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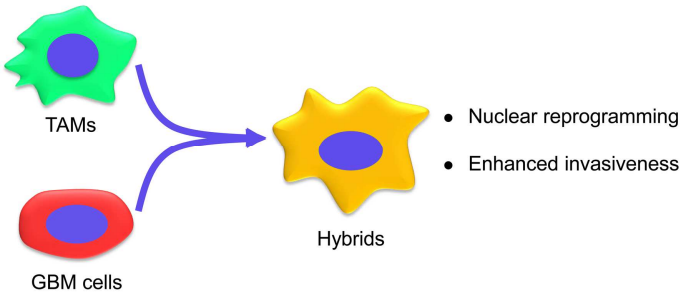
Abstract

Hybrid formation is a fundamental process in normal development and tissue homeostasis, while the presence and the biological role of hybrids between tumor-associated macrophages (TAMs) and glioblastoma (GBM) cells remain elusive. In this study, we observed that TAM-GBM cell hybrids existed in human GBM specimens as demonstrated by co-expression of glioma biomarkers (GFAP, $IDH1^{R132H}$ and PDGFRA) and macrophage biomarkers (CD68 and CD14). Furthermore, TAM-GBM cell hybrids could also be found in C57BL/6 mice orthotopically inoculated with mouse GBM cells labeled with RFP and after co-culture of bone marrow-derived macrophages from GFP-expressed mice with RFP-labeled GBM cells. The hybrids underwent nuclear reprogramming with unique gene expression profile as compared to parental cells. Moreover, glioma invasion-associated genes were enriched in the hybrids that possessed higher invasiveness, and more hybrids in the invasive margin of GBM were observed as compared to GBM core area. Our data demonstrate the presence of TAM-GBM cell hybrids that enhances GBM invasion. With a better understanding of TAM-GBM cell hybrids, new therapeutic strategies targeting GBM will be developed to treat GBM patients.



Hybrids by tumor-associated macrophages × glioblastoma cells entail nuclear reprogramming and glioblastoma invasion

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Abstract

Hybrid formation is a fundamental process in normal development and tissue homeostasis, while the presence and the biological role of hybrids between tumor-associated macrophages (TAMs) and glioblastoma (GBM) cells remain elusive. In this study, we observed that TAM-GBM cell hybrids existed in human GBM specimens as demonstrated by co-expression of glioma biomarkers (GFAP, $IDH1^{R132H}$ and PDGFRA) and macrophage biomarkers (CD68 and CD14). Furthermore, TAM-GBM cell hybrids could also be found in C57BL/6 mice orthotopically inoculated with mouse GBM cells labeled with RFP and after co-culture of bone marrow-derived macrophages from GFP-expressed mice with RFP-labeled GBM cells. The hybrids underwent nuclear reprogramming with unique gene expression profile as compared to parental cells. Moreover, glioma invasion-associated genes were enriched in the hybrids that possessed higher invasiveness, and more hybrids in the invasive margin of GBM were observed as compared to GBM core area. Our data demonstrate the presence of TAM-GBM cell hybrids that enhances GBM invasion. With a better understanding of TAM-GBM cell hybrids, new therapeutic strategies targeting GBM will be developed to treat GBM patients.

Keywords: tumor microenvironment; tumor immunity; glioblastoma invasiveness.

Abbreviations: BMDMs-GFP, green fluorescent protein-labeled bone marrow-derived macrophages; DAPI, 4,6-diamidino-2-phenylindole; EdU, 5-ethynyl-2'-deoxyuridine; FACS, fluorescence-activated cell sorter; FCM, flow cytometry; FISH, fluorescence in situ hybridization; GBM, glioblastoma; GL261-RFP, red fluorescent protein-labeled murine glioblastoma cell line GL261; GSEA, gene set enrichment analysis; IDH, isocitrate dehydrogenase; $IDH1^{R132H}$, isocitrate dehydrogenase 1 with R132H mutation; PDGFRA, platelet derived growth factor receptor alpha; TAMs, tumor-associated macrophages.

1. Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain malignant tumor. The median survival time for GBM is less than 15 months despite development in therapeutics [1]. To a certain extent, the poor prognosis results from the interaction of GBM cells with tumor microenvironment [2]. In the tumor microenvironment, tumor-associated macrophages (TAMs) are the major infiltrating immune cells which confer protumoral functions in GBM [2]. TAMs are recruited and activated by the factors secreted by GBM cells such as CSF-1 and POSTIN [3,4]. In turn, TAMs promote GBM progression through TGFB1-TGFBR2 or PTN-PTPRZ1 signaling pathways [5,6]. Additionally, formation of hybrids by TAMs and tumor cells has been reported as an alternative way to promote tumor progression [7,8].

Hybrid formation is a fundamental process in normal development and tissue homeostasis [9]. As to tumor cell hybrids, perhaps the best-known are hybridomas formed by myeloma cells and lymphocytes to produce monoclonal antibodies. Indeed, the hypothesis of tumor cell hybrids was firstly proposed in 1911 by Otto Aichel that tumor cells might form hybrids with motile leucocytes to confer the malignant cells with enhanced invasiveness and metastasis [7,8]. To date, hybrids between tumor cells and TAMs have been reported in multiple cancers including melanoma [10-15], renal carcinoma [16,17], ovarian carcinoma [18], intestinal adenoma [19], and pancreatic ductal adenocarcinoma [14], but most of these studies just identified TAM-tumor cell hybrids without further describing the gene expression characteristics and the biological role of hybrids. Therefore, further research on TAM-tumor cell hybrids is needed.

In the current study, we aimed to explore the presence of TAM-GBM cell hybrids and investigate the transcriptome characteristics as well as the biological role of them. We found that TAM-GBM cell hybrids existed in human GBM specimens, animal allografts and *in vitro* co-culture system. Compared to parental GBM cells and TAMs, the hybrids generated novel gene expression characteristics after nuclear reprogramming. Besides, gene set enrichment analysis (GSEA) showed that hybrids were enriched with glioma invasion-associated genes. Moreover, hybrids were higher invasive than GBM cells as analyzed by invasion assay, and

there were more hybrids in the invasive margin as compared to the GBM core area. Our results demonstrate the presence of TAM-GBM cell hybrids that bear nuclear reprogramming with unique gene expression characteristics and contribute to GBM invasion.

2. Materials and methods

2.1. Clinical GBM specimens

Surgical GBM specimens were obtained from patients in the Department of Neurosurgery, Southwest Hospital with written consent. The tumors were independently diagnosed by at least two neuropathologists according to the 2016 WHO classification of tumors of the central nervous system [20], and the clinicopathologic features of these patients were summarized in Supplementary Table 1. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Southwest Hospital.

2.2. Cell culture

Murine GBM cell line GL261 was a gift from Prof. Zi-ling Wang (Beijing Institute of Transfusion Medicine, Beijing, China), which was originally from NCI (National Cancer Institute, Frederick, Maryland) [21]. The cell line was authenticated through STR testing (Beijing Microread Genetics Co., Ltd., Beijing, China) and maintained in DMEM (Hyclone, GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania) with 10% FBS (Hyclone). Murine fibroblast cell line L-929 was purchased from the Cell Bank of Shanghai Institute of Cell Biology and Biochemistry, Chinese Academy of Sciences (Shanghai, China), cultured in RPMI-1640 (Hyclone) containing 10% FBS (Hyclone) to collect L-929 conditioned medium. All the cells were not contaminated by mycoplasma and incubated in a humidified incubator with 5% CO_2 and 37 °C temperature.

2.3. Isolation and IL-4 treatment of GFP-labeled bone marrow-derived macrophages (BMDMs-GFP)

BMDMs-GFP were isolated and differentiated according to the classic protocol [22]. Briefly, GFP-expressing mice [C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb] (aged 6 to 8 week) [23,24], purchased from Model Animal Research Center of Nanjing University (Nanjing, China), were used to obtain bone marrows. After culture for 7 days in the macrophage complete medium (RPMI-1640 containing 10% FBS supplemented with 20% L-929 conditioned medium), the mature BMDMs-GFP were stimulated with 20 ng/ml IL-4 (PeproTech China, Suzhou, China) for 6 h for measuring mRNA, and for 24 h for flow

cytometry and *in vitro* co-culture experiments.

2.4. In vitro co-culture experiments

In the co-culture system, RFP-labeled GL261 (GL261-RFP) cells were mixed with IL-4 primed BMDMs-GFP at various ratios and co-cultured in tissue-treated culture dishes with RPMI-1640 containing 10% FBS. After co-culture for 48 h, the percentage of hybrids was analyzed through fluorescence-activated cell sorter (FACS) (BD FACSAria II, BD Biosciences, San Jose, California). Time-lapse imaging of the co-culture system was performed using the Cell Observer microscope (Carl Zeiss AG, Oberkochen, Germany) equilibrated to 5% CO_2 and 37 ℃ temperature and the associated AxioVision software (Carl Zeiss AG). Fluorescent images were taken every 20 minutes using RFP and GFP detectors with a 40X objective, and the video was produced at 3 frames per second.

2.5. 5-ethynyl-2'-deoxyuridine (EdU)-labeling and detection

BMDMs-GFP were labeled with EdU as previously described with minor modification [14]. Briefly, bone marrows isolated from the GFP-expressing mice were cultured in the macrophage complete medium for 7 days with 10 μM EdU (Beyotime Biotechnology, Shanghai, China) supplemented at the 6th day for 24 h. Then the EdU-labeled BMDMs-GFP were treated with 20 ng/ml IL-4 (PeproTech China) for 24 h, and were incubated with non-EdU-labeled GL261-RFP cells for additional 48 h. In turn, GL261-RFP cells were pretreated with 10 μM EdU for 24 h and incubated with non-EdU-labeled BMDMs-GFP for additional 48 h. The cells in the co-culture systems were FACS-sorted, labeled with Alexa Fluor 647 according to the protocol of BeyoClick™ EdU Cell Proliferation Kit (Beyotime Biotechnology), and detected by Zeiss 800 confocal microscope.

2.6. GBM allograft implantation

GL261-RFP cells were orthotopically injected into the brains of 6-wk-old female C57BL/6 mice (1 x 10^5/mouse) (Laboratory Animal Center, Southwest Hospital) as previously described [5,6]. For the sex-mismatched GL261-RFP allograft model, the male mice were used as the hosts. The tumor-bearing mice were sacrificed at the 14th day after transplantation

and the allografts were obtained for further flow cytometry analysis, immunofluorescence staining and fluorescence in situ hybridization (FISH). The animal studies were approved by the Institutional Animal Care and Use Committee of the Southwest Hospital according to the Guide for the Care and Use of Laboratory Animals.

2.7. Flow cytometry (FCM) and cell sorting

Single-cell suspensions were prepared from a portion of human GBM specimens or entire GL261-RFP allografts as described previously [5], and then the human and mouse GBM single-cell suspensions were incubated with Human BD Fc Block (BD Pharmingen, 564219) (BD Biosciences, San Jose, California) or Mouse BD Fc Block (BD Pharmingen, 553142) respectively for 10 min at 4 °C to block Fc receptors. Subsequently, for FCM analysis of the percentage of hybrids in a portion of human GBM specimens, the human single cells were incubated with PE-conjugated mouse anti-GFAP (BD Pharmingen, 561483) and Alexa Fluor 647-conjugated mouse anti-human CD68 (BD Pharmingen, 562111) according to the intracellular staining protocol of BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Pharmingen). Alternatively, in preparation for the following double immunofluorescence staining on FACS-sorted cells, antibodies from different species were chosen, *i.e.*, purified rabbit anti-GFAP (Abcam, ab33922) (Cambridge, United Kingdom) with the secondary anti-rabbit antibody (Thermo Fisher Scientific, A-11034) (Waltham, Massachusetts) and the above mouse anti-CD68 were used for intracellular staining for cell sorting. While the single-cell suspensions of GL261-RFP allografts were incubated with APC-conjugated rat anti-mouse CD11b (BD Pharmingen, 553312) for 30 min on ice. To detect the invasion marker CXCR4, APC-conjugated rat anti-mouse CXCR4 (Biolegend, 146507) was used together with FITC-conjugated rat anti-mouse CD11b (BD Pharmingen, 561688). For all staining, isotype controls were used. Doublets were discriminated using pulse-width parameter and dead cells were gated out by the LIVE/DEAD® Fixable Dead Cell Stain Kits (Thermo Fisher Scientific). Data acquisition and cell sorting were performed on BD FACSAria II and data were analyzed through FlowJo X 10.0.7 (Treestar) (BD Biosciences, San Jose, California).

2.8. Statistical analyses

Unpaired Student's t-test and one-way ANOVA were applied respectively to compare two groups and multiple groups. All experiments were performed independently at least three times and data were presented as mean ± SEM. Statistical significance was assigned at $p<0.05$ (*), $p<0.01$ (**) or $p<0.01$ (***). These statistical analyses were performed by SPSS 19.0 or GraphPad Prism 6.0.

2.9. Lentiviral transduction, invasion assay, immunofluorescence staining, FISH, RNA-seq and quantitative RT-PCR

See Supplementary Materials and Methods.

3. Results

3.1. Hybrids between TAMs and GBM cells in human GBM specimens

Hybrid formation is a fundamental process in normal development and tissue homeostasis [9]. However, the existence of TAM-GBM cell hybrids remains elusive. We detected TAM-GBM cell hybrids in human GBM specimens on the basis of the established glioma biomarker GFAP and human macrophage biomarker CD68 [25,26]. As shown in Fig. 1A, FSC and SSC were firstly used for gating debris-free cells from cell suspensions made from a portion of surgical GBM specimens. Then SSC-Height and SSC-Width were used for gating single cells. Next, dead cells before fixation were gated out. At last, we analyzed the percentages of $GFAP^+CD68^+$ hybrids. We found that there was a small percentage of $GFAP^+CD68^+$ hybrids in human GBM, varying from 1.35% to 1.96% (Fig. 1B). Some of FACS-sorted $GFAP^+CD68^+$ hybrids exhibited multi-nucleated as validated by immunofluorescence analysis (Fig. 1C, Fig. S1 and S2), while Fig. S1A indicated single GFAP positive cells and single CD68 positive cells and Fig. S1B exhibited a low magnification scope to show the purity of FACS-sorted $GFAP^+CD68^+$ hybrids. The $GFAP^+CD68^+$ hybrids were also detected on human GBM tissues through immunofluorescence analysis (Fig. 1D, Movie S1 and S2). Besides, we conducted double staining of $IDH1^{R132H}$ and CD68 on IDH1 mutant GBM tissues and found $IDH1^{R132H}$/CD68 positive hybrids (Fig. S3, Movie S3 and S4). As platelet derived growth factor receptor alpha (PDGFRA) amplification is common in GBM [27], we also analyzed expression of PDGFRA and macrophage biomarker CD14 on GBM patient tissues, and observed $PDGFRA^+$ $CD14^+$ hybrids (Fig. S4, Movie S5 and S6). Taken together, these data demonstrate that there are TAM-GBM cell hybrids in clinical GBM samples.

3.2. Hybrids formed by TAMs and GBM cells in vivo and in vitro

To further confirm the presence of TAM-GBM cell hybrids, the sex-mismatched GL261-RFP transplanted tumor model was conducted. The female GL261-RFP cells were implanted orthotopically into the male C57BL/6 mice (Fig. 2A, Fig. S5), and then TAM-GBM cell hybrids together with GBM cells and TAMs were sorted from the GBM-bearing mice on the basis of RFP labeled on GBM cells and CD11b expressed by murine macrophages (Fig. S6). The FACS-sorted cells were validated by FISH (Fig. 2A, Supplementary Table 2), and

immunofluorescence staining (Fig. 2B, Supplementary Table 3). Of note, multiple X chromosomes and one Y chromosome such as XXY and XXXY were included in the same nucleus of the RFP^+CD11b^+ cells, revealing the existence of hybridization between TAMs and GBM cells (Fig. 2A, Supplementary Table 2). Further immunofluorescence and FISH analyses on the same cells confirmed the presence of TAM-GBM cell hybridization (Fig. 2C). Hybrids were also detected on the tissues of murine GBM allografts as demonstrated by co-expressing of both RFP and CD11b (Fig. 2D, Movie S7 and S8).

Besides, we established the co-culture system of GL261-RFP cells and IL-4 induced BMDMs-GFP to detect TAM-GBM cell hybrids (Fig. 3A, Fig. S5, Fig. S7), and observed the cell expressing both RFP and GFP, referred to as TAM-GBM cell hybrid (Fig. 3B and 3C, Fig. S8, Movie S9). Among the various mixed ratios of GL261-RFP to BMDMs-GFP, one GL261-RFP to four BMDMs-GFP was the optimal ratio to make the greatest contribution to the percentage of hybrids (Fig. 3D). To explore whether hybrids in the RFP^+GFP^+ gate were really double-positive, we conducted flow cytometry analysis and immunofluorescence staining on the FACS-sorted cells from the co-culture system. As shown in Fig. S9, hybrids from the RFP^+GFP^+ gate expressed both RFP and GFP. Additionally, we found that the hybrid could exhibit one EdU^+ nucleus together with one EdU^- nucleus in the EdU-labeled BMDMs-GFP/non-EdU-labeled GL261-RFP co-culture system (Fig. 3E), as well as in the EdU-labeled GL261-RFP/non-EdU-labeled BMDMs-GFP co-culture system (Fig. S10). Collectively, these data demonstrate that TAM-GBM cell hybrids exist *in vivo* and *in vitro*.

3.3. TAM-GBM cell hybrids exhibit unique transcriptome characteristics

Hybrid formation may result in generation of novel gene expression after nuclear programming [19]. Therefore, to analyze the gene expression characteristics of TAM-GBM cell hybrids, we conducted RNA-seq in the FACS-sorted hybrids along with parental cells from GBM allografts which were validated through immunofluorescence staining before sequencing (Fig. 2B, Fig. S6). As compared to GBM cells, hybrids up-regulated 3038 genes and down-regulated 2694 genes (Fig. 4A and 4B), while they had 316 up-regulated genes and 490 down-regulated genes as compared to TAMs (Fig. 4A and 4B). There were 39

up-regulated genes and 39 down-regulated genes of hybrids as compared to both parental GBM cells and TAMs (Fig. 4B and 4C, Supplementary Table 4 and 5). The top 10 from the up-regulated genes or the down-regulated genes of hybrids were further validated by qRT-PCR (Fig. 4D). GO and KEGG pathways analyses showed that the differently expressed genes of hybrids were enriched in chemokine-mediated signaling pathway, positive regulation of JAK-STAT cascade, digestive system development, *etc*. (Fig. 4E). Taken together, these data suggest that TAM-GBM cell hybrids undergo reprogramming and thus acquire unique gene expression characteristics.

3.4. TAM-GBM cell hybrids are enriched with cell hybridization-associated genes

There are many genes involved in cell hybridization, although currently none of them have been demonstrated as fusogens except for Syncytins in mouse placenta and F proteins in *Caenorhabditis elegans* [9]. Thus we established a gene set of cell hybridization-associated genes (referred to as the genes involved in cell hybridization) (Supplementary Table 6), which was followed by GSEA analysis and qRT-PCR analysis. As compared to GBM cells, hybrids were enriched with cell hybridization-associated genes (NES=1.33, p<0.001) (Fig. 5A). However, there was no statistical significance between hybrids and TAMs (NES=-0.75, p=0.878) (Fig. S11). Validation by qRT-PCR analysis revealed that three top enriched cell hybridization-associated genes (Adam8, Atp6v0d2 and Ocstamp) were up-regulated in hybrids as compared to both GBM cells and TAMs (Fig. 5B). Collectively, these results demonstrate that TAM-GBM cell hybrids are enriched with cell hybridization-associated genes, indicating that cell hybridization-associated genes may participate in hybrid formation.

3.5. TAM-GBM cell hybrids contribute to GBM invasiveness

As macrophages entail strong migratory ability, formation of hybrids between macrophages and tumor cells may confer tumor cells with enhanced invasiveness and metastasis [7,8]. Therefore, we analyzed the invasion phenotype of TAM-GBM cell hybrids. As shown in Fig. 6A, hybrids were enriched with glioma invasion-associated genes (Fig. 6A, Supplementary Table 7). The top 10 invasion-associated genes of hybrids were further validated by qRT-PCR and FCM (Fig. 6B and 6C). As qRT-PCR analysis demonstrated, three invasion-associated

genes, Adam8, Mmp12 and Mmp13, were statistically significantly enhanced in hybrids as compared to those in both GBM cells and TAMs, while other two genes, Epha2 and St3gal1, remained higher in TAMs with statistical significance than those in both GBM cells and hybrids (Fig. 6B). Invasion assay showed that hybrids exhibited higher invasive ability than GBM cells (Fig. 6D). Importantly, there were more hybrids in the invasive margin of GBM as compared to the GBM core area (Fig. 6E and 6F, Movie S10 and S11), implying that TAM-GBM cell hybrids may participate in GBM invasiveness. Taken together, these data demonstrate that hybrids formed by TAMs and GBM cells may contribute to GBM invasion.

4. Discussion

Previous studies of interaction between TAMs and tumor cells mainly focus on how paracrine signaling pathways promote tumor progression [2,28,29]. However, whether hybrid formation between TAMs and tumor cells, an alternative way to contribute tumor malignancy [7,8], exists in GBM and its biological role in GBM development remain elusive. In the current study, we uncover the existence of TAM-GBM cell hybrids in human specimens, orthotopically transplanted allografts and *in vitro* cell co-culture system. The TAM-GBM cell hybrids undergo nuclear reprogramming and generate unique gene expression profile. Moreover, they are enriched with glioma invasion-associated genes and exhibit enhanced invasive ability.

Hybrids formed by TAMs and tumor cells in human specimens have been reported in multiple tumors. For example, TAM-tumor cell hybrids have been observed in lymph node metastasis [12,13], brain metastasis [10], and the blood of melanoma patients [11]. Donor-patient hybrids have been detected in the patients after bone marrow transplantation bearing with renal carcinoma [16,17], or pancreatic ductal adenocarcinoma [14]. TAM-tumor cell hybrids have also been found in the ascites of ovarian carcinoma patients [18]. In our study, we identified TAM-GBM cell hybrids on a portion of surgical GBM specimens from seven patients based on detection of co-expression of GBM biomarker GFAP and macrophage biomarker CD68. The percentage of hybrids was ranged from 1.35% to 1.96%. Apart from detection of hybrids on GFAP and CD68, we also detected hybrids via other GBM biomarkers (such as $IDH1^{R132H}$ and PDGFRA) and another macrophage biomarker CD14. To further confirm the presence of hybrids between TAMs and GBM cells, we conducted experiments on sex-mismatched GL261-RFP derived allografts and the co-culture system, and found that there were TAM-GBM cell hybrids. Thus, taking evidence from human specimens, animal models and co-culture experiment together, we demonstrate the presence of hybrids between TAMs and GBM cells.

Upon hybrid formation, reprogramming will take place so that novel gene expression profile will emerge [19]. We found that hybrids exhibited unique transcriptome characteristics as

compared to both parental cells, indicating that they underwent reprogramming after hybrid formation. In the process of reprogramming, hybrids may gradually lose extra chromosomes through reductive mitosis [30], and eventually stable hybrids with enhanced malignant behaviors will emerge based on Darwin's theory of evolution: "survival of the fittest" [31]. Because of the chromosomal instability and genomic instability, the survival rate of hybrids is very low [32], which is also the difficulty to do research in hybrids.

TAM-tumor cell hybrids could adopt macrophages' migratory property while still growing in the uncontrolled manner of parental tumor cells [7,8]. In the present study, hybrids were higher invasive than GBM cells as analyzed by invasion assay, and there were more hybrids in the invasive margin of GBM, indicating that formation of hybrids between TAMs and GBM cells confers GBM cells with enhanced invasiveness. Importantly, three glioma invasion-associated genes (Adam8, Mmp12 and Mmp13) and three enriched cell hybridization-associated genes (Adam8, Atp6v0d2 and Ocstamp) were up-regulated in the hybrids as compared to both GBM cells and TAMs, implying that Adam8 may play an important role in the hybrid formation and hybrid-mediated GBM invasion. Adam8, a member of Adam (a disintegrin and metalloprotease domain) family, is implicated in hybrid formation during bone morphogenesis via NF-κB, Erk and Akt pathways [33]. Additionally, it has been reportedly associated with invasiveness and metastasis in pancreatic cancer, gastric cancer, osteosarcoma and GBM [34-37]. Therefore, it is necessary to investigate the role of Adam8 in hybrid-mediated invasion in future studies.

Indeed, there are a number of approaches for cells to express biomarkers of both tumor cells and TAMs. For example, tumor cells could obtain macrophage biomarkers through cell fusion [18,19]. Besides, macrophages could confer tumor cells with macrophage biomarkers through cytoplasmic transfer [15], or exosome transduction [38]. Other ways such as phagocytosis of tumor cells by macrophages [39], tumor reversion [40], tumor plasticity [41], and gene expression deregulation [42], may also result in the cells with co-expression of proteins from different cell types. Quantification of the percentages of sex chromosomes in double-positive cells derived from sex-mismatched allografts (Supplementary Table 2) revealed that 78.75%

of double-positive cells exhibited genotype of X chromosome amplification with one Y chromosome, indicating that they were yielded from fusion. However, there were also some double-positive cells showing “XX” (6.25%) or “XY” (15.00%), which might originate from cytoplasmic transfer, exosome transduction, gene expression deregulation, phagocytosis, *etc*. We demonstrate that TAM-tumor cell hybrids, regardless of their origins, exist in GBM and play an important role in GBM invasiveness.

In summary, our study reveals the existence of the hybrids formed by TAMs and GBM cells with unique gene expression characteristics and enhanced invasiveness phenotype. Further studies are essential to find the targets of TAM-GBM cell hybrids. New therapeutic strategies for GBM may emerge from a better understanding of TAM-GBM cell hybrids.

Conflicts of interest: The authors declare no conflicts of interest.

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Figure legends

Fig. 1. Hybrids formed by TAMs and GBM cells in human GBM specimens. A, Representative presentation of flow cytometry analysis showing the existence of $GFAP^+CD68^+$ hybrids in GBM patients. B, The percentages of $GFAP^+CD68^+$ hybrids in the resected GBM samples from 7 patients by FCM analysis. C, Confocal images of double-staining GFAP and CD68 on FACS-sorted $GFAP^+CD68^+$ hybrids of GBM patient 4. Scale bar, 5 μm. D, Confocal images of double-staining GFAP and CD68 on frozen GBM tissue from patient 6. Area marked with square is magnified on the right. The yellow arrow indicates the single $CD68^+$ cell, while the white arrow indicates the $GFAP^+CD68^+$ hybrid. Scale bar, 20 μm (left), 5 μm (right).

Fig. 2. TAM-GBM cell hybrids in murine orthotopically transplanted GBM. A, Experimental design for FISH analysis of FACS-sorted RFP^+CD11b^- GBM cells, RFP^-CD11b^+ TAMs and RFP^+CD11b^+ hybrids from sex-mismatched GL261-RFP derived allografts (up) and the corresponding results (down). Chromosome X (red) and chromosome Y (green) are showed in the DAPI stained nuclei (blue). ♀, female. ♂, male. Scale bar, 5 μm. B, Confocal images of double-staining of RFP and CD11b on FACS-sorted GBM cells, TAMs and hybrids from GL261-RFP derived tumors. Scale bar, 10 μm. C, Analyses of FACS-sorted GBM cells, TAMs and hybrids from sex-mismatched GL261-RFP derived allografts on confocal microscope by both immunofluorescence and FISH. Scale bar, 5 μm. D, Confocal images of double-staining of RFP and CD11b on frozen tissues from GL261-RFP allografts. Area marked with square is magnified on the right. The yellow arrow indicates the single $CD11b^+$ cell, while the white arrow indicates the RFP^+CD11b^+ hybrid. Scale bar, 20 μm (left), 5 μm (right). Animal experiments are conducted independently at least three times and allografts from five tumor-bearing mice are used each time.

Fig. 3. TAM-GBM cell hybrids in *in vitro* co-culture system. A, Schematic presentation of co-culture experiments of GL261-RFP cells with BMDMs-GFP and the followed micrographs analysis as well as flow cytometry analysis. B, Representative micrographs of live TAM-GBM cell hybrid in the co-culture system of GL261-RFP cells with BMDMs-GFP

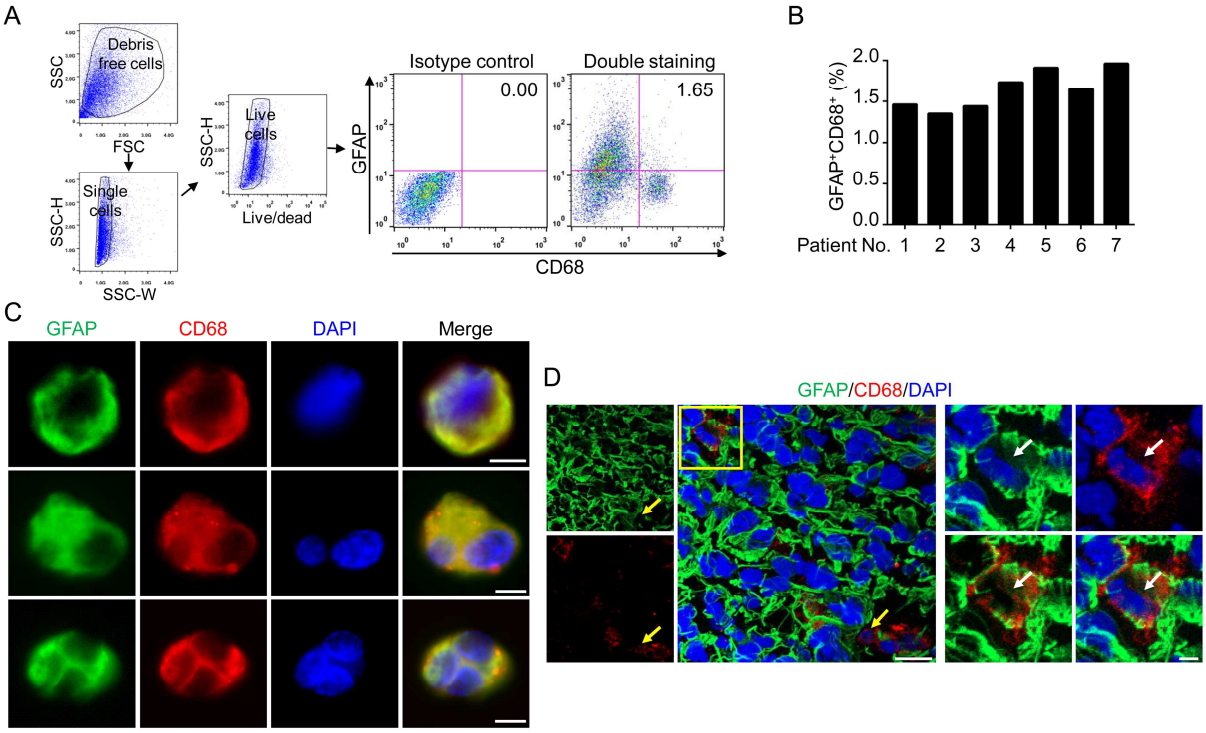
detected by Cell Observer microscope. The arrow indicates the hybrid. Scale bar, 20 μm. See also Fig. S8. C, Time-lapse images detected by Cell Observer microscope revealing the process of hybrid formation between GL261-RFP and BMDM-GFP in the co-culture system. See also Movie S9. D, Flow cytometry analyses of the percentage of hybrids in the co-culture system of various mixed ratios of GL261-RFP cells to BMDMs-GFP. Data are shown as mean ± SEM from three independent experiments. E, Schematic presentation of co-culture experiments (up) and the followed confocal microscopy analysis of expression of RFP, GFP and EdU for FACS-sorted cells from the co-culture system (down). Non-EdU-labeled GL261-RFP cells and EdU-labeled BMDMs-GFP were co-cultured for 48 h, and then RFP^+GFP^+ hybrids, RFP^+GFP^- GL261 cells and RFP^-GFP^+ BMDMs were sorted from the co-culture system for analysis of the expression of RFP, GFP and EdU. Scale bar, 5 μm. All experiments are performed independently for three times.

Fig. 4. Unique gene expression characteristics of TAM-GBM cell hybrids. A, the volcano plots showing the differently expressed genes in RFP^+CD11b^+ hybrids *versus* RFP^+CD11b^- GBM cells and in RFP^+CD11b^+ hybrids *versus* RFP^-CD11b^+ TAMs sorted from GL261-RFP derived allografts. B, The Venn diagrams showing the gene numbers of the up-regulated and down-regulated genes of hybrids as compared with GBM cells and TAMs. C, Heatmaps of the up-regulated and down-regulated genes of hybrids *versus* parental cells. No.1, 2, 3 indicate samples from three independent experiments with allografts from five tumor-bearing mice used in each independent experiment. D, qRT-PCR analyses of the top 10 up-regulated (left) and down-regulated (right) genes of hybrids. Data are shown as mean ± SEM from three independent experiments with allografts from five tumor-bearing mice used in each independent experiment. *p<0.05, **p<0.01, ***p<0.001, ns, not significant. E, GO and KEGG pathways analyses of TAM-GBM cell hybrids conducted through the Cytoscape software with ClueGO plug-in. The same node color indicates the grouped GO and KEGG terms and the node size represents the statistical significance.

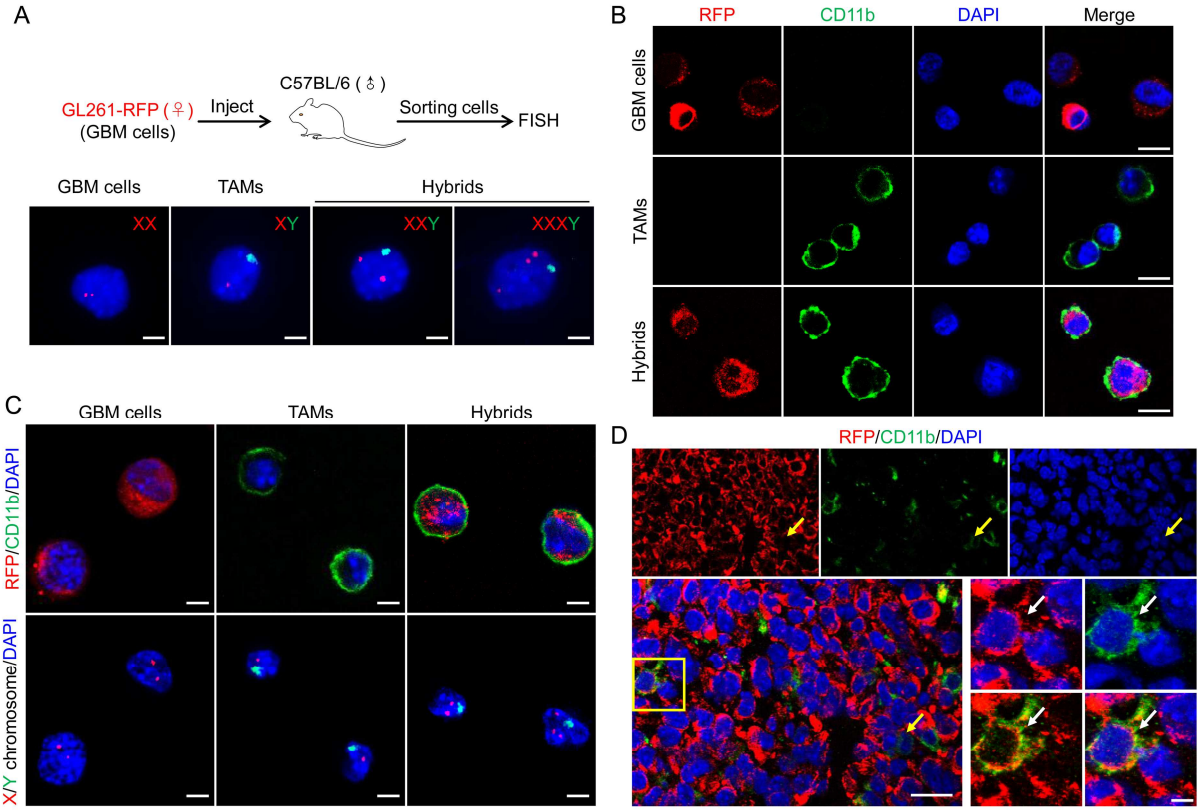
Fig. 5. TAM-GBM cell hybrids are enriched with cell hybridization-associated genes. A, GSEA analysis of cell hybridization-associated genes in TAM-GBM cell hybrids and GBM

cells sorted from GL261-RFP derived allografts. NES, normalized enrichment score. B, qRT-PCR analyses of the top 10 cell hybridization-associated genes of TAM-GBM cell hybrids. Data are shown as mean ± SEM from three independent experiments with allografts from five tumor-bearing mice used in each independent experiment, $**p<0.01$, $***p<0.001$, ns, not significant.

Fig. 6. TAM-GBM cell hybrids contribute to GBM invasion. A, GSEA analysis of glioma invasion-associated genes in TAM-GBM cell hybrids and GBM cells sorted from GL261-RFP derived allografts. NES, normalized enrichment score. B, qRT-PCR analyses of the top 10 glioma invasion-associated genes of TAM-GBM cell hybrids. $*p<0.05$, $**p<0.01$, $***p<0.001$, ns, not significant. C, flow cytometry analysis of Cxcr4 expression in the isotype control, GBM cells, TAMs and hybrids from GL261-RFP derived allografts. D, Representative images of invasive GBM cells (left and up) and invasive hybrids (left and down) from invasion assay, and quantification of invasive cells shown in the right. Cells used for invasion assay were sorted from GL261-RFP derived allografts. Scale bar, 50 μm. $***p<0.001$. E, Confocal images showing the existence of RFP^+CD11b^+ hybrid in the invasive margin of GBM allografts. The irregular dashed line indicates the border between GBM core area and normal brain tissue. Area within a distance of 200 μm from the border is referred as invasive margin, otherwise it is demarcated as tumor core area. Region indicated with square is magnified on the right. The white arrow indicates the RFP^+CD11b^+ hybrid. Scale bar, 20 μm (left), 5 μm (right). F, Quantification of RFP^+CD11b^+ hybrids in the invasive margin and in the tumor core area of GBM allografts. $*p<0.05$. Data are shown as mean ± SEM from three independent experiments with allografts from five tumor-bearing mice used in each independent experiment (B, D and F).



Cao M.-F., et al. Figure 1



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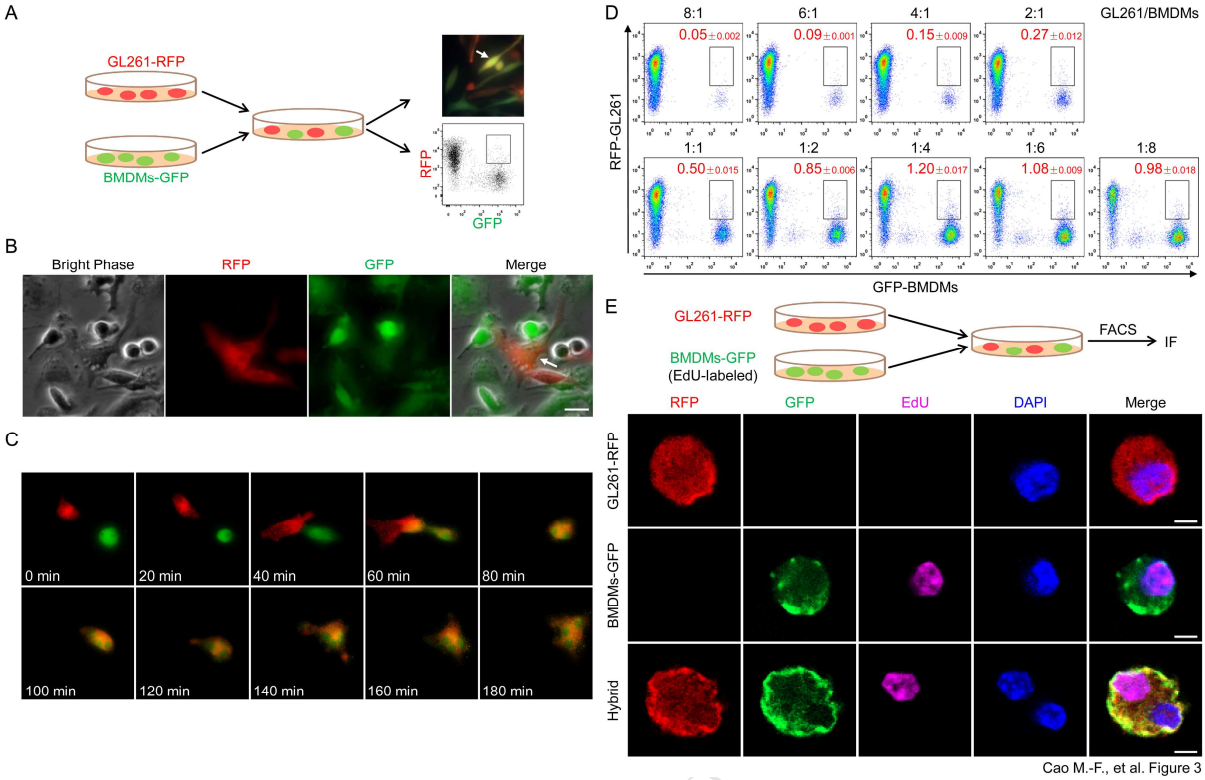
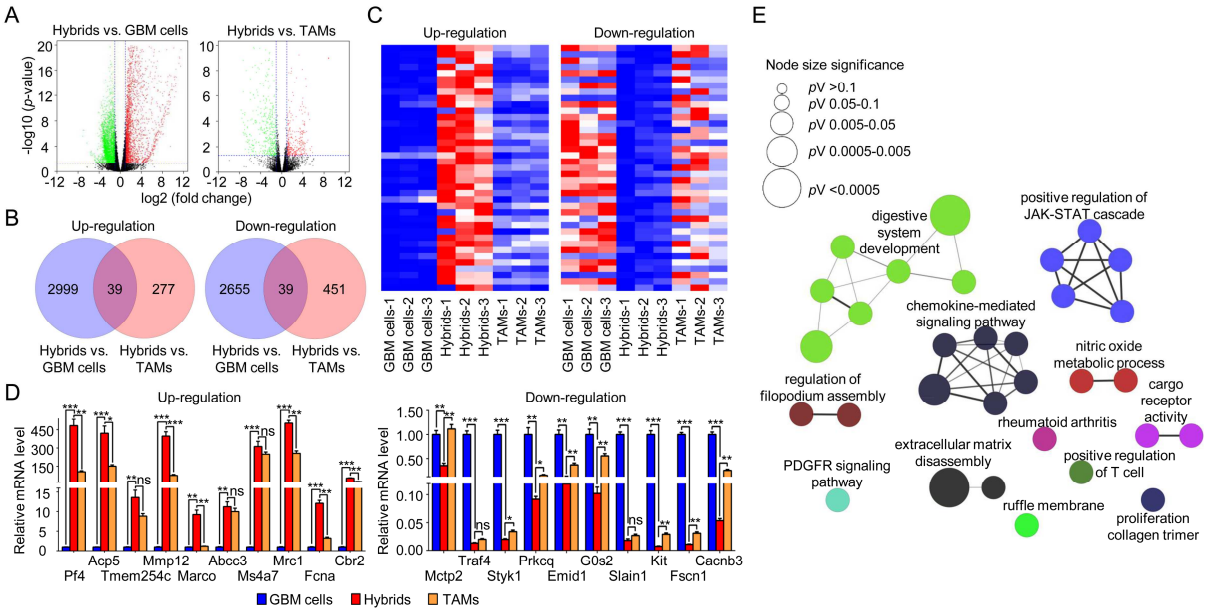


Cao M.-F., et al. Figure 3



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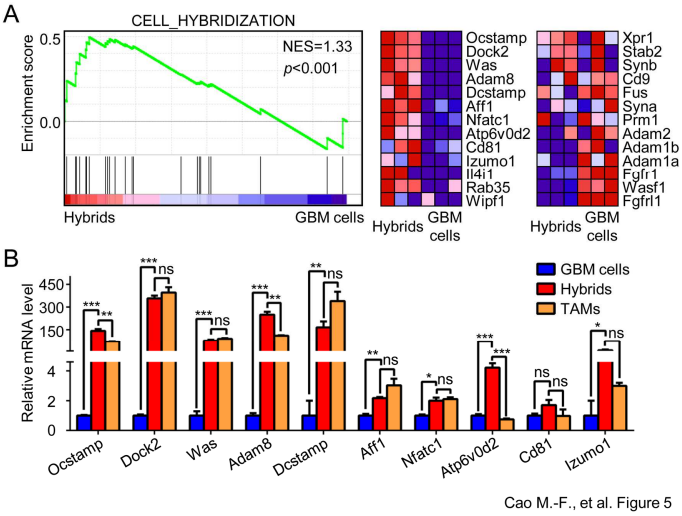
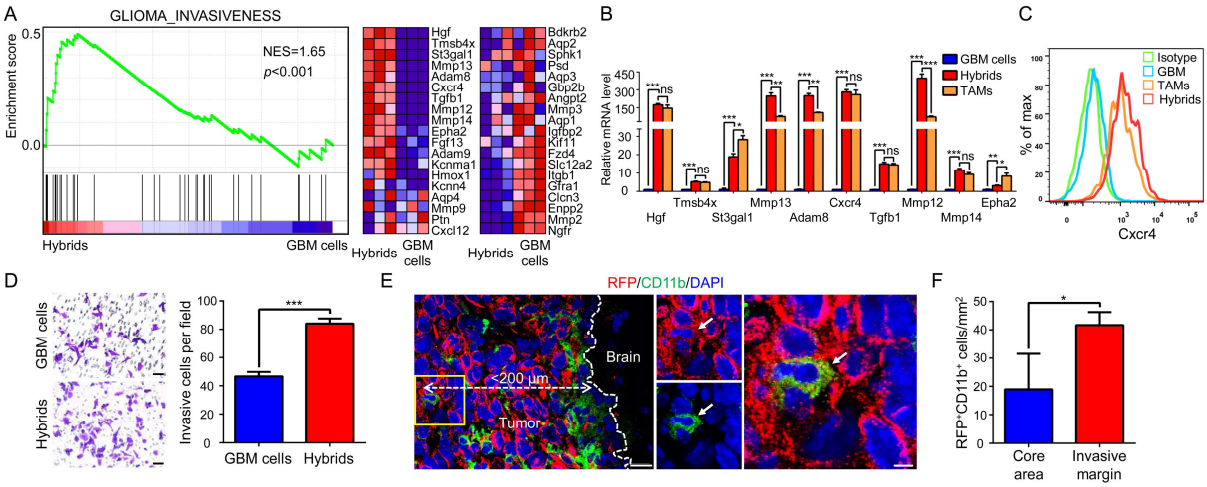


Cao M.-F., et al. Figure 5



Cao M.-F., et al. Figure 6

Highlights

- Hybrids formed by TAMs and tumor cells exist in GBM
- TAM-GBM cell hybrids bear nuclear reprogramming with unique gene expression profile
- TAM-GBM cell hybrids contribute to GBM invasion