines were plated into T75flasks (TPP, USA) at a density of 6,000 cell /cm² in alpha-MEM (GIBCO,USA) + 5% platelet lysate (40). A day before cells reached 80% confluency, monolayers were washed twice with PBS (pH= 7.4) and once with serum free media -SFM (DM-F12). Next GSM was added in the flasks and after 48 hours this conditioned media (CM) was collected, centrifuged at 300xg for 10 minutes at 4°C, filtered through 0.2µm filters(BD, Biosciences, USA) and aliquoted. The CM was stored at -80°C and fresh aliquots were used prior to each experimental assay.

2.3.2. Preliminary data

In order to investigate the effect of condition media (CM) on GSCs, initially one sample of each of BM-MSC and WJ-MSC was used to generate CM and different concentrations of CM were tested with and without addition of fresh GSM. The results are shown in figure (S1.a). Serum free media (DM-F12 only) was kept as another control. Based on the preliminary tests, 100% CM was selected to perform the further experiments in a ratio of 1:9 with fresh gliosphere media (10%GSM: 90%CM).

2.3.3. Assessment of the effects of conditioned medium (CM) on GSCs (gliospheres)

2.3.3.1 Morphological assessment.

Briefly 10³/cm² cells from dissociated gliospheres at passage 13(G13) were plated in ultralow attachment six well plates (Corning, USA) containing CM. The experiment was conducted for 7 days and cells growing in normal GSM were kept as control. CM was added every 48 hours and changes in sphere forming ability of GSCs were captured by phase contrast microscopy (Zeiss, Germany).

2.3.3.2 3D cell proliferation and viability assessment

The proliferation rate of GSCs (at G13) was evaluated by CellTiter-Glo[®] 3D Cell Viability Assay (Promega, USA). In brief, the cells were cultured at a seeding density of 5x10³ cells/well in 100 µl of CM in 96 well ultralow attachment plates (Corning,USA). Every 48 hours fresh CM was added along with control and experiment was conducted for 7 days. The proliferation rate was determined at days 3 and 7 by CellTiter-Glo[®] 3D Cell Viability reagent according to the manufacturer's instruction (Promega, USA). Luminescence

was measured using a microplate reader (GloMax[®]-multi, Promega, USA). The cell survival percentage was measured by dividing the mean value of treated well by the mean value of control wells multiply by 100.

% viability = mean value of treated well / mean value of control wells x100

The decline in viability was measured by deducting the percent viability of treatment from the control viability at that time point.

Decline in viability = 100% viability of control- % viability of treated well.

2.3.3.3 Invasion assay.

In order to determine the effect of CM on the invasive ability of GSCs, invasion assay was performed using Cultrex[®] BME Cell Invasion Assay (Trevigen, USA) with a slight modification . Briefly, 5×10^4 cells (in triplicate) were treated with CM from both types of MSCs in 6 well ultralow attachment plates (Corning, USA) for 7 days. The media was refreshed every 48 hours. At day 7 the spheres were collected and harvested by accutase (GIBCO, USA), centrifuged at 200xg for 4 minutes and 50^4 cells (in triplicate) were seeded in the upper chamber of BME 96 well plate. The lower chamber was filled with serum media (DM-F12 +10%FBS, GIBCO,USA). After two days the plates were processed according to manufacturer's instruction and the luminescence was measured using microplate reader (GloMax[®]-multi, Promega, USA).

2.3.3.4 Clonogenic assay

In order to investigate if the effect exerted by CM from both types of MSCs is reversible or irreversible, clonogenic assay was performed for both adherent and spheroid system.

- ***Colony Forming Efficiency (CFE)***

Briefly, 5×10^3 cells/well were treated in 24 well ultralow (Corning, USA) with CM from both types of MSCs for 7 days along with control. After harvesting, single cell suspensions were obtained. 50 cells from each sample were seeded in 6 well plates (TPP, USA) in standard culture media (DM-F12+10%FBS) for two weeks. The medium was exchanged every three days. Colonies were stained using 0.5% crystal violet dye (Sigma-Aldrich, USA) according to the manufacturer's instructions and counted by a light microscope (Zeiss, Germany).

- ***Sphere Forming Efficiency (SFE)***

Similarly, 2×10^3 cells/ well were cultured in 24 well ultralow plates (Corning, USA) in GSM for about 7days. GSM was added every 48 hours and sphere formation was monitored under light microscope (Zeiss, Germany). On day 8 the spheres were counted and photographed using Zeiss microscope. Spheres were dissociated as described before and were plated again for successive passage. Fresh GSM was added every 48 hours and passage time was kept for 7 days.

2.4. Whole Transcriptome Microarray

2.4.1. RNA extraction.

Briefly, 10^4 cells/cm² (G13) were seeded in 25 ml ultralow attachment flasks (Corning, USA) containing CM from two types of MSCs and control (gliospheres). Sorted population that did not make spheres was taken as a negative control. On day 7 the spheres were harvested using the accutase (Gibco,USA). Total RNA was extracted using trizol[®] reagent (Invitrogen, CA, USA) and cleaned up with RNeasy Mini kit (Qiagen, USA) following the instructions of the manufacturer. Extracted RNA was quantified using NanoDrop 2000c spectrophotometer system (ThermoFisher Scientific, USA). RNA quality analysis was performed using Agilent 2100 Bioanalyzer instrument with Agilent RNA 6000 Nano Kit according to the manufacturer's instructions.

2.4.2. Global gene expression profiling (Transcriptome analysis)

Whole Transcriptome analysis was performed in triplicate for gliospheres , control group (-Ve and cell line) ,gliospheres treated with; CM from BM-MSCs (BMT) and CM from WJ-MSCs (WJT). GeneChip[®] Human Transcriptome Array (HTA) 2.0 (Affymetrix. Inc., Santa Clara, CA, USA) was used for gene expression profile analysis. The procedure was followed as described by manufacturer. The microarray data can be accessed via Gene Expression Omnibus-GEO (GSE149216) (www.ncbi.nlm.nih.gov/geo). For simplicity, we will use terms BMT and WJT for treated gliospheres from here onward.

2.4.3. Data Analysis

Raw CEL files normalization was performed using the signal Space Transformation-robust multi-array analysis (SST-RMA) using Affymetrix Expression Console software version (TAC) 4.0.1 (Affymetrix). Differentially expressed genes (DEGs), at fold change (log2) of ≥ 2 or ≤ -2 with a statistical significance level ($p < 0.05$) (gliospheres vs. BMT gliospheres, gliospheres vs WJT gliospheres and gliospheres vs control) were picked up. Gene ontology (GO) term and pathway enrichment analyses were conducted to determine the roles of these DEGs by Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA) , NetworkAnalyst (3.0) (www.networkanalyst.ca) and using the open source Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (<https://david.ncifcrf.gov/home.jsp>).

2.5. RT² Profiler[™] PCR Array

In order to validate further the specific genes and pathways involved in certain mechanisms exhibited by transcriptomic analysis and particularly the effect on stemness, CSC arrays were performed and analysed according to manufacturer's instruction (Qiagen, USA). Briefly cDNA was synthesized using the RT² First Strand Kit[®] Cat. No. (330404, Qiagen, USA). A diluted cDNA aliquot was mixed with RT² SYBR Green qPCR Mastermix Cat No. (330503, Qiagen, USA) and loaded into the 96-well array plate of RT² Profiler[™] PCR Array Human Cancer Stem Cells Cat No. (PAHS-176Z). Q-PCR reactions were performed using the CFX96 C1000 Touch thermal cycler (Bio-Rad, USA) with the following temperature setting: (i) 95 °C for 10

min, (ii) 40 cycles of 95 °C for 15 seconds, (iii) 60 °C for 1 minute. The data analysis was performed using the 2- $\Delta\Delta C_t$ method available by the SABiosciences company (Qiagen, USA) web portal, www.SABiosciences.com/pcrarraydataanalysis.php. The data were normalized, across all plates, to the average of arithmetic mean of the following housekeeping genes: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Beta-2-microglobulin (B2M) and actin beta (ACTB). The threshold cycle values of the control wells were all within the ranges recommended by the PCR array user manual.

2.6. Statistical analysis

All experiments were performed in triplicates ($n=3$). Data were analyzed using Microsoft Excel and GraphPrism software. Quantitative data were expressed as mean $\pm$ standard deviation. Data were evaluated by two way ANOVA and Dunnet's post-test was used to analyze multiple comparisons ($P<0.05$).

3. Results

3.1. CM concentration optimization assays

Figure (S1.a) shows the results of preliminary tests for different concentrations of CM. It was noted that cells treated with different concentrations of CM changed their morphology at 48 and 72 hrs and started to become linear rather than making spheres. It was also noted that GSCs kept on growing even in serum-free conditions (DM-F12 only) without the addition of growth factors and supplements under ultralow attachment culture conditions (3D). This showed the plasticity of GSCs, modifying them according to the change in the environment (41). However, to determine the effect of CM from MSCs specifically for GSCs in 3D culture system under normal growth conditions, we no longer used the SFM as a control for further assays.

3.2. Enrichment and characterization of CD133 positive population.

Figure (S2.a) depicts the flow cytometric evaluation of the CD133⁺ population present in U87 cell line (P3-P7) before sorting and enrichment of a positive population after sorting. Before cell sorting, we found that the average percentage of CD133⁺ population was 5.1% in cell line. After sorting CD133 positive population was enriched to 48% while the negative population still showed 2.1% of CD133⁺ cells (Table. 2). In order to determine if successive cultures of sorted cells have affected the enrichment of the CD133⁺ population in cancer stem cell media (GSM), we also determined the enrichment of sorted positive population at a random passage (G5), which was above 90% with almost uniform sphere morphology (Fig. S2.b). The enriched glioma stem cell line with 90 % CD133 positive population was used for all the experiments.

3.3 Morphological characteristics of sorted populations

Sorted populations were divided into positive and negative fractions and were cultured in adherent (2D) and sphere system (3D). In adherent cultures, CD133 positive population made a mesh network and

started forming connections from passage 1, and at passage 3, cells showed a conspicuous stellar morphology, which is a typical morphological feature of U87 cells (Fig1.a P3, arrow). While CD133 negative population showed more large cells indicating the presence of a differentiated progeny (Fig1.a P3, arrow). Moreover, the negative population showed a slight stellar morphology at later passages (P5-P6), which might be due to the presence of residual CD133 positive cells in negative fraction or some CD133 negative cells may have acquired the state of CD133 positive cells (42).

Similarly, in spheroid culture, positive fraction started making spheres from G1 and continues till passage G12. However, the negative fraction did not make spheres after G3, and at latter passages cells tend to be adherent rather than spheroids (same as in Fig S1.b). This negative population, failed to form spheres, was kept as a negative control for molecular analysis.

3.4. Immunocytochemistry of Gliospheres

Figure (1.c) shows three panels of markers selected for the characterization of the glioma stem cells in actively growing spheres (gliospheres). The markers were; glioma stem cell markers (CD133 and Msh1), neuronal lineage markers (nestin, sox1, sox2 and pax6) and differentiated markers (GFAP, Tuj1 and Olig123). Since the glioma spheroids contain heterogeneous populations (43) comprising of stem and differentiated cells, so we found the substantial presence of all the selected markers in various combinations.

3.5. CM inhibited sphere formation, proliferation, invasion and clonogenicity of GSCs.

Gliospheres were treated with CM from two types of MSCs for 7 days and the results are shown in Figure 2. Morphologically, spheroids tend to change their form as early as on day 3 (D3) where cells started to become linear rather than spheroids. At D7, cells started to branch out just like adherent culture, despite growing in ultralow stem cell environment (Fig. 2.a.) In relation to this, 3D Glow viability assay showed that cells' proliferative ability was significantly decreased at D7 ($p < 0.0005$) as compared to control (Fig. 2.b) and the effect was same from all individual biological samples. On average, CM from both WJ-MSCs and BM-MSCs reduced the viability of glioma stem cells around 36% and 35% respectively (Fig.S 1.b). Additionally, CM from both types of MSCs significantly reduced the invasive ability of GSCs (Fig 2.c) As a fraction of total, the reduction in invasion was 27% (WJ) and 26% (BM) as compared to 45% from control (Fig. S1.c).

In order to investigate if the effect of CM from both types of MSCs is irreversible, a clonogenic assay was performed. It was noted that the colony-forming efficiency of GSCs was significantly reduced both adherently and in spheroids compared to control ($p < 0.005$, Fig.2 d,e). It was found that the average sphere/colony formation from control was 39/19, while the reduction in sphere/colony formation was 5/11 from BM-MSCs and 7/6 from WJ-MSCs (Fig.S1.d).

3.6. Molecular Analysis.

3.6.1. CM inhibited the metabolism and cell cycle while activated the immune response at the Molecular level

Principal component analysis (PCA) was performed to demonstrate global gene-expression changes among different treatments (BMT vs. gliospheres, WJT vs. gliospheres, gliospheres, and -VeS) and to identify any outliers. PCA (Fig 3.a) shows that each type of group clustered together with a clear separation between them, and no outlier was observed. Both BMT and WJT gliospheres are closer to the gliospheres (+VeS) while -Ve control has its unique ancestor.

To generate a list of differentially expressed genes (DEGs) between different types of treatments in comparison to controls, eBayes ANOVA method was used, and a filtering criterion of; Fold Change (FC) ≤ -2 or ≥ 2 and p-value ≤ 0.05 were applied. The detailed description of DEGs has been provided in supplementary file and shown in Venn diagram (excel 1, Fig.3b).

Gliospheres. For 1483 DEGs between gliospheres and negative control (Fig.3c), 81 significant canonical pathways were identified by Ingenuity Pathways Analysis software (IPA, Qiagen, USA). Based on the z-score evaluation, supremely activated pathways included cholesterol biosynthesis, mevalonate pathway 1, systemic lupus erythematosus in T cells signaling pathway, Toll-Like receptor signaling while *PD-1/PD-L1* cancer immunotherapy pathway was inhibited.

WJT gliospheres. For 579 DEGs between WJT and gliospheres applied to IPA (Fig 4.a), 57 canonical pathways were identified as significant. These include *TREM1* signaling, *GP6* signaling, and *NF- κ B* activation by virus as activated, while Oxidative phosphorylation was inhibited.

BMT gliospheres. For 611 DEGs between BMT and gliospheres applied to IPA (Fig 4.b), 36 significant canonical pathways were identified. Based on z-score, topmost pathways include *PD-1/PD-L1* cancer immunotherapy (*activated*), calcium-induced T lymphocyte apoptosis and Oxidative phosphorylation (*inhibited*).

It was noted that both treatments commonly inhibited oxidative phosphorylation at complexes IV-V (green, down-regulated Fig 4.c).

3.6.1.1 . Gene ontology and pathway analysis

To investigate the functional importance and biological processes associated with the differentially expressed signature genes with each treatment (CM from BM and WJ MSCs) as well as in gliospheres (+VeS vs -Ve), GO analysis was performed using open source DAVID gene annotation website (<http://david.abcc.ncifcrf.gov>) and NetworkAnalyst(3.0). The signature up- and down-regulated genes were analyzed separately. Based on the significance (p-value ≤ 0.05 and Hits), top KEGG pathways and biological processes for each type of treatment group are shown in supplementary Figures (S3.S4.S5).

Gliospheres. For up-regulated genes in gliospheres, Ribosome biogenesis in eukaryotes, Steroid biosynthesis, Antigen processing and presentation, Asthma, and Terpenoid backbone biosynthesis were

identified as significantly up-regulated Kegg pathways. While the down-regulated genes were involved in *HIF-1* signaling pathway, Protein export, Protein processing in the endoplasmic reticulum, Ferroptosis, Glycosphingolipid biosynthesis - ganglio series, and Autophagy (Fig. S3.).

Similarly, up-regulated genes were represented by top biological processes of lipid, steroid and cholesterol metabolic process, antigen processing and presentation, fatty acid biosynthesis and metabolic process, and immune response while the down-regulated genes were represented by top biological processes of negative regulation of the apoptotic process, cell death, angiogenesis, cell adhesion, rhythmic process, and synaptic vesicle exocytosis (Fig. S3).

BMT and WJT gliospheres. From both types of treatment (CM of BM-MSCs and WJ- MSCs), we found almost the same significantly up-regulated kegg pathways such as: ECM-receptor interaction, *AGE-RAGE* signaling pathway in diabetic complications, Focal adhesion, Protein digestion and absorption, *PI3K-Akt* signaling pathway and proteoglycans in cancers. While Ribosome biogenesis in eukaryotes, Systemic lupus erythematosus, Asthma, and antigen processing and presentation were significantly down-regulated or inhibited (Fig. S4 and S5)

In the same manner, GO terms for up-regulated biological processes were enriched in cell adhesion, angiogenesis, receptor-mediate endocytosis, heart and skeletal system development, cellular defense response, cell differentiation and blood coagulation while the down-regulated genes were represented by top biological processes of DNA replication, regulation of cell cycle, RNA splicing, DNA repair, cell cycle, antigen processing and presentation, RNA splicing, and protein transport (Fig. S4 and S5).

Based on pathway analysis and biological processes ,it was noted that CM from both types of MSCs inhibited the metabolism, arrested cell cycle and activated immune response in GSCs.

3.7. RT² Profiler™ PCR Array

3.7.1. CM inhibited cell proliferation, pluripotency of glioma stem cells and induced cell differentiation and immune response.

In order to validate the findings of microarray analysis, specific human CSC array was used to investigate if the cell cycle was arrested and multipotency or pluripotency of glioma stem cells was affected and which specific genes and pathways are involved in causing this effect. The results of CSC array for gliospheres are summarized in (Table.3a). After normalization, out of 84 genes of the arrays, 29 genes were identified as DEGs (24-upregulated, 5- down-regulated) in gliospheres. A Scatter plot for gliospheres (gliospheres vs. Control, Fig.5a) shows the distribution of gene expression changes along the central diagonal line. Significant up-regulated genes ($p < 0.05$) were *DNMT1*, *GSK3B*, *IKBKB*, *ITGA6*, *LATS1*, *LIN28B*, *WWC1*, *ZEB2*, and *YAP1*. While the genes observed with higher fold regulation were *LIN28B*, *JAG1*, *EPCAM*, *TWIST2*, *ATM*, *NANOG* and *CD38* (Table 3a). However, one gene was found significantly up-regulated ($p < 0.05$) and with high fold regulation *LIN28B* (7.72).

Scatter plots for WJT and BMT gliospheres have shown the same pattern as depicted in Figure (5.b and c, Table.3b). Based on fold regulation, it was noted that both treatments down-regulated most of the genes that were up-regulated in gliospheres. We found almost the same up-regulated genes by both treatments such as *CXCL8* and *FOXP1*, while *PLAUR* was up-regulated by WJT, and *NANOG* remained unchanged by BMT. However, we found some variations in down-regulated genes by both treatments. Common genes significantly ($p < 0.05$) down-regulated by both treatments were, *DKK1*, and *KITLG*. Since *DKK1* was already down-regulated ($FC < -4.45$) in gliospheres too, so only one gene was significantly ($p < 0.05$) down-regulated by both treatments, which is *KITLG* while *DKK1* seems to be significantly up-regulated ($FC < -2.80$ and -2.69) as compared to gliosphere (Tables. 3 a,b).

Uniquely down-regulated genes based on fold regulation by WJT were *ABCG2*, *EPCAM* and *LIN28B*, while uniquely down-regulated genes by BMT were *JAG1* and *POU5F1* (Table 3.b).

3.7.1.1. Pathway analysis and GO terms of CSC arrays

Gliospheres. Figure 6 shows the activated and inhibited pathways and biological processes of significantly DEGs in gliospheres. It was noted that most significant activated pathways in gliospheres were *Hippo* signaling, *P13/Akt* signaling, Pathways in cancer and MicroRNAs in cancer (Fig. 6 a). In relation to this, highly significant biological processes (based on p values) in gliospheres were protein phosphorylation, negative regulation of apoptotic processes, glycogen and carbohydrate metabolic processes, cell proliferation, circadian rhythm, chromatin organisation and cell matrix adhesion (Fig. 6 b).

Based on high fold regulation, the top activated pathways were, Proteoglycans in cancer, Notch signaling pathway, P53 signaling pathway, Endocrine resistance, Th1, and Th2 cell differentiation, Haematopoietic cell lineage, homologous combinations, platinum drug resistance etc (Fig. 6 c). In addition to this, top activated biological processes were related to cell communication, hemopoiesis, negative regulation of apoptotic processes, regulation of cell cycle, DNA replication, angiogenesis, cell proliferation, and DNA repair (Fig. 6 d).

WJT and BMT gliospheres. Figure 7 gathers the information about activated and inhibited pathways and biological processes of DEGs of treated gliospheres with two types of CM of MSCs. We found similar activated KEGG pathways (non significant) from both treatments (Fig S6 a,b) except complement and coagulant cascade pathway in WJT gliospheres. It was found that both treatments significantly down-regulated almost similar pathways with a slight variation (Fig. 7). Among down-regulated pathways from WJT, the significant ones were *Rap1* signaling, *Hippo* signaling, *ABC* transporters, Signaling pathways regulating pluripotency of stem cells and *TGF-beta* signaling pathways etc. In relation to this, the top most down-regulated biological processes were negative regulation of apoptotic processes, regulation of cell cycle, cell proliferation and angiogenesis.

In WJT gliospheres up-regulated biological processes related to genes (*PLAUR*, *FOXP1*, *CXCL8*) were macrophage activation, inhibition of apoptotic process, heart development, angiogenesis, immune

response, and blood coagulation.

Similarly, Figure 7 b shows the up and down-regulated pathways and biological processes of DEGs of BMT gliospheres. It was noted that there was a slight difference in up-regulated biological processes with respect to genes (*FOXP1, CXCL8, NANOG*). *Nanog* also up-regulated cell differentiation, cell proliferation and transcription by RNA polymerase II while *FOXP1* and *CXCL8* remained the same as in WJT gliospheres. However, none of these up-regulated genes from both treatments were statistically significant ($p < 0.05$).

On the other hand, the significant down-regulated pathways observed from both treatments were signaling pathways regulating pluripotency of stem cells, *Rap 1* signaling, pathways in cancer, *Notch* signaling and *TGF-beta* signaling etc. Similarly, significantly down-regulated biological processes noted were the same, with the highlighted ones being the negative regulation of apoptotic processes, cell communication, angiogenesis and regulation of cell cycle etc.

Among up-regulated pathways, *AGE-RAGE* signaling and *Nf-kB* signaling were the same as shown by microarray analysis as well. This shows that the results of the CSC array are consistent with the findings from the microarray and are being validated. Overall, CSCs array analysis depicted the inhibition of cell proliferation, pluripotency, induced differentiation and activated immune response in GSCs.

3.7.3. Significant genes down-regulated by both treatments in CSC array

It was noted that two genes *KITLG* and *DKK1*, were significantly down-regulated by CM of two types of MSCs. The details of their relevant pathways and biological processes are shown in Figure 8 (a & b). Significantly down-regulated pathways found with *KITLG* were, hematopoietic cell lineage, melanogenesis, *Rap1* signaling, *MAPK signaling*, *P13/AKT* signaling, pathways in cancer, *PLD* signaling and *Ras* signaling. On the other hand, *DKK1* was involved in the down-regulation of Wnt signaling pathways. With regard to this, the main down-regulated biological processes were negative regulation of the apoptotic process, cell proliferation, cell adhesion, and endoderm development.

Overall, CM from both MSCs inhibited the cell proliferation, invasion, and pluripotency of GSC, but illicit the immune response that can activate angiogenesis by modulating the tumor microenvironment (Table 4).

Discussion

In Glioblastoma, GSCs were first identified by Singh et al., as a population of cells capable of initiating tumor growth *in vivo* (44). The crucial role played by GSCs in tumor initiation, progression, recurrence, and resistance to therapy indicates that new therapeutic strategies require the eradication of this population (45,46,47). It is also important to note that tumor cells are heterogeneous; therefore, it may be more advantageous to target multiple elements of various cellular pathways, to eradicate GBM (48). A possible solution to specifically target GSCs might be to force them to acquire a non-self-renewing state. In this

non-stem cell-like state, the cells should lose their tumorigenic nature and become vulnerable to therapies. Many therapies fail to have the expected beneficial effects due to the blood–brain barrier and the presence of active efflux pumps that prevent drug entry into the brain. New treatment modalities, including novel agents and small molecule inhibitors, are currently under investigation. Remotely, mesenchymal stem cells (MSCs) and their soluble factors are reported to exhibit beneficial anticancer effects (49,50,51). In order to investigate further if the soluble factors of MSCs may affect different pathways related to proliferation and stemness of GSC to transform them into non-stem-like cells prone to therapies, we planned this study.

In the first step CD133 positive cells were sorted and characterized according to morphological and immunocytochemical assays. These cells exhibited a high expression of all the known markers established for GSC profile. Actively growing GSCs in spheroids were treated with paracrine factors of manipulated MSCs and showed morphological changes, reduced proliferation, viability and invasion. Clonogenic assay revealed a remarkable decrease in self renewal ability of GSCs signifying the efficacy of paracrine factors of MSCs. Gene ontology results of DEGs from microarray analysis were further evaluated by specific human CSC array and we found consistent results of arrested cell cycle, inhibited metabolism, inhibited pluripotency of GSC and activated immune response. IPA analysis of treated spheres indicated inhibited oxidative phosphorylation from both treatments. However, slightly variable canonical pathways were up-regulated for immune response, from two types of treatments.

Metabolic reprogramming has been the hallmark of cancer stem cells. Growing evidence has demonstrated that slow-cycling GSCs possess a preference for mitochondrial oxidative metabolism. Mitochondrial function plays a crucial role in maintaining CSCs stemness and drug resistance (52,53,54). Few studies have demonstrated that CSCs can rely on fatty acid oxidation for their maintenance and function (55) and lipid catabolism seems critical for CSCs self-renewal (56). Similarly, the mevalonate pathway is an essential metabolic pathway in providing cells with bioactive molecules, crucial for different cellular processes, including cell proliferation, differentiation, survival (57) and CSCs enrichment (58). Since CSCs have been shown to be enriched in mitochondrial mass and relying heavily on OXPHOS, disrupting this pathway has become an attractive therapeutic strategy. Oxidative phosphorylation (OxPhos) plays a central role in cellular energy. The OxPhos electron transport chain (ETC) constitutes four complexes (CI-CIV) that transfer electrons from donors generated by the TCA cycle and fatty acid oxidation to oxygen. Complex V (ATP synthase) uses the stored energy in the proton gradient to generate ATP (59,60,61). As shown by our results of IPA, the main activated metabolic pathways involved in gliospheres were those with fatty acid and mevalonate pathways that were inhibited due to disruption at C4 and C5 complexes of ETC in OxPhos (Fig. 4.c). This may have, in turn, inhibited the proliferation, viability, invasion and sphere-forming ability of GSC consistent with the study done by (62).

IPA analysis of WJT gliospheres showed activation of *TREM1* signaling, *GP6* signaling and *Nf-kB* signaling. It has been shown that *TREM1* had been up-regulated only in infectious inflammatory responses (63). In tumors, *TREM1* seems to be induced on tumor-associated macrophages, which has been correlated with cancer recurrence and poor survival (64). Immunohistochemical analysis of breast

tumor tissues confirmed co-localization of *TREM1* protein expression with the pan-macrophage marker, CD68. These findings established the role of tumor infiltrating macrophages in promoting inflammation by immune evasion (65). It has also been investigated that *TREM1* expression is regulated by *Nf-kB* at the transcriptional level [66], emphasizing the contribution of *Nf-kB* pathway activation in bridging inflammation and tumor promotion and progression (67). Hypoxia regulated genes mediate blood vessel formation by stimulating encoding of chemotactic molecules such as *CCL2*, *IL8* and *VEGF* that recruit macrophages and exert tumor-promoting effects such as angiogenesis (68,69,70). Our results are in agreement with the above-mentioned studies in view of macrophage activation and angiogenesis, as confirmed by the biological processes of treated spheres (Fig. 7). We propose that CM from WJ-MSCs might have caused a hypoxic effect, which may contribute to angiogenesis through the involvement of macrophages in the tumor microenvironment.

GPVI (Glycoprotein VI) is exclusively expressed on platelets and megakaryocytes and together with integrin $\alpha_2\beta_1$ mediates collagen-induced aggregation and adhesion (71,72,73). The role of platelets in the pathophysiology of GBM appears to be two-edged. On the one hand, activated platelets and their secretome can modulate immune responses, thereby prolonging overall survival in a GBM model in mice (74). On the other hand, platelet activation needs to be avoided since GBM patients have an increased risk for systemic cardiovascular events, and the intratumoral occlusion of numerous vessels leads to a hypoxia-induced tumor progression (75). We found that WJT spheres have up-regulated a coagulation cascade to recruit platelets as a survival strategy. Therefore suitable antithrombotic and antiplatelet concepts may be a valuable addition to future individualized, targeted therapies (76). However, the details of the molecular mechanism in platelet activation require further studies.

IPA analysis of BMT spheres showed up-regulation of *PD1/PDL1* pathway and inhibition of calcium-induced T lymphocyte apoptosis. *PD-L1* is not constitutively expressed in tumor cells but rather is inducibly expressed (i.e., adaptive immune resistance) in response to inflammatory signals (77,78). Immune checkpoint inhibitors, *PD-1* and *PD-L1*, have shown clinical efficacies against many different solid and hematologic malignancies (79). Binding of *PD-L1* to its receptor suppresses T cell migration, proliferation, and secretion of cytotoxic mediators, and restricts tumor cell killing. Inhibitors of PD-1 and *PD-L1* disrupt PD-1 axis, thereby reverses T cell suppression and enhances endogenous antitumor immunity to unleash long-term antitumor responses in a wide range of cancers (80). Our results show that CM from BM-MSCs has exerted an adaptive immune response, which induced *PDI/PDL1* expression on cancer cells.

Ca^{2+} signaling plays an essential role throughout vertebrate development, from fertilization to organogenesis. It has been shown that the main checkpoints controlling the fate of a cell are mainly controlled by Ca^{2+} signaling pathways (81). Few studies have shown that some tumors develop an immune evasion strategy based on FasL-mediated destruction of invading lymphocytes (82,83,84). Invading T lymphocytes that express Fas is stimulated to apoptosis by tumor cells that express FasL. The expression of FasL has recently been demonstrated in GBMs (85). It has also been reported that T lymphocytes were present in GBMs and would account for the aggressive growth of tumors (86). Our

results show that the calcium-induced T lymphocyte apoptosis pathway has been inhibited, which indicates that CM from BM-MSCs might have affected the Fas-FasL combination and inversed the reaction of immune evasion by GSCs which in turn has up-regulated apoptosis in GSC as shown by biological process analysis. (Fig. 7)

From gene ontology results of specific CSC array, we found two common genes significantly down-regulated from CM of both MSCs such as; *KITLG* and *DKK1*.

DKK1 has downregulated the Wnt pathway (Fig. 9a). A large number of studies have suggested that WNT signaling is aberrantly activated in GBM, and it promotes GBM growth and invasion via the maintenance of stem cell properties (87,88,89,90). *Dickkopf (DKK)* acts as an antagonist of WNT signaling via binding to its co-receptor LRP (91,92). Interestingly, the MSC-induced pro-tumorigenic effect seems to be regulated by the Wnt/ β -catenin signaling in breast cancer (93,94), whereas the inhibition of tumor proliferation occurs by MSC induced secretion of *DKK-1*, an inhibitor of the same pathway (95,96). Furthermore, the MSC-derived CM exerts its effect by targeting the *Wnt/ β -catenin* signaling pathway (97,98). Our in silico results are in accordance with these studies as we found inhibition of growth and stemness properties of GSC through downregulation of the Wnt pathway (Fig. 8a).

KITLG gene encodes the ligand of receptor tyrosine kinases (RTKs) by the KIT locus. RTKs are a family of cell surface receptors, which upon activation, signal through two major downstream pathways *Ras/MAPK/ERK* and *Ras/PI3K/AKT*. These pathways are involved in the regulation of cell proliferation, survival, differentiation, and angiogenesis (99). We found that CM from MSCs inhibited these pathways through the downregulation of the *KITLG* gene. In addition, *Phospholipase D (PLD)* activity has been suggested to function as a sensor of metabolites, including lipid pools (100) and a critical regulator of autophagy (101). Keeping this in mind, we predict that the metabolism of the GSCs was deregulated by inhibition of *PLD* pathway, which might have up-regulated apoptosis and differentiation also shown by biological processes (Fig. 8.b) mediated by *KITLG* gene.

Among uniquely up-regulated genes of CSC array of gliospheres *LIN28B* was found to be the most significant. Lin28, along with Oct4, Sox2, and Nanog, has corroborated its role in pluripotent stem cells (102). In addition to tumor initiation, *LIN28B* is necessary for the maintenance of cancers too (103). Recent advances have shown that Lin28 regulates *let-7* microRNA biogenesis and mRNA translation, to coordinate both cellular metabolism and proliferative growth pathways for stem cell self-renewal (104). Our results of WJT spheres show the down-regulation of *LIN28B* that has contributed to the inhibition of metabolism and the self-renewal capacity of GSC. In addition, we also found the downregulation of *ABCG2* related to chemoresistance and SP formation (105) and *EPCAM* regulating the proliferation and invasion (106).

From BMT spheres, uniquely down-regulated genes were *JAG1* and *POU5F1*. *JAG1* is the ligands of the Notch signaling and has been shown to promote glioma-initiating cells (GICs) in glioblastoma. Notch signaling mediates direct cell-cell interactions and plays a crucial part in cell fate maintenance and self-renewal of GICs (107). Moreover, studies have shown that down-regulation of Jagged1 induces apoptosis

and inhibits proliferation in glioma cell lines (108). Similarly, different variants of *OCT4 (POU5F1)* have been related to colony formation and regulation of cell survival in GSC (109). We found inhibition of these two vital stem cell markers from CM of BM-MSCs.

One common pathway that has been identified by both arrays was the *AGE-RAGE* signaling pathway. *RAGE* was first identified as a receptor for *AGEs* in relation to diabetes, renal diseases, and aging (110,111). In glioblastomas, *RAGE* is expressed on tumor cells, endothelial cells, stromal cells, and tumor-associated macrophages comprising microglia and myeloid-derived macrophages(112). *RAGE* binding activates downstream signaling pathways that stimulate cell proliferation, survival, and migration via increased angiogenesis, inflammation, and reduced apoptosis, While blocking *RAGE* signaling suppresses tumor growth and metastasis (113–116). Despite the inhibition of oncogenic mechanisms at the cellular level, we found the macrophage activation and immune response as activated biological processes in treated gliospheres. We presume that *AGE-RAGE* signaling might be the contributing factor for this response, which can modulate the tumor microenvironment for angiogenesis. Combining inhibition of *RAGE* signaling with CM might be a novel strategy to inhibit tumor growth.

There are several challenges involved in treating glioma, including the immunosuppressive nature of GBM itself with high inhibitory checkpoint expression, the immunoselection blood brain barrier impairing the ability for peripheral lymphocytes to traffic to the tumor microenvironment and the high prevalence of corticosteroid use which suppress lymphocyte activation. However, by simultaneously targeting multiple costimulatory and inhibitory pathways, it may be possible to achieve an effective antitumoral immune response(117). This is where a combination of manipulated MSCs-secreted factors has the most significant potential.

Conclusion

Taken together, the results of microarray and CSC arrays in vitro elucidate the possible mechanism of action by which MSCs secretome inhibited the 3D formation of GSCs observed in culture. The inhibition was translated into decreased oncogenic activities, including stemness of GSCs through different pathways mediated by *KITLG* and *DKK1* genes. We conclude that CM from two sources of MSCs holds the antitumor properties, which are mediated by different routes of signaling pathways while causing the same effect. It has been shown that neurotrophic factors in CM could access affected neurons in central nervous system (CNS) by either directly crossing the blood-brain barrier or through the retrograde transport mechanism in CNS. Since CM has already been implicated for many neurodegenerative diseases (118,119), therefore, CM from MSCs can be a subject of combinatorial therapy for gliomas. Regarding the preference of choice, we propose that CM from BM-MSCs may contribute more valuable effect as a combinatorial therapy in conjunction with antitumor immune therapy to treat gliomas. This study provided valuable information regarding the potential ability of acclimatized MSCs with GSC environment to interrupt the growth and pluripotency of GSCs with the potential for translation to new treatment options for GBM in human patients. Further, in vivo studies along with proteomics are warranted to translate the apparent therapeutic efficacy of MSCs' CM in the treatment of glioblastoma.

List Of Abbreviations

Cancer stem cells CSC

Glioblastoma multiforme GBM

Glioma stem cells GSCs

Mesenchymal stem cells MSCs

Gliosphere Media GSM

Serum free media SFM

Wharton Jelly- WJ-MSCs

Bone Marrow- BM-MSCs

Condition Media CM

Treated gliospheres with CM-WJ-MSCs WJT

Treated gliospheres with CM-BM-MSCs BMT

Differentially expressed genes DEGs

Ingenuity Pathway Analysis IPA

Principal component analysis PCA

Colony forming efficiency CFE

Sphere forming efficiency SFE

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee at the Cell Therapy Center. No animal or human was included in this study so consent to participate is not applicable.

Consent for publications

Not applicable

Availability of data and materials

Microarray data has been submitted and can be accessed via Gene Expression Omnibus-GEO (GSE149216) (www.ncbi.nlm.nih.gov/geo). Additional excel file of DEGs-PCA has been provided as supplementary material.

Competing interest.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1
Primary and secondary antibodies used for Immunocytochemistry.

Primary antibody	Cat number	Manufacturer
Anti-CD133	Ab19898	Abcam/UK
Rabbit recombinant monoclonal mushashi1/Msi1	ab199781	Abcam/UK
GFAP monoclonal antibody (ASTRO6)	MA512023	Invitrogen
OLIG1/OLIG2/OLIG3 Monoclonal Antibody (257224)	MA523805	Invitrogen
beta-3 Tubulin Monoclonal Antibody (2 28 33)	322600	Invitrogen
Nestin Monoclonal antibody (10C2)	14-9843-82	Thermo Fisher Scientific
Sox 2 antibody	48-1400	Thermo Fisher Scientific
Anti-PAX6 antibody	ab5790	Abcam/Uk
Anti-Sox1 antibody	ab87775	Abcam/UK
Secondary antibody	Cat number	Manufacturer
Goat anti-Mouse IgG1 AlexaFluor 488	A21121	Thermo Fisher Scientific
Goat anti-Rabbit IgG (H + L) AlexaFluor 546	A11035	Thermo Fisher Scientific
Goat anti-Mouse IgG (H + L) AlexaFluor 546	A11003	Thermo Fisher Scientific

Table 2
Percentage of CD133 population before and after sorting with Mean $\pm$ SD (standard deviation).

	CD133 before Sorting (%)	After sorting + ve population (%)	After sorting- ve population (%)
1	4.17	49.75	0.62
2	7.24	45.02	4.85
3	5.4	40.45	0.65
4	3.7	56.3	2.3
Mean $\pm$ SD	5.127 $\pm$ 1.58	47.88 $\pm$ 6.77	2.105 $\pm$ 1.99

Table.3 a . Up and down regulated genes in Gliospheres comprised of glioma stem cells sorted from cell line (U87MG ATCC-HTB14). Human Cancer Stem Cell array (RT² Profiler[™] PCR Array catalog No.PAHS-176Z) was used to identify differentially expressed genes in Gliospheres compared to Control (cell line and -Ve population).

Gene Symbol	Fold Regulation	p-Value
ATM	3.42	0.078675
CD38	7.36	0.050531
DNMT1	2.43	0.046934
EGF	2.58	0.264680
EPCAM	5.66	0.174573
GATA3	2.31	0.249860
GSK3B	2.27	0.026471
IKBKB	2.07	0.041685
ITGA6	2.19	0.016071
JAG1	4.48	0.126777
JAK2	2.57	0.232893
KITLG	2.16	0.115002
LATS1	2.22	0.033813
LIN28B	7.72	0.031777
MAML1	2.20	0.088189
NANOG	3.36	0.134051
NOTCH1	2.15	0.141905
POU5F1	2.36	0.252004
PROM1	2.35	0.298908
TAZ	2.28	0.071685
TWIST2	3.35	0.142327
WWC1	2.09	0.030530
YAP1	2.17	0.032586
ZEB2	2.50	0.000967
DKK1	-4.45	0.063695
FOXP1	-3.44	0.156756
ID1	-2.81	0.104330
CXCL8	-3.43	0.483770
NOS2	-3.39	0.275178

Table. 3 b. Up and down regulated differentially expressed genes in treated Gliospheres with condition media of WJ-MSCs (WJT) and BM-MSCs (BMT) in comparison to Gliospheres (control) using Human Cancer Stem Cell array (RT² Profiler[™] PCR Array catalog No.PAHS-176Z).

	WJ-Treated		BM-Treated	
Gene Symbol	Fold Regulation	p-Value	Fold Regulation	p-Value
FOXP1	4.80	0.070962	2.58	0.096343
CXCL8	3.02	0.678317	2.16	0.832641
PLAUR	2.03	0.228839	-	
NANOG	-	-	3.35	0.106006
ABCG2	-2.05	0.066120	-	
DKK1	-2.80	0.032991	-2.69	0.045981
ID1	-2.94	0.088560	-4.85	0.056106
KITLG	-2.16	0.038884	-2.06	0.040332
EPCAM	-2.34	0.143145	-	-
LIN28B	-2.35	0.083791	-	-
JAG1	-	-	-2.43	0.199360
POU5F1	-	-	-2.29	0.180843

Table 4. Summary of the effects of CM from MSCs on different mechanisms of GSCs growing as gliospheres.

	Gliospheres (Control)	Gliospheres treated with CM from BM-MSCs (BMT vs gliospheres)	Gliospheres treated with CM from WJ-MSCs (WJT vs gliospheres)
Morphology	Actively growing as spheroids	Expanded morphology like cell line	Expanded morphology like cell line
Proliferation	Highly proliferative	Inhibited proliferation	Inhibited proliferation
Viability	Viable(100%)	Reduced Viability 35%	Reduced viability 36%
Invasion	45%	26%	25%
Self-renewal	39 spheres, 19 colonies	5.0 spheres, 11 colonies	7 spheres, 6.0 colonies
Microarray	Metabolism, Proliferation,	Inhibited metabolism Activated immune response	Inhibited metabolism Activated immune response
CSC array	Metabolism, Proliferation, pluripotency	Inhibited metabolism, proliferation, Pluripotency, Activated differentiation ,immune response	Inhibited metabolism, proliferation, pluripotency, resistance Activated differentiation, immune response

Figures

Figure 1

Characterization of sorted population from U87MG ATCC-HTB14 based on self renewal and immunocytochemical assay. a. Morphological evaluation of sorted CD133 positive and negative population in adherent culture from passage 1 to 3 (P1-P3) Scale bar (100µm). Red arrow shows the mesh network and stellar morphology of U87 cells in CD133 positive population at passage 3(P3). Negative population showed large cells indicating the presence of differentiated progeny at P3 (blue arrow). b. Morphological evaluation of sorted CD133 positive and negative population in sphere culture (3D). CD133 positive population showed self renewal ability by forming spheres till passage (G10). While CD133 negative population stops making spheres at third passage (G3). Scale bar.100µm. c. Immunocytochemistry of sorted CD133 positive population for GSC(CD133,Msh1), neuronal lineage(nestin,sox1,sox2,pax6) and differentiated (GFAP,Tuj1,Olig123) markers in combinations at G13. Scale bar (50µm).

Figure 2

Effect of CM from MSCs on GSCs' (gliospheres) morphology, proliferation, invasion and clonogenicity. a. Representative images of the morphological changes occurring in gliospheres with CM at day 3 and 7. Scale bar (100µm). Cells started adherence at day 3 and become branched out at day7 rather than spheroids as compared to control. b. Effect of CM from two types of MSCs on the proliferation of gliospheres at day 3 and 7. Proliferative ability of GSCs' was significantly inhibited at day7($P < 0.0005$) in comparison to control. c. Effect of CM from two types of MSCs on the invasive ability of gliospheres at Day 7. Both treatments significantly inhibited the invasive potential of GSCs ($p < 0.05$). Clonogenicity of treated glioma stem cells was significantly reduced both in d) sphere culture, SFE e) adherent culture, CFE. This shows that treatment of condition media from both MSCs caused irreversible effect on the self renewal ability of glioma stem cells.

Figure 3

Microarray results of gliospheres and treated gliospheres using TAC analysis. a. PCA showing the distribution of clusters of control and treated gliospheres. Blue colour shows the negative control group. Red colour shows the gliosphere group and purple and green depict the treated group. Treated groups are clustered together at the same position close to gliospheres while control group has its unique ancestor. b. Venn diagram showing the distribution of DEG among different groups. c. Top activated and inhibited canonical pathways in gliospheres vs control (cell line vs -Ve) by Ingenuity Pathway Analysis (IPA). Significantly enriched canonical pathways were identified with a right-tailed Fisher's Exact Test that calculates the P-values. The P-values were corrected for multiple testing using the Benjamini-Hochberg method for correcting the FDR. The z-score indicates predicted activation state of the canonical pathway. Blue color or lighter shades of blue indicate a negative z-score and down-regulation of the pathway, and orange color or lighter shades of orange indicate a positive z-score and up-regulation of the pathway.

Fig 4.

Figure 4

IPA analysis showing significantly enriched canonical pathways in treated gliospheres with CM from two types of MSCs. Significantly enriched canonical pathways were identified with a right-tailed Fisher's Exact Test that calculates the P-values. The P-values were corrected for multiple testing using the Benjamini-Hochberg method for correcting the FDR. The z-score indicates predicted activation state of the canonical pathway. Blue color or lighter shades of blue indicate a negative z-score and down-regulation of the pathway, and orange color or lighter shades of orange indicate a positive z-score and up-regulation of the pathway. a. Activated and inhibited canonical pathways in treated gliospheres with CM fromWJ-MSCs (WJT vs gliospheres). b. Activated and inhibited canonical pathways in treated gliospheres with CM fromBM-MSCs (BMT vs gliospheres). c. Dysregulation of the oxidative phosphorylation pathway in treated gliospheres by IPA analysis .The schematic figure illustrates complex I-V of the mitochondrial electron transport chain. (green, downregulated complexes vi-v).

Fig .5.

a.

b.

c.

Figure 5

Scatter plots of DEGs in Gliospheres and treated Gliospheres using Human Cancer Stem Cell array (RT² Profiler™ PCR Array catalog No.PAHS-176Z). Gliospheres comprising of glioma stem cells sorted from U87MG ATCC-HTB14 cell line. A standard 2-fold change in expression was used as an arbitrary cut-off and significance at $p < 0.05$. The up-regulated genes are marked with red points, while the down-regulated genes are marked with green points, and the unchanged genes are marked with black points. A. Gliospheres comprised of glioma stem cells sorted from U87MG ATCC-HTB14 compared to Control (U87MG ATCC-HTB 14 cell line and –Ve control). B. Treated Gliospheres with condition media of Wharton's Jelly Mesenchymal stem cell (WJT) in comparison to Gliospheres. C. Treated Gliospheres with condition media of Bone Marrow Mesenchymal stem cell (BMT) in comparison to Gliospheres.

Fig.6

Figure 6

Gene ontology terms of gliospheres from human CSC arrays. Activated KEGG pathways according to a. significant genes (p values) and c. high fold regulation in gliospheres. Up-regulated biological processes according to b.) significant genes (p values) d.) high fold regulation in gliospheres. Colour variations shows the significance level with red being the highest ($p < 0.0005$) and white the lowest ($p < 0.05$).

Fig. 7

Figure 7

Gene ontology terms of gliospheres treated with CM of WJ-MSCs and BM-MSCs . WJT gliospheres a. Down-regulated KEGG pathways, b. Down-regulated biological processes BMT gliospheres c. Down-regulated KEGG pathways , d. Down-regulated biological processes. Both treatments significantly affected the oncogenic activities of GSCs.

Fig 8.

Figure 8

Significant genes affected by both treatments. a. Significantly down regulated genes (p value) and KEGG pathways by both treatments (CM from WJ-MSCs and BM-MSCs). b. Significantly down regulated biological processes from two treatments (CM from WJ-MSCs and BM-MSCs).

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