Anti-oncogenic activities exhibited by paracrine factors of MSCs can be mediated by modulation of KITLG and DKK1 genes in glioma SCs in vitro

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Cancer stem cells (CSCs) use their stemness properties to perpetuate their lineage and survive chemotherapy. Currently cell-based and cell-free therapies are under investigation to develop novel anti-cancer treatment modalities. We designed this study to investigate how cell extracts of mesenchymal stem cells affect the growth of glioma stem cells in vitro. Gliospheres were generated from U87MG cell line and treated with conditioned media of Wharton’s jelly and bone marrow mesenchymal stem cells. The effects were investigated at the functional and molecular levels. Our results showed that conditioned media from both types of mesenchymal stem cells changed the morphology of spheres and inhibited the proliferation, invasion, and self-renewal ability of glioma stem cells. At the molecular level, metabolism interruption at oxidative phosphorylation, cell cycle arrest, cell differentiation, and upregulation of the immune response were observed. Furthermore, this effect was mediated by the upregulation of the DKK1 gene inhibiting the Wnt pathway mediated by growth factor activity and downregulation of the KITLG gene activated by growth factor and cytokine activity, inhibiting multiple pathways. We conclude that different types of mesenchymal stem cells possess anti-tumor properties and their paracrine factors, in combination with anti-immune modalities, can provide practical therapeutic targets for glioblastoma treatment.

INTRODUCTION

The modern theory of carcinogenesis focuses on the presence of malignant transformations in adult tissue stem cells. The theory of cancer stem cells (CSCs) is old, as it was first described in 1973. Later, in 1997, the existence of a heterogeneous tumor cell population was mentioned in leukemia for the first time. Analysis of this cell population revealed stem cell properties, such as self-renewal capacity, high proliferation rate, and maintenance of the tumor cell population. These properties form the basis of the modern accepted hypothesis of CSCs. The CSC hypothesis gained credibility because all main cancer-origin theories (genetic/epigenetic events and chemical-, infection-, and virus-induced carcinogenesis) indicated that the tissue stem cell is involved in the generation of cancer. Moreover, recent studies also suggest that CSCs are a major driving force for tumorigenesis, metastasis, aggressiveness, and resistance to treatment. 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).

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. The highly infiltrative growth of GBM and its resistance to chemotherapies/radiotherapies prevent complete elimination of tumor cells, despite improvements made in surgical techniques and therapeutic protocols. The highly tumorigenic subpopulation of glioblastoma CSCs (GSCs) is thought to be one of the reasons for a high GBM recurrence rate. 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 microRNAs (miRNAs), are presently being investigated in the development of novel therapeutic targets.

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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. Therefore, GSCs would only be vulnerable to treatments while they are in an actively growing state.

Cellular therapy for cancer is being revisited because of using mesenchymal stem cells (MSCs) for cancer treatment. MSCs are multipotent stem cells mostly isolated from perinatal sources such as umbilical cord Wharton’s jelly (WJ-MSCs), placenta (PL-MSCs), and adult tissues such as bone marrow (BM-MSCs) and adipose tissue (AT-MSCs). They have been shown to have wide therapeutic potential because of their immunomodulatory ability, wound- and neoplasm-directed homing, and tissue repair ability. 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. Recently, pre-clinical studies demonstrated that MSCs might suppress the growth of GBM cells, although conflicting studies have also found the opposite effect. However, the mechanisms by which MSCs may suppress GBM growth have not been illustrated yet.

It has been reported that paracrine factors of MSCs showed enhanced beneficial effects on recovery from injury or disease in some experimental models. MSCs 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. To improve the therapeutic ability of MSCs, the composition of the secretome of MSCs can be modulated by preconditioning the MSCs during in vitro culture. Preconditioning of MSCs by hypoxia, inflammatory stimulus, and other factors/conditions before their use in therapy is a new strategy currently being investigated.

Based on this knowledge, we sought to investigate the effects of the conditioned media (CM) from two different types of MSCs preconditioned with a GSC microenvironment to explore the same biological mechanisms and signaling pathways that are associated with anti- or pro-tumorigenic effects in vitro. In doing so, we evaluated the effects of factors secreted (cytokines, chemokines, miRNAs, or growth factors in CM), under the same culture conditions, from two kinds of MSCs, BM-MSCs (BM1, BM2, BM3) and WJ-MSCs (WJ1, WJ2, WJ3), on GSC proliferation, invasion, and self-renewal capability. Furthermore, the genetic changes at signaling pathway levels were explored by microarray analysis followed by validation of the effect on the pluripotency of GSCs with human CSC arrays in vitro.

RESULTS
Preliminary data
Figure S1 shows the results of preliminary experiments for different concentrations of CM (50% and 100%) on the CD133+ population sorted from the U87MG cell line. It was noted that cells treated with different concentrations of CM changed their morphology at 48 and 72 h and started to become linear rather than making spheres (Figure S1A). Similarly, we found that gliosphere CM (GSCM) harvested from BM1-MSCs showed statistically significant inhibition of proliferation of GSCs in any formulation of CM concerning both controls at 96 h (p < 0.01, p < 0.0001). However, CM from WJ3-MSCs showed significant inhibition from 50% concentration of serum-free CM (SFCM) + gliosphere media (GSM) only (p < 0.05) (Figure S1B). This prompted us to stretch the experiment for the usual passage time of 7 days. It was also noted that GSCs kept on growing even in serum-free conditions (Dulbecco’s modified Eagle’s medium [DMEM]-F12 only) without the addition of growth factors and supplements under ultralow attachment culture conditions (3D) (Figures S1A and S1B). This showed the plasticity of GSCs, modifying themselves according to the change in the environment. However, to determine the effect of CM from MSCs specifically for GSCs in the 3D culture system under normal growth conditions, we no longer used the serum-free media (SFM) as a control for further assays. Based on the preliminary data of morphology and proliferation, 100% CM (GSCM) was selected to perform the further experiments in a ratio of 1:9 with fresh GSM (10% GSM/90% CM) to replenish the growth factors.

Enrichment and characterization of CD133+ population
Figure S2A depicts the flow cytometric evaluation of the CD133+ population present in the U87MG cell line (P3–P7) before sorting and enrichment of a positive population after sorting. Before cell sorting, we found that the average percentage of the CD133+ population was 5.1% in the cell line. After sorting, the CD133+ population was enriched to 48% while the negative population still showed 2.1% of CD133+ cells (Table 2). To determine whether successive cultures of sorted cells have affected the enrichment of the CD133+ population in CSC media (GSM), we also determined the enrichment of the sorted positive population at a random passage (G5), which was above 90% with almost uniform sphere morphology (Figure S2B). The enriched glioma stem cell line with a 90% CD133+ population was used for all of the experiments.

Morphological characteristics of sorted populations
Sorted populations were divided into positive and negative fractions and were cultured in adherent (2D) and sphere (3D) systems. In adherent cultures, the CD133+ population made an interconnected mesh network from passage 1, and at passage 3, cells showed a conspicuous stellar morphology as for U87MG cells (Figure 1A, P3, arrow), whereas the CD133– population showed larger cells indicating the presence of a differentiated progeny (Figure 1A, P3,
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 as for adherent culture, despite growing in ultralow stem cell environment (Figure 2A). Concerning this, a 3D Glo viability assay showed that cells’ proliferative ability was significantly decreased at D7 (p < 0.0001) as compared to control (Figure 2B) and the effect was same from all individual biological samples. Additionally, CM from both types of MSCs (averages) significantly reduced the invasive ability of GSCs (p < 0.05) (Figure 2C) As a fraction of total, the reduction in invasion was 27% (WT) and 26% (BM) as compared to 45% from control (Figure S1C).

To investigate whether 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 the control (p < 0.05, Figures 2D and 2E). 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 (Figure S1D).

Molecular analysis

CM inhibited the metabolism and cell cycle while activating the immune response at the molecular level (microarray)

Principal-component analysis (PCA) demonstrating global gene-expression changes among different treatments (BMT versus gliospheres, WJT versus gliospheres, gliospheres, and −VeS) and any outliers (Figure 3A) 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 the −Ve control (−VeS) has its unique ancestor.

To generate a list of differentially expressed genes (DEGs) between different types of treatments in comparison to controls, the eBayes ANOVA method was used, and a filtering criterion of fold change (FC) –2 or less or ≥2 and a p value ≤ 0.05 were applied. The detailed description of DEGs has been provided in the supplementary file (Excel 1) and shown in the Venn diagram (Figure 3B).

Gliospheres. For 1,483 DEGs between gliospheres and the negative control (Figure 3C), 81 significant canonical pathways were identified by Ingenuity Pathway Analysis (IPA) software (QIAGEN, USA). Based on the Z score evaluation, supremely activated pathways included cholesterol biosynthesis, mevalonate pathway 1, systemic lupus erythematosus in the T cell signaling pathway, and Toll-like receptor signaling while PD-1/PD-L1 cancer immunotherapy pathway was inhibited (Table 3).

WJT gliospheres. For 579 DEGs between WJT and gliospheres applied to IPA (Figure 4B), 57 canonical pathways were identified as significant. These include TREM1 signaling, GP6 signaling, and nuclear factor kB (NF-kB) activation by virus as activated, while oxidative phosphorylation was inhibited (Table 3).

<table>
<thead>
<tr>
<th>Antibodies</th>
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<th>Manufacturer</th>
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<td>Goat anti-mouse IgG (H+L)-Alexa Fluor 546</td>
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Table 1. Primary and secondary antibodies used for immunochemistry
BMT gliospheres. For 611 DEGs between BMT and gliospheres applied to IPA (Figure 4B), 36 significant canonical pathways were identified. Based on the Z score, the topmost pathways include PD-1/PD-L1 cancer immunotherapy (activated), calcium-induced T lymphocyte apoptosis, and oxidative phosphorylation (inhibited) (Table 3).

It was noted that both treatments commonly inhibited oxidative phosphorylation at complexes IV–V (green, downregulated, Figure 3C).

Gene Ontology (GO) and pathway analysis (microarrays)
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 versus –Ve), GO analysis was performed using the open-source DAVID gene annotation website (https://david.abcc.ncifcrf.gov/) and NetworkAnalyst (3.0). The signature upregulated and downregulated genes were analyzed separately. Based on the significance (p ≤ 0.05 and hits), the top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and biological processes for each type of treatment group are shown in Figures S3–S5.

Gliospheres. For upregulated genes in gliospheres, ribosome biogenesis in eukaryotes, steroid biosynthesis, antigen processing and presentation, asthama, and terpenoid backbone biosynthesis were identified as significantly upregulated KEGG pathways. The downregulated genes were involved in the HIF-1 signaling pathway, protein export, protein processing in the endoplasmic reticulum, ferroptosis, glycosphingolipid biosynthesis ganglio series), and autophagy (Figure S3).

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

BMT and WJT gliospheres. From both types of treatment (CM of BM-MSCs and WJ-MSCs), we found almost the same significantly upregulated KEGG pathways such as ECM-receptor interaction, the AGP-RAGE signaling pathway in diabetic complications, focal adhesion, protein digestion and absorption, the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway, and proteoglycans in cancers, while ribosome biogenesis in eukaryotes, systemic lupus erythematosus, asthama, and antigen processing and presentation were significantly downregulated or inhibited (Figures S4 and S5).

In the same manner, GO terms for upregulated biological processes were enriched in cell adhesion, angiogenesis, receptor-mediated endocytosis, heart and skeletal system development, cellular defense response, cell differentiation, and blood coagulation, while the downregulated 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 (Figures S4 and S5).

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

RT2 Profiler PCR array
CM inhibited cell proliferation and the pluripotency of glioma stem cells and induced cell differentiation and the immune response
To validate the findings of microarray analysis, a specific human CSC array was used to investigate whether the cell cycle was arrested and multipotency or pluripotency of glioma stem cells was affected and which specific genes and pathways were involved in causing this effect. The results of the CSC array for gliospheres are summarized in (Table 4). After normalization, out of 84 genes of the arrays, 29 genes were identified as DEGs (24 upregulated, 5 downregulated) in gliospheres. A scatterplot for gliospheres (gliospheres versus control, Figure 4A) shows the distribution of gene expression changes along the central diagonal line. Significantly upregulated genes (p < 0.05) were DNM1T1, GSK3B, IKBKB, ITGA6, LATS1, LIN28B, WWC1, ZEB2, and YAPI, while the genes observed with higher fold regulation were LIN28B, JAG1, EPCAM, TWIST2, ATM, NANO3, and CD38 (Table 4). However, one gene was found significantly upregulated (p < 0.05) and with high fold regulation LIN28B (7.72).

Scatterplots for WJT and BMT gliospheres have shown the same pattern as depicted in Figures 4B and 4C and Table 5. Based on fold regulation, it was noted that both treatments downregulated most of the genes that were upregulated in gliospheres. We found almost the same upregulated genes by both treatments such as CXCL8 and FOXP1, while PLAU was upregulated by WJT, and NANO3 remained unchanged by BMT. However, we found some variations in downregulated genes by both treatments. Common genes significantly (p < 0.05) downregulated by both treatments were DKK1 and KITLG. Since DKK1 was already downregulated (FC less than –4.45) in gliospheres too, only one gene was significantly (p < 0.05) downregulated by both treatments, which is KITLG.

| Table 2. Percentage of CD133 population before and after sorting with mean ± SD |
|---------------------------------|---------------------------------|---------------------------------|
| CD133 before sorting (%)       | After sorting +Ve population (%)| After sorting – Ve population (%)|
| 1                              | 4.17                            | 49.75                           |
| 2                              | 7.24                            | 45.82                           |
| 3                              | 5.4                             | 40.45                           |
| 4                              | 3.7                             | 56.3                            |
| Mean ± SD                      | 5.127 ± 1.58                    | 47.88 ± 6.77                    |
|                                | 2.105 ± 1.99                    |                                  |

SD, standard deviation.
while DKK1 seemed to be significantly upregulated (FC less than $-2.80$ and $-2.69$) as compared to gliosphere (Table 4).

Uniquely downregulated genes based on fold regulation by WJT were ABCG2, EPCAM, and LIN28B, while uniquely downregulated genes by BMT were JAG1 and POU5F1 (Table 5).

Pathway analysis and GO terms of CSC arrays

Gliospheres. Figure 5 shows the activated and inhibited pathways and biological processes of significantly upregulated DEGs in gliospheres. It was noted that the most significant activated pathways in gliospheres were Hippo signaling, P13/Akt signaling, pathways in cancer, and microRNAs in cancer (Figure 5A). With 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 organization, and cell-matrix adhesion (Figure 5B).

Based on high fold regulation, the top activated pathways were proteoglycans in cancer, the Notch signaling pathway, the p53 signaling pathway, endocrine resistance, T helper (Th1 and Th2) cell differentiation, hematopoietic cell lineage, homologous combinations, and platinum drug resistance (Figure 5C). In addition to this, top activated biological processes were related to cell communication, hematopoiesis, negative regulation of apoptotic processes regulation of cell cycle, DNA replication, angiogenesis, cell proliferation, and DNA repair (Figure 5D).
**WJT and BMT gliospheres.** Figure 6 shows 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 (Figures S6A and S6B) except complement and coagulant cascade pathways in WJT gliospheres. It was found that both treatments significantly downregulated almost similar pathways with a slight variation (Figure 6). Among downregulated pathways from WJT, the significant ones were Rap1 signaling, Hippo signaling, ABC transporters, signaling pathways regulating pluripotency of stem cells, and transforming growth factor β (TGF-β) signaling pathways (Figure 6A). To this, the topmost downregulated biological processes were negative regulation of apoptotic processes, regulation of cell cycle, cell proliferation, and angiogenesis (Figure 6B).

In WJT gliospheres upregulated biological processes related to genes PLAUR, FOXP1, and CXCL8 were macrophage activation, inhibition of apoptotic process, heart development, angiogenesis, immune response, and blood coagulation (Figure S6C).

Similarly, Figures 6B and 6D show the downregulated pathways and biological processes of DEGs of BMT gliospheres. It was noted that there was a slight difference in upregulated biological processes for genes FOXP1, CXCL8, and NANOG (Figure S6D). Nanog upregulated 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 upregulated genes from both treatments was statistically significant.
Alternatively, the significantly downregulated pathways observed from both treatments were signaling pathways regulating pluripotency of stem cells, Rap1 signaling, pathways in cancer, Notch signaling, and TGF-β signaling. Similarly, significantly downregulated 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.

Among upregulated pathways, AGE-RAGE signaling and NF-κB 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, CSC array analysis depicted the inhibition of cell proliferation, pluripotency, induced differentiation, and activated immune response in GSCs.

Significant genes downregulated by both treatments in CSC array

It was noted that two genes, KITLG and DKK1, were significantly downregulated by CM of two types of MSCs. The details of their relevant pathways and biological processes are shown in Figures 7A and 7B. Significantly downregulated pathways found with KITLG were hematopoietic cell lineage, melanogenesis, Rap1 signaling, mitogen-activated protein kinase (MAPK) signaling, PI3/AKT signaling, pathways in cancer, PLD signaling, and Ras signaling. Alternatively, DKK1 has been involved in the downregulation of Wnt signaling
paths. About this, the main downregulated biological processes were negative regulation of the apoptotic process, cell proliferation, cell adhesion, and endoderm development. In addition to this, we found that significant cellular components involved to modulate these genes were extracellular region and space, plasma membrane, cytoskeleton, and cellular projections. At the molecular function level, growth factor activity significantly upregulated the DKK1 gene while growth factor and cytokine activity both significantly inhibited the KITLG gene (Figure 7D).

**DISCUSSION**

In glioblastoma, GSCs were first identified by Singh et al.\(^44\) as a population of cells capable of initiating tumor growth *in vivo*. 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.\(^45\)\(^-\)\(^47\) Importantly, also note that tumor cells are heterogeneous; therefore, it may be more advantageous to target multiple elements of various cellular pathways to eradicate GBM.\(^38\) 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 BBB 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, MSCs and their soluble factors are reported to exhibit beneficial anticancer effects.\(^49\)\(^-\)\(^51\) To investigate further whether the soluble factors of MSCs may affect different pathways related to proliferation and stemness of GSCs to transform them into non-stem-like cells prone to therapies, we planned this study. An additional objective was to identify whether different kinds of MSCs can exhibit the same anti-tumoral potential at the functional and molecular levels.

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

**Table 3. Significantly enriched canonical pathways (IPA)**

<table>
<thead>
<tr>
<th>Table 3. Significantly enriched canonical pathways (IPA)</th>
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<tbody>
<tr>
<td>Top canonical pathways based on significance</td>
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<tr>
<td>Gliospheres versus control (cell line and (-)e population)</td>
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<tr>
<td>Superpathway of cholesterol biosynthesis</td>
</tr>
<tr>
<td>Cholesterol biosynthesis I</td>
</tr>
<tr>
<td>Cholesterol biosynthesis II (via 24,25-dihydrolanosterol)</td>
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<tr>
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<td>Systemic lupus erythematosus T cell signaling pathway</td>
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<td>Calcium-induced T lymphocyte apoptosis</td>
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Metabolic reprogramming has been the hallmark of CSCs. Growing evidence has demonstrated that slow-cycling GSCs possess a preference for mitochondrial oxidative metabolism. Mitochondrial function plays a crucial role in maintaining stemness and drug resistance of GSCs.\(^52\)\(^-\)\(^54\) Few studies have demonstrated that CSCs can rely on fatty acid oxidation for their maintenance and function,\(^25\) and lipid catabolism seems critical for CSC 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 CSC enrichment.\(^58\) Since CSCs are enriched in mitochondrial mass and rely heavily on oxidative phosphorylation (OXPhos), disrupting this pathway has become an attractive therapeutic strategy. 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 tricarboxylic acid (TCA) cycle and fatty acid oxidation to oxygen. Complex V (ATP synthase) uses the stored energy to synthesize ATP.
energy in the proton gradient to generate ATP. As shown by our results of IPA (Table 3), 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 the ETC in OxPhos (Figure 3C). This may have, in turn, inhibited the proliferation, invasion, and sphere-forming ability of GSCs consistent with the study of Shi et al.

IPA analysis of WJT gliospheres showed activation of TREM1 signaling, GP6 signaling, and NF-κB signaling. It has been shown that TREM1 had been upregulated only in infectious inflammatory responses. In tumors, TREM1 seems to be induced on tumor-associated macrophages, which has been correlated with cancer recurrence and poor survival. Immunohistochemical analysis of breast tumor tissues confirmed the 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. It has also been investigated that TREM1 expression is regulated by NF-κB at the transcriptional level, emphasizing the contribution of NF-κB pathway activation in bridging inflammation and tumor promotion and progression. Hypoxia-regulated genes mediate blood vessel formation by stimulating encoding of chemotactic molecules such as CCL2, interleukin-8 (IL8), and VEGF that recruit macrophages and exert tumor-promoting effects such as angiogenesis. Our results are in agreement with the above-mentioned studies given macrophage activation and angiogenesis, as confirmed by the biological processes of treated spheres (Figure 6).

GPVI (glycoprotein VI) is exclusively expressed on platelets and megakaryocytes and together with integrin α2β1 mediates collagen-induced aggregation and adhesion. 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.97 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.95 Immune checkpoint inhibitors, PD-1 and PD-L1, have shown clinical efficacies against many different solid and hematologic malignancies.96 Binding of PD-L1 to its receptor suppresses T cell migration, proliferation, and secretion of cytotoxic mediators, and it restricts tumor cell killing. Inhibitors of PD-1 and PD-L1 disrupt the PD-1 axis, thereby reversing T cell suppression and enhancing endogenous antitumor immunity to unleash long-term antitumor responses in a wide range of cancers.97 Our results show that CM from BM-MSCs have exerted an adaptive immune response, which may have induced PD-L/PD-L1 expression on cancer cells.

Ca2+ 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 Ca2+ signaling pathways.81 A few studies have shown that some tumors develop an immune evasion strategy based on FasL-mediated destruction of invading lymphocytes.82–84 Invading T lymphocytes that express Fas are stimulated to apoptosis by tumor cells that express FasL. The expression of FasL has recently been demonstrated in GBM.85 It has also been reported that T lymphocytes were present in GBM 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 reversed the reaction of immune evasion by GSCs, which in turn upregulated apoptosis in GSCs, as shown by biological process analysis (Figure 6).

From GO results of a specific CSC array, we found two common genes significantly downregulated from CM of both MSCs, such as KITLG and DKK1.

DKK1 has downregulated the Wnt pathway (Figure 7A). Many 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.87–90 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 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 exert effects by targeting the Wnt/β-catenin signaling pathway.97,98 Our in silico results followed these studies, as we found inhibition of growth and stemness properties of GSCs through downregulation of the Wnt pathway mediated by growth factor activity at the molecular level (Figures 7A and 7D).

KITLG, also known as the stem cell factor (SCF) 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

### Table 4. Upregulated and downregulated genes in gliospheres comprised of glioma stem cells sorted from cell line using human cancer stem cell array compared to control (cell line and –Ve population)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold regulation</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>3.42</td>
<td>0.078675</td>
</tr>
<tr>
<td>CD16</td>
<td>7.36</td>
<td>0.050531</td>
</tr>
<tr>
<td>DNMT1</td>
<td>2.43</td>
<td>0.046934</td>
</tr>
<tr>
<td>EGF</td>
<td>2.58</td>
<td>0.264680</td>
</tr>
<tr>
<td>EPCAM</td>
<td>5.66</td>
<td>0.174573</td>
</tr>
<tr>
<td>GATA3</td>
<td>2.31</td>
<td>0.249860</td>
</tr>
<tr>
<td>GSK3B</td>
<td>2.27</td>
<td>0.028471</td>
</tr>
<tr>
<td>IKKβ</td>
<td>2.07</td>
<td>0.041685</td>
</tr>
<tr>
<td>ITGA6</td>
<td>2.19</td>
<td>0.016071</td>
</tr>
<tr>
<td>JAG1</td>
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<td>0.126777</td>
</tr>
<tr>
<td>JAK2</td>
<td>2.57</td>
<td>0.232893</td>
</tr>
<tr>
<td>KITLG</td>
<td>2.16</td>
<td>0.115002</td>
</tr>
<tr>
<td>LAT51</td>
<td>2.22</td>
<td>0.033813</td>
</tr>
<tr>
<td>LIN28B</td>
<td>7.72</td>
<td>0.031777</td>
</tr>
<tr>
<td>MAML1</td>
<td>2.20</td>
<td>0.088189</td>
</tr>
<tr>
<td>NANO5</td>
<td>3.36</td>
<td>0.134051</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>2.15</td>
<td>0.141905</td>
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<tr>
<td>POU5F1</td>
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<td>0.252004</td>
</tr>
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<td>PROM1</td>
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<tr>
<td>TAZ</td>
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<td>0.071685</td>
</tr>
<tr>
<td>TWIST2</td>
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<td>WWC1</td>
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<td>YAP1</td>
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<td>ZEB2</td>
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<td>0.080967</td>
</tr>
<tr>
<td>DKK1</td>
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<tr>
<td>FOXP1</td>
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</tr>
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<td>CXCL8</td>
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<td>0.483770</td>
</tr>
<tr>
<td>NOS2</td>
<td>–3.39</td>
<td>0.275178</td>
</tr>
</tbody>
</table>

Cancer stem cells were determined with the RT²Profiler PCR array (catalog no. PAHS-176Z, Qiagen, USA).

IPA analysis of BMT spheres showed upregulation of the PD-L1/PD-L1 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 it restricts tumor cell killing. Inhibitors of PD-1 and PD-L1 disrupt the PD-1 axis, thereby reversing T cell suppression and enhancing endogenous antitumor immunity to unleash long-term antitumor responses in a wide range of cancers.70 Our results show that CM from BM-MSCs have exerted an adaptive immune response, which may have induced PD-L1/PD-L1 expression on cancer cells.
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.\(^9\) We found that CM from MSCs inhibited these pathways through the downregulation of the KITLG gene mediated by the growth factor and cytokine activity. Also, phospholipase D (PLD) activity has been suggested to function as a sensor of metabolites, including lipid pools,\(^1\) and a critical regulator of autophagy.\(^1\) Keeping this in mind, we predict that the metabolism of the GSCs was deregulated by inhibition of the PLD pathway, which might have upregulated apoptosis and differentiation also by biological processes (Figure 7B) mediated by the KITLG gene.

Among uniquely upregulated genes of the 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.\(^10\) In addition to tumor initiation, LIN28B is necessary for the maintenance of cancers as well.\(^10\) Recent advances have shown that Lin28 regulates let-7 miRNA biogenesis and mRNA translation, to coordinate both cellular metabolism and proliferative growth pathways for stem cell self-renewal.\(^10\) Our results of WJT spheres showed the downregulation of LIN28B that might have contributed to the inhibition of metabolism and the self-renewal capacity of GSCs and can alone be considered as a biomarker of GBM stemness after further evaluation at the protein level and using an in vivo model.

From BMT spheres, uniquely downregulated genes were JAG1 and POU5F1. JAG1 is the ligand 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.\(^10\) Moreover, studies have shown that the downregulation of Jagged1 induces apoptosis and inhibits proliferation in glioma cell lines.\(^10\) Similarly, different variants of OCT4 (POU5F1) have been related to colony formation and regulation of cell survival in GSCs.\(^10\) 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 is the AGE-RAGE signaling pathway. RAGE was first identified as a receptor for AGE to diabetes, renal diseases, and aging.\(^10,10\) In glioblastomas, RAGE is expressed on tumor cells, endothelial cells, stromal cells, and tumor-associated macrophages, comprising microglia and myeloid-derived macrophages.\(^10\) 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.\(^111-113\) 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 while sensitizing CSCs 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 BBB impairing the ability for peripheral lymphocytes to traffic to the tumor microenvironment, and the high prevalence of corticosteroid use, all of which suppress lymphocyte activation. However, by simultaneously targeting multiple costimulatory and inhibitory pathways, it may be possible to achieve an effective antitumoral immune response.\(^114\) This is where a combination of manipulated MSC-secreted factors has the most significant potential for cell-free-based anticancer therapies.

### Conclusions

Taken together, the results of microarray and CSC arrays, in vitro, elucidate the possible molecular targets by which secreted factors of MSCs 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 growth factors and cytokines in CM from two sources of MSCs hold 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 the central nervous system (CNS) by either directly crossing the BBB or through the retrograde transport mechanism in the CNS. CM have already been implicated for many neurodegenerative diseases,\(^115-117\) and 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 a more valuable effect as a combinatorial therapy in conjunction with antitumor immune therapy to treat gliomas since it has inhibited the T lymphocyte apoptosis pathway to facilitate the immune reaction and also inhibited the pluripotent stem cell markers. This study has provided valuable information regarding the potential ability of acclimatized MSCs with the GSC environment to interrupt the growth and pluripotency of GSCs with the potential for translation to practical treatment options for GBM in human patients. Furthermore, in vivo studies along with proteomics are warranted to translate the apparent therapeutic efficacy of CM of MSCs in the treatment of glioblastoma. Moreover, identification of the specific growth factors and cytokines responsible for the inhibitory response will provide useful information for developing an effective paradigm for glioblastoma treatment.

MATERIALS AND METHODS

Isolation of GSCs from GBM cell line U87MG

Monolayer generation of cell line

The GBM cell line U87MG (ATCC HTB14TM) was expanded according to the protocol described by ATCC with a slight modification. Briefly, the cell line was expanded in standard culture media (DMEM-F12 1:1, l-glutamine 200 mM, 10,000 U/mL penicillin/streptomycin, 25 μg/mL amphotericin B, and 10% heat-inactivated fetal bovine serum [FBS]; Gibco/Invitrogen, USA). The cells were

Figure 5. Gene ontology terms of gliospheres from human CSC arrays

(A and C) Activated KEGG pathways according to (A) significant genes (p values) and (C) high fold regulation in gliospheres. (B and D) Upregulated biological processes according to (B) significant genes (p values) and (D) high fold regulation in gliospheres. Color variations shows the significance level, with red being the highest (p < 0.0001) and white the lowest (p < 0.05).
seeded at $1 \times 10^4$ cells/cm$^2$ in T75 cultures flasks and maintained at 37°C and 5% CO$_2$. The culture medium was exchanged every 2–3 days. FACS sorting and enrichment of glioma stem cells

The cell line (U87MG) expanded at different passages (P3–P9) was investigated for the percentage of the CD133$^+$ population, which was sorted by using a fluorescence-activated cell sorting (FACS) JAZZ cell sorter (BD Biosciences, USA) according to the manufacturer’s instruction. Briefly, upon reaching 80%–90% confluence, U87MG cells were harvested using 0.25% trypsin EDTA (Invitrogen, USA), washed twice with cold PBS (Invitrogen, USA), and centrifuged at 300 × g for 5 min. The cell pellet was re-suspended in 100 μL of BD FACS staining buffer (BD Biosciences, USA), and 10 μL of CD133 antibody (eBioscience, USA) was added, mixed well, and put at 4°C for about 30 min in the dark. Next, 500 μL of cold PBS was added to wash the cells and centrifuged at 300 × g for 5 min, and the pellet was re-suspended in 500 μL of PBS for sorting. Sorted populations were divided into positive and negative fractions and were evaluated for the enrichment of CD133$^+$ cells using a FACSCanto II and analyzed by FACS-Diva software version 7 (BD Biosciences, USA).

Characterization of sorted populations

First gliosphere generation

Sorted tumor cells from both positive and negative populations were directly seeded in CSC media (termed herein as GSM) (DMEM-F12 buffer (BD Biosciences, USA), and 10 μL of CD133 antibody (eBioscience, USA) was added, mixed well, and put at 4°C for about 30 min in the dark. Next, 500 μL of cold PBS was added to wash the cells and centrifuged at 300 × g for 5 min, and the pellet was re-suspended in 500 μL of PBS for sorting. Sorted populations were divided into positive and negative fractions and were evaluated for the enrichment of CD133$^+$ cells using a FACSCanto II and analyzed by FACS-Diva software version 7 (BD Biosciences, USA).

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supplemented with N2 [Gibco, USA], epidermal growth factor [EGF] [20 ng/mL, Invitrogen], basic fibroblast growth factor [bFGF] [20 ng/mL, Gibco, USA], leukemia inhibitory factor [LIF] [10 ng/μL, Chemicon, Germany], and B27 [1:50; Life Technologies, USA] in six-well ultralow attachment plates (Corning, USA) to enrich for glioma stem cells (gliospheres) and were maintained at 37°C 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.

CD133+ and CD133− selected cell populations, derived from first gliospheres, were dissociated using StemPro Accutase cell dissociation reagent (Gibco, USA) according to the manufacturer’s instructions. Cells were subsequently re-suspended in GSM and seeded in 24-well ultralow plates (Corning, USA) at 1 × 10^3 cells/well. The formation of free-floating sub-spheres was observed by phase-contrast microscopy (Zeiss, Germany), and the experiment was conducted until passage 12 (G12, gliosphere). Cells in each passage were kept in culture between 7 and 10 days.

**Monolayer generation**

Similarly, small fractions of sorted cells from positive and negative populations were cultured in standard culture media (DMEM-F12 + 10% FBS, Gibco, USA) to investigate their expansion ability adherently after sorting. The morphological changes were observed

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**Figure 7. Significant genes affected by both treatments (CM from WJ-MSCs and BM-MSCs)**

(A) Significantly downregulated genes (p value) and KEGG pathways by both treatments. (B) Significantly downregulated biological processes from two treatments. (C) Significant cellular components activated in response to the effect of two treatments. (D) Significant molecular functions depicting the response of two genes as a result of activation by growth factor and cytokine activity.
under a phase-contrast microscope (Zeiss, Germany). The experiment was conducted for six passages, and one passage time was kept between 5 and 7 days.

**Immunofluorescence assay of CD133⁺ population**

A panel of GSC markers, neuronal lineage markers, and late differentiated neuronal markers were selected (Table 1) to characterize the sorted CD133⁺ population by immunocytochemistry. Briefly, sub-spheres at passage 13 (G13) were harvested by treatment with StemPro Accutase cell dissociation reagent (Gibco, USA) and washed with PBS (pH 7.4, Gibco, USA), followed by centrifugation. After resuspension, cells were seeded on poly-l-lysine-coated coverslips at a seeding density of 2 × 10⁵ cells/coverslip and were immersed in GSM with 2% FBS (Gibco, USA). After 2 days the cells were fixed with 4% formaldehyde for 20 min followed by washing. Coverslips were then permeabilized with 0.3% Triton X-100 for 15 min at room temperature. Blocking was performed with 10% normal goat serum (Gibco, USA) for 1 h at room temperature. Cells were stained with the primary antibodies in 1% blocking buffer overnight at 4°C with shaking followed by washing with 0.1% Triton X-100 in PBS three times for 5 min each. Nuclei were stained with DAPI (Thermo Fisher Scientific, USA) for 5 min and coverslips were mounted onto slides. Cell imaging was performed on an inverted phase-contrast fluorescence microscope (Zeiss Axio Observer Z1, Zeiss, Germany). The description of the primary and secondary antibodies used for the assay is given in Table 1.

**Functional assays**

**Preparation of MSC CM under GSM conditions**

BM-MSCs and WJ-MSCs were acquired from the Cell Therapy Center, The 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 G3 were used to generate CM. All six cell lines were passaged for both adherent and spheroid system. To investigate whether the effect exerted by CM from both types of MSCs is reversible or irreversible, clonogenic assays (colony-forming efficiency [CFE] and sphere-forming efficiency [SFE]) were performed for both adherent and spheroid system.

**Optimization of CM concentrations**

Initially different concentrations of CM were tested on morphology and proliferation potential of glioma stem cells (Figures S1A and S1B). As described above, GSM and SFM were tested as 100% and 50% with fresh GSM, from one sample of BM1-MSCs and WJ3-MSCs, and SFM and GSM were kept as controls. These experiments were conducted for 4 days (96 h) only. Based on these results, further experiments were planned from 100% GSCM for 7 days, and to replenish the growth factors in the GSCM, we used the CM in a ratio of 10%:90% (GSM/GSCM). For simplicity, we have used the term CM herein.

**Assessment of the effects of CM on GSCs (gliospheres)**

**Morphological assessment**

Briefly, 1 × 10⁴ cells/cm² from dissociated gliospheres at 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 a control. CM was added every 48 h, and changes in the sphere-forming ability of GSCs were captured by phase-contrast microscopy (Zeiss, Germany).

**3D cell proliferation and viability assessment**

The proliferation rate of GSCs (at G13) was evaluated by a CellTiter-Glo 3D cell viability assay (Promega, USA). In brief, the cells were cultured at a seeding density of 5 × 10⁵ cells/well in 100 μL of CM in 96-well ultralow attachment plates (Corning, USA). Every 48 h fresh CM were added along with control and the 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 detection system, Promega, USA).

**Invasion assay**

To determine the effect of CM on the invasive ability of GSCs, an invasion assay was performed using a Cultrex BME cell invasion assay (Trevena, USA) with a slight modification. Briefly, 5 × 10⁴ cells were treated with CM from both types of MSCs in six-well ultralow attachment plates (Corning, USA) for 7 days. The media were refreshed every 48 h. On day 7 the spheres were collected and harvested by Accutase (Gibco, USA), centrifuged at 200 × g for 4 min, and 5 × 10⁴ cells were seeded in the upper chamber of the BME 96-well plate. The lower chamber was filled with serum media (DMEM-F12 + 10% FBS, Gibco, USA). After 2 days the plates were processed according to the manufacturer’s instruction and the luminescence was measured using a microplate reader (GloMax-multi detection system, Promega, USA).

**Clonogenic assay**

To investigate whether the effect exerted by CM from both types of MSCs is reversible or irreversible, clonogenic assays (colony-forming efficiency [CFE] and sphere-forming efficiency [SFE]) were performed for both adherent and spheroid system.

**CFE.** Briefly, 5 × 10³ cells/well were treated in 24-well ultralow plates (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 six-well plates (TPP, USA) in standard culture media (DMEM-F12 + 10%...
FBS) for 2 weeks (14 days). The medium was exchanged every 3 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).

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

**Molecular assays (microarrays and CSC arrays)**

**RNA extraction**

Brieﬂy, 1 × 10^6 cells/cm^2 (G13) were seeded in 25 mL of ultralow attachment ﬂasks (Corning, USA) containing CM from two types of MSCs and control (gliospheres). The sorted population that did not make spheres was taken as a negative control. On day 7 the spheres were harvested using Accutase (Gibco, USA). Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) and cleaned up with the RNeasy mini kit (QIAGEN, USA) following the instructions of the manufacturer. Extracted RNA was quantified using the NanoDrop 2000c spectrophotometer system (Thermo Fisher Scientiﬁc, USA). RNA quality analysis was performed using an Agilent 2100 Bioanalyzer instrument with an Agilent RNA 6000 Nano kit according to the manufacturer’s instructions.

**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, Santa Clara, CA, USA) was used for gene expression proﬁle analysis. The procedure was followed as described by the manufacturer. The microarray data can be accessed via Gene Expression Omnibus (GEO: GSE149216). For simplicity, we use the terms BMT and WJT for treated gliospheres herein.

**Data analysis**

Raw CEL file normalization was performed using the signal space transformation-robust multi-array analysis (SST-RMA) using Affymetrix Expression Console software (transcription analysis console [TAC]) version 4.0.1 (Affymetrix). DEGs at FC (log_2) of ≥2 or ≤−2 or less with a statistical signiﬁcance level of p < 0.05 (gliospheres versus BMT gliospheres, gliospheres versus WJT gliospheres, and gliospheres versus control) were picked up. GO term and pathway enrichment analyses were conducted to determine the roles of these DEGs by IPA software (Ingenuity Systems, Redwood City, CA, USA), NetworkAnalyst (3.0) (https://www.networkanalyst.ca/), and using the open-source Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/home.jsp).

**RT² Profiler PCR array**

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 analyzed according to the manufacturer’s instructions (QIAGEN, USA). Brieﬂy, cDNA was synthesized using the RT² First Strand Kit (catalog no. 330404, QIAGEN, USA). A diluted cDNA aliquot was mixed with RT2 SYBR Green qPCR mastermix (catalog no. 330503, QIAGEN, USA) and loaded into the 96-well array plate of RT2 Profiler PCR array human CSCs (catalog no. PAHS-176Z, QIAGEN, USA). qPCR reactions were performed using the CFX96 C1000 Touch thermal cycler (Bio-Rad, USA) with the following temperature setting: (1) 95°C for 10 min, and (2) 40 cycles of 95°C for 15 s and 60°C for 1 min. The data analysis was performed using the 2^(-ΔΔCt) method available by the SA Biosciences company (QIAGEN, USA). The data were normalized, across all plates, to the average of the arithmetic mean of the following housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-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.

**Statistical analysis**

All experiments were performed in triplicates (n = 3). Data were analyzed using Microsoft Excel and GraphPad Prism software. Quantitative data were expressed as mean ± standard deviation. Data were evaluated by two-way ANOVA, and Dunnett’s post-test was used to analyze multiple comparisons (p < 0.05). All procedures performed in this study were in accordance with the instructions from institutional review board of the Cell Therapy Center.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.11.005.

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

The authors declare no competing interests.
REFERENCES


