Frizzled 4 Regulates Stemness and Invasiveness of Migrating Glioma Cells Established by Serial Intracranial Transplantation

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Tumor and Stem Cell Biology

Abstract

One of the most detrimental hallmarks of glioblastoma multiforme (GBM) is cellular invasiveness, which is considered a potential cause of tumor recurrence. Infiltrated GBM cells are difficult to completely eradicate surgically and with local therapeutic modalities. Although much effort has focused on understanding the various mechanisms controlling GBM invasiveness, its nature remains poorly understood. In this study, we established highly serial intracranial transplantation. U87R4 cells were highly invasive and displayed stem cell-like properties, as compared to noninvasive but proliferative U87L4 cells. Microarray analysis during serial transplantation revealed that apoptosis-inducing genes (caspase3 and PDCD4) were downregulated whereas several cancer stem cell–relevant genes [Frizzled 4 (FZD4) and CD44] were upregulated in more invasive cells. U87R4 cells were resistant to anticancer drug–induced cell death, partly due to downregulation of caspase3 and PDCD4, and they retained activated Wnt/β-catenin signaling due to upregulation of Frizzled 4, which was sufficient to control neurosphere formation. We also found that FZD4 promoted expression of the epithelial to mesenchymal transition regulator SNAIL, along with acquisition of a mesenchymal phenotype. Taken together, our results argue that Frizzled 4 is a member of the Wnt signaling family that governs both stemness and invasiveness of glioma stem cells, and that it may be a major cause of GBM recurrence and poor prognosis. 

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Introduction

Glioblastoma multiforme (GBM; WHO grade IV) is one of the most common and malignant central nervous system tumors in humans, with an estimated median survival time of less than 1 year (1). Although much information about the clinicopathological roles of various causal factors for GBM genesis continues to accumulate, human GBM still remains one of the most incurable human malignancies.

The infiltrative property of GBM cells is one of the major causes of tumor recurrence and patient lethality, because GBM cells that infiltrate toward normal parenchymal tissues are hard to eradicate with local therapeutic modalities and are extremely resistant to various anticancer therapies (2). Recently, several histopathological features of GBM suggested that this tumor arises from cancer stem cells (or cancer-initiating cells) that retain many properties similar to normal neural stem cells, such as self-renewal and the potential for multidifferentiation, giving rise to tumor heterogeneity (3). A growing body of evidence has demonstrated that multiple stemness factors, such as epidermal growth factor (EGF; 4), hedgehog (5), notch (6), Wnt (7), and Id4 (8), which play pivotal roles in genesis and maintenance of embryonic and adult stem cells, are activated in many types of malignancies and are sufficient to generate cancer stem cells in cell culture and mouse model systems.

In particular, Wnt signaling is frequently activated in colorectal cancers, melanomas, breast cancers, and gliomas due to loss of adenomatous polyposis coli (APC) function and/or gain of β-catenin activity. The Wnt/β-catenin pathway also induces cell migration, which is necessary for pattern formation and differentiation during embryonic development (9) and tumor progression (10). Wnt signaling is also considered a stemness
regulator that promotes cellular invasiveness through regulation of epithelial to mesenchymal transition (EMT) in many types of malignancies (7, 11–13). Such evidence suggests at least 2 possibilities: (i) invasive glioma stem cells (GSC) may be a major cause of tumor recurrence, and (ii) Wnt signaling may be a potential driver that leads to acquisition of both stemness and invasiveness in GBM cells. However, these possibilities are not well understood in relation to GBM recurrence.

In this study, to understand the potential invasiveness of GSCs and tumor recurrence, we established 2 different cell lines: a highly invasive glioma cell line (U87R4 cells) and a noninvasive cell line (U87L4 cells) from U87MG glioma cells following 4 rounds of serial in vivo orthotopic transplantation. We found that U87R4 cells retained several GSC features, such as aggressive tumorigenicity, robust invasiveness, neurosphere formation, neural stem cell marker expression, and resistance to anticancer drug-induced cell death. Furthermore, we also demonstrated that increased expression of the signaling regulator Frizzled 4 (FZD4), upregulated Wnt signaling, and decreased caspase3 and PDCD4 expression in U87R4 cells were responsible for acquisition of GSC-like properties and resistance to apoptosis.

Materials and Methods

Cell culture and neurosphere formation

U87MG cells [which are purchased from American Type Culture Collection (ATCC)] were maintained in Dulbecco’s modified Eagle’s medium (DMEM) enriched with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin (GIBCO-BRL), and 2 mmol/L l-glutamine (GIBCO-BRL) for fewer than 6 months after receipt. This cell line was authenticated by ATCC with the use of various tests, including DNA profiling, and cytogenetic analyses. For the neurosphere formation assay, cells were plated into 96-well plates at a density of 1 cell per well and maintained in neural stem cell culture conditions (Neurobasal, a medium enriched with 20 ng/mL EGF, 20 ng/mL basic fibroblast growth factor, 1 × 10^7/mL N2, and 1% penicillin/streptomycin) for 10 to 14 days.

Intracranial transplantation and establishment of invasive cell lines

Cultured cells were harvested with 0.25% trypsin/0.02% EDTA for 2 minutes, washed twice with Hank’s balanced salt solution (HBSS), and resuspended in Ca^2+/Mg^2+-free HBSS. Cell viability was determined by trypan blue exclusion. Only single-cell suspensions with more than 90% viability were used for in vivo studies. Cells (2 × 10^5) in 5 μL HBSS were stereotactically injected into the left striata of nude mice (6-week-old BALB/c nu/nu; coordinates: anterior–posterior, +2.2; medial–lateral, +2.5; dorsal–ventral, −2.2 mm from Bregma). When body weight had decreased more than 25%, mice were considered to have GBM. Brains with tumors were mechanically and enzymatically dissociated into single cells, red blood cells were removed by differential centrifugation. The remaining cells (10^6 cells) were labeled with 1 μL fluorescence isothiocyanate (FITC)-conjugated anti-H-2Kb antibody (BD Pharmingen). H-2Kb-populations were sorted using a FACSAria machine (BD Biosciences), and tumor cells were cultured in the conditions described above. This procedure was repeated 4 times to enrich for nonmigratory and migratory glioma cells. In addition, RT-PCR (reverse transcription-polymerase chain reaction) was performed to determine the purity of human cell population by amplifying a human b-actin cDNA fragment with human-specific b-actin primer set. R1 and R3 cells were cultured for 3 consecutive passages (~3 weeks) to attain...
sufficient numbers of cells (2 × 10^6 cells) for intracranial injections, while R2 and R4 cells were cultured for 5 to 6 passages to perform intracranial injections and in vitro studies. All mouse experiments were performed in accordance with government and institutional guidelines and regulations.

Results

In vivo selection of highly invasive GBM cells

To establish highly invasive subclones from human GBM cell lines, U87MG glioma cells were stereotactically injected into the left hemisphere of brains of immunocompromised mice (BALB/c nude). When GBMs formed, the brains were harvested and divided in half coronally. GBM subclones derived from primary culture of the left and right hemispheres after intracerebral implantation were denoted L1 (noninvasive cells derived from left brain hemispheres) and R1 (highly invasive cells established from right brain hemispheres), respectively. To further enrich cells with an invasive potential, we repeated this procedure 3 more times, establishing L4 from L1 and R4 from R1 (Fig. 1A). At each step, H-2Kb^+ mouse cells were excluded from the recovered cultures (<2%) by fluorescence-activated cell sorting to ensure that the L4 and R4 subclones were of human origin (data not shown).

U87L4 cells were mainly comprised of cells showing epithelial-like rounding morphology (Fig. 1B; left photograph), whereas most U87R4 cells showed mesenchymal-like cell features, such as a long spindle shape (Fig. 1B; right photograph). Furthermore, histological examination demonstrated that U87R4 cell-derived tumors displayed peripheral invasion of the surrounding brain as single cells and cell clusters, compared to U87L4-driven tumors (Fig. 1C). Of note, when cultured continuously for more than 15 passages under conventional culture conditions (DMEM + 10% FBS), the majority of U87L and U87R cell populations retained mixed epithelial- and mesenchymal-like cell types, similar to U87MG parental cells. Therefore, we used relatively early passage U87L and U87R cells (less than 5 passages) to compare their cellular and genetic characteristics. When determining tumorigenic capability in vivo, we observed that the survival periods of tumor-bearing mice that were intracranially implanted with U87L2 and U87R2 cells were significantly shorter than those of mice bearing tumors derived from U87MG parental cells (Fig. 1D). This result may be due to enrichment of tumor cells that retained a highly tumor-initiating potential during serial in vivo transplantation. Although the median survival of U87R2-injected mice was relatively shorter than that of U87L2-injected mice, there was no significant difference in the
markers, such as CD133 and nestin (15) in neurospheres. Although both U87L4 and U87R4 cells retained a population of cancer stem cells, the number of glioma stem-like cells (14) that although both U87L4 and U87R4 cells retained a population of cancer stem cells, the number of glioma stem-like cells (14) was higher in U87R4 cells than U87L4 cells. We examined GSC characteristics by investigating osteogenesis (17), but its role in cancer stem cells has not been studied. Because U87R4 cells showed phenotypes resembling glioma stem-like cells, we focused on CD44 and FZD4 among the upregulated genes, and found that expression levels of all these genes were significantly upregulated in U87R4 cells compared to U87L4 cells. Among the downregulated genes in U87R4 cells, we found that survival periods of U87R4-injected mice were longer than those of U87L4-injected mice and U87L2- and U87R2-injected mice (Fig. 1D). Although we did not determine a precise reason for this observation, the longer survival of U87R4-injected mice might be attributed to a lesser proliferative property of U87R4 cells compared to U87L4. In addition, it is likely that U87L4 cells displayed rapid tumor growth compared to U87R4, and increased U87L4-driven tumor burden might lead to earlier death by impairing normal brain function (data not shown). Conversely, the invasive U87R4 cells generated relatively dispersed tumors with smaller tumor mass (Fig. 1C) that might exert a lesser damage to normal brain function than U87L4-injected mice.

**Cellular characteristics of U87L4 and U87R4 cells**

To validate the in vivo invasive properties of U87R4 cells, we conducted an in vitro invasion assay using a transwell culture system. U87R4 cells were more invasive than U87L4 cells in Matrigel-coated transwell cultures (Fig. 2A). U87R4 cells showed a significant reduction in cell proliferation compared to U87L4 cells (Fig. 2B). Because a recent study reported that human U87MG and T98G glioma cells contain a subpopulation of glioma stem-like cells (14), we compared glioma stem-like cell properties of U87L4 and U87R4 cells by investigating their serial neurosphere forming ability and GSC marker expression. U87R4 cells formed more neurospheres that were derived from U87L4 and U87R4 cells using immunofluorescence and found that U87R4-derived neurospheres showed increased nestin and decreased CD133 expression compared to U87L4-derived neurospheres (Fig. 2D), suggesting that neurospheres derived from U87R4 and U87L4 cells may contain different types of glioma stem-like cells.

**Gene expression signature in highly invasive U87R4 cells**

To examine the signaling pathways promoting the invasiveness of GBM cells, gene expressions in invasive subclones (U87R2 and U87R3 cells) and noninvasive, proliferative subclones (U87L2 and U87L3 cells) were compared using Affymetrix Human Genome U133 Plus 2.0 arrays containing more than 47,000 human transcripts. We found that 4 genes (RANKL, DISC1, CD44, and FZD4) were significantly upregulated, and 4 genes (caspase3, SMAD6, MAML3, and PDCD4) were downregulated in U87R4 cells compared to U87L4 cells ($P < 0.05$; Table 1). Next, we validated the expression levels of differentially expressed genes in U87R4 and U87L4 cells using real-time RT-PCR. FZD4 are involved in Wnt/β-catenin signaling, which plays crucial roles in cancer stem cell genesis and cell migration by inducing EMT in many types of malignancies, such as colorectal and breast cancers (7). CD44 is a cancer stem cell marker expressed in various cancer types, including breast cancers (16). RANKL is associated with osteogenesis (17), but its role in cancer stem cells has not been studied. Because U87R4 cells showed phenotypes resembling glioma stem-like cells, we focused on FZD4, CD44, and RANKL among the upregulated genes, and found that expression levels of all these genes were significantly upregulated in U87R4 cells compared to U87L4 cells (Fig. 3A). Among the downregulated genes in U87R3 cells, SMAD6 is an inhibitor of TGF-β signaling that is associated with cell cycle arrest.

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**Table 1.** Partial list of differentially expressed genes between the U87R cell line vs. the U87L cell line.

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Gene name</th>
<th>Probe</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increased in U87R2/R3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANKL</td>
<td>Tumor necrosis factor (ligand) superfamily, member 11</td>
<td>211153_s_at</td>
<td>0.023</td>
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<tr>
<td>DISC1</td>
<td>Disrupted in schizophrenia 1</td>
<td>206090_s_at</td>
<td>0.003</td>
</tr>
<tr>
<td>CD44</td>
<td>CD44 antigen (homing function and Indian blood group system)</td>
<td>217330_at</td>
<td>0.004</td>
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<td>FZD4</td>
<td>Frizzled homolog 4 (Drosophila)</td>
<td>217523_at</td>
<td>0.032</td>
</tr>
<tr>
<td><strong>Decreased in U87R2/R3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASP3</td>
<td>Caspase 3, apoptosis related cysteine protease</td>
<td>202763_at</td>
<td>0.045</td>
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<tr>
<td>SMAD6</td>
<td>MAD, mothers against decapentaplegic homolog 6 (Drosophila)</td>
<td>213565_s_at</td>
<td>0.005</td>
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<td>MAML3</td>
<td>Mastermind-like 3 (Drosophila)</td>
<td>228508_at</td>
<td>6E-05</td>
</tr>
<tr>
<td>PDCD4</td>
<td>Programmed cell death 4 (neoplastic transformation inhibitor)</td>
<td>212593_s_at</td>
<td>3E-07</td>
</tr>
</tbody>
</table>

*NOTE: Genes that were significantly (*, $P < 0.05$) increased and decreased in highly invasive U87R2/R3 compared to noninvasive, proliferative U87L2/L3 cells.*

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Caspase3 and PDCD4 are well-known factors driving cell death in a variety of normal and cancer cells (20, 21). We found that expression levels of caspase3 and PDCD4, but not SMAD6, were significantly reduced in U87R4 cells compared to U87L4 cells (Fig. 3B). To evaluate the gene expression patterns in the U87R cells derived from serial in vivo intracranial transplantation assays, we compared expression of FZD4, RANKL, CD44, SMAD6, caspase3, and PDCD4 in U87R2 and U87R4 cells, and found that FZD4 and PDCD4 levels in U87R4 cells were higher than those in U87R2 cells, whereas caspase3 levels in U87R4 cells were lower than U87R2 cells (Fig. 3C), implying that FZD4-high and caspase3-low expression pattern might be an advance signature of U87R4 cells, which is achieved during serial in vivo intracranial transplantation. Taken together, these results suggest that U87R4 cells display cellular features resembling glioma stem-like cells, such as a stemness and resistance to apoptosis, via activation of Wnt signaling and inactivation of cell death regulators.

**Decreased caspase3 and PDCD4 expression and resistance to an anticancer drug in U87R4 cells**

Because highly invasive cancer cells often retain resistance to cell death from various physiological and pharmacological insults (22), and our mRNA expression data showed decreased expression of caspase3 and PDCD4 in U87R4 cells, we examined whether U87R4 cells were resistant to anticancer drug-induced cell death compared to U87L4 cells. U87R4 cells were significantly more resistant to staurosporine than U87L4 cells (Fig. 4A). To examine whether this resistance was associated with downregulation of caspase3 and PDCD4, we stably depleted expression of caspase3 or PDCD4 in U87L4 cells using short hairpin RNA interference (shRNAi). As shown in Figure 4B, over 50% of caspase3 and PDCD4 expression was successfully reduced in U87L4 cells transduced with caspase3- or PDCD4-shRNAi, as compared to U87L4 cells transduced with nonspecific scrambled shRNAi. Individual depletion of caspase3 or PDCD4 in U87L4 cells transduced with nonspecific scrambled shRNAi, compared to U87L4 cells transduced with nonspecific scrambled shRNAi, led to marked increases in resistance to staurosporine- or temozolomide-induced cell death (Fig. 4C and Supplementary Fig. S1), whereas the transient overexpression of caspase3 in U87R4 cells led to relative decrease in resistance to staurosporine (STS)- or temozolomide-driven apoptotic stimulus (Fig. 4D and Supplementary Fig. S1). However, STS, but not temozolomide, significantly increased sensitized cell death in U87R4 cells that are transiently overexpressing PDCD4 (Supplementary Fig. S1). Together, our data suggest that resistance to anticancer drugs may result from decreased expression of caspase3 and PDCD4 in U87R4 cells.
FZD4 leads to acquisition of glioma stem cell properties through activation of canonical Wnt/β-catenin signaling

Several previous studies have demonstrated that FZD4 activates the canonical Wnt pathway, which plays a pivotal role in controlling stemness in normal and malignant tissues (23–25). To evaluate whether the canonical Wnt pathway was activated and FZD4 was involved in activation of Wnt signaling in U87R4 cells, we examined activation of β-catenin, a crucial regulator in the canonical Wnt pathway, in U87R4 cells and in U87L4 and U87R4 cells subjected to gain- and loss-of-functions of FZD4, respectively. In the canonical Wnt pathway, β-catenin is released from destruction complex (Axin APC and GSK3) via phosphorylation by CKIα and GSK3β and becomes stabilized and accumulates in the nucleus (26). Nuclear β-catenin then forms a nuclear complex with T-cell factor (TCF)/lymphoid enhancer factor to activate transcription of target genes (27). We found that the expression level and transcriptional activity of β-catenin were markedly increased in U87R4 cells compared to U87L4 cells, as determined by Western blotting and TOP/FOP luciferase reporter assays (28; Fig. 5A). Furthermore, overexpression of FZD4 in U87L4 cells resulted in a significant increase in neurosphere formation compared to U87L4-control cells, whereas depletion of FZD4 in U87R4 cells resulted in a marked decrease in neurosphere formation compared to control U87R4-shScramble cells (Fig. 5C). Thus, overexpression of FZD4 in U87R4 cells is sufficient to promote β-catenin expression and neurosphere formation.

Next, we examined expression of FZD4, nestin, CD133, and β-catenin in the margin and core regions of tumors derived from U87R4 and U87L4 cells, as well as 2 human primary GBM specimens. Expression of FZD4 and nuclear β-catenin was detected in tumor cells restricted to the margin of U87R4-derived tumors and human primary GBM specimens, whereas CD133 expression was mainly dramatically decreased nuclear accumulation of β-catenin (Fig. 5B). When grown in neural stem cell culture conditions that promote GSCs to form neurospheres (30), neurosphere numbers from U87R4 cells were markedly increased when compared to neurospheres from U87L4 cells. Furthermore, overexpression of FZD4 in U87L4 cells resulted in a significant increase in neurosphere formation compared to U87L4-control cells, whereas depletion of FZD4 in U87R4 cells resulted in a marked decrease in neurosphere formation compared to control U87R4-shScramble cells (Fig. 5C). Thus, overexpression of FZD4 in U87R4 cells is sufficient to promote β-catenin expression and neurosphere formation.

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detected in tumor cells localized in the core region of U87L4-derived tumors. Therefore, FZD4 high/Nestin high/CD133 low cells may represent a ‘mesenchymal’ subtype of GSCs (see the Discussion section).

**FZD4 regulates a mesenchymal-like cell phenotype via SNAI1 induction**

Because Wnt/β-catenin signaling promotes EMT in many types of malignancies (32, 33), we assessed whether FZD4 was associated with glioma cell invasiveness. U87L4-FZD4 cells showed a significant increase, whereas U87R4-shFZD4 cells displayed a marked decrease, in cell invasion in Matrigel-coated transwell cultures, compared to their corresponding control-transfected cells (Fig. 6A). EMT is regulated by several transcriptional factors such as twist, SNAIL, and slug during normal development and in malignant states (34). Thus, we investigated whether FZD4 regulates expression of EMT-regulating transcriptional factors. We found that U87R4 cells expressed higher levels of SNAIL protein (Fig. 6B), but not mRNA (data not shown), than U87L4 cells, and furthermore, SNAIL protein expression was increased in U87L4-FZD4 cells and decreased in U87R4-shFZD4 cells (Fig. 6B). However, twist expression was not altered in U87L4 and U87R4 cells by gain or loss of FZD4 expression. Slug was not expressed at detectable levels by Western blotting in either U87L4 or U87R4 cells (data not shown).

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**Figure 5.** FZD4 regulates stemness properties through canonical Wnt signaling. A, Wnt signaling activity was detected by Western blotting (top) and a luciferase reporter gene assay (bottom graph) in U87L4 and U87R4 cells following treatment with or without a GSK3 inhibitor (30 mmol/L LiCl, 10 hours). Western blotting was performed to detect P-GSK3 and β-catenin. α-Tubulin was used as a loading control. The luciferase reporter gene assay was performed by calculating the relative luciferase activity of pTOP-FLASH vs. pFOP-FLASH. ***, P < 0.01 (n = 3). B, expression levels of FZD4 mRNA were detected with real-time RT-PCR in U87L4-Hygro, U87L4-FZD4, U87R4-shScramble, and U87R4-shFZD4 cells. Data represent the relative luciferase activity of pTOP-FLASH vs. pFOP-FLASH in 293T cells transduced with or without the FZD4 expression vector. *, P < 0.05 (n = 3); **, P < 0.01 (n = 3). Immunofluorescence images show expression and localization of β-catenin (red). C, single-cell neurosphere formation assay. **, P < 0.01 (n = 3). D, representative immunohistochemical images show expression of FZD4, β-catenin, nestin, and CD133 in the margin and core regions of tumors derived from U87R4 and U87L4 cells, as well as 2 human GBM specimens. Immunofluorescence images showing localization of CD31 (red) and nestin (red) in perivascular region derived from human GBM specimen. DAPI (blue) was used to stain nuclei.
Consistent with SNAI1 expression, expression of the mesenchymal marker vimentin was increased in FZD4-over-expressing cells (U87L4 and U87L4-FZD4 cells), whereas vimentin was decreased in cells with reduced FZD4 expression (U87L4 and U87R4-shFZD4 cells; Fig. 6B). Furthermore, vimentin was abundantly expressed in tumor cells restricted to the margin of U87R4-derived tumors and human primary GBM specimens compared to the core of the same tumor, and both the margin and core regions of U87L4-derived tumors (Fig. 6C). To determine whether FZD4-mediated acquisition of mesenchymal characteristics in U87R4 cells was due to SNAI1 induction, we depleted SNAI1 expression in these cells using SNAI1-specific siRNA. Decreased SNAI1 expression in U87R4 cells was associated with the loss of expression of various mesenchymal marker mRNAs: vimentin, fibroblast-specific protein 1 (FSP1), smooth muscle alpha actin (SMA), and vitronectin (Fig. 6D). Furthermore, we compared expression levels of EMT-relevant genes from microarray, and found that expression levels of several EMT-relevant genes, including vimentin, YKL40, MMP3, microRNA-21, fibroblast activating protein (FAP), Twist1, and fibronectin (FN1), increased more than 2-fold in U87R3 cells compared with U87L3 cells (data not shown). Therefore, U87R4 cells appear to display a mesenchymal-like cell phenotype and highly invasive features that may be regulated by FZD4-SNAI1 expression.

Finally, we examined whether FZD4 is also implicated in invasive features of GSCs derived from 3 GBM patients (35). The FACS and transwell culture assays revealed that FZD4-positivity in GSCs correlated with their invasiveness (Supplementary Fig. S2A). Furthermore, FZD4\textsuperscript{high} GSC4 showed a significantly increased invasion compared to FZD4\textsuperscript{low} GSC4 (Supplementary Fig. S2B). We also examined expression levels of GSC (nestin and CD133) and EMT (vimentin and E-cadherin) markers in 3 FZD4\textsuperscript{high} GSCs using FACS analysis. All 3 FZD4\textsuperscript{high} GSCs displayed high levels of nestin and vimentin, while low levels of CD133 and E-cadherin (Supplementary Fig. S2C). Taken together, our findings that FZD4 regulates cancer stemness and invasiveness have implication for the cellular origin of mesenchymal subtype of GSCs in experimental and clinical trials setting.

Discussion

Despite advances in multimodal treatments such as surgery, radiation, and chemotherapy, the poor prognosis of patients with GBM is largely attributed to the highly invasive feature of GBM cells. Invasive GBM cells infiltrate into the surrounding brain parenchyma and escape surgical resection and local therapeutic modalities, and are considered a principle reason for tumor recurrence (36). Because identifying mechanisms and/or driving factors governing the invasiveness of GBM cells
is mandatory for development of therapeutic strategies that inhibit tumor recurrence, establishment of a highly invasive glioma cell model by serial in vivo orthotopic transplantation provides more insight into glioma cell invasiveness.

Our results indicate that the GSC-like feature of U87R4 cells was regulated by increased FZD4 expression, which activated canonical Wnt/β-catenin signaling. Although Wnt signaling plays a pivotal role in the genesis and maintenance of cancer stem cells in many types of malignancies including colorectal cancers (37), its function is less characterized in human GBM. Although we were not able to directly validate FZD4 expression in patients with GBM due to a lack of GBM specimens containing the marginal region of the tumor and normal tissues compared to GBM specimens containing the tumor core region, our results indicate that FZD4 is a critical Wnt signaling component that is activated in highly invasive GBM cells.

In this study, we demonstrated that the highly invasive U87R4 cell line retained several GSC properties, such as neurosphere formation, nestin expression, and resistance to proapoptotic drugs. Interestingly, U87R4 cells showed decreased CD133 and increased CD44 expression, which is consistent with our previous study showing that primary CD133-negative GBM cells give rise to neurospheres and heterogeneous tumors and show more invasive growth and a more ‘mesenchymal‘ subtype genetic signature compared to CD133-positive GBM cells (35). Moreover, other groups have also suggested that primary CD133<sup>low</sup> and CD44<sup>high</sup> GSCs possess a ‘mesenchymal‘ gene signature and cellular phenotypes similar to adult neural stem cells compared to CD133<sup>high</sup> and CD44<sup>low</sup> GSCs that show ‘pro-neural‘ transcriptional profiles and less invasiveness in vitro culture conditions, similar to fetal neural stem cells (38–40). Furthermore, invasive mesenchymal GSCs (CD133<sup>low</sup> and CD44<sup>high</sup>) express a higher level of FZD6 and possess activated Wnt signaling compared to noninvasive proneural GSCs (CD133<sup>high</sup> and CD44<sup>low</sup>; 38).

Although we did not address how FZD4 regulates post-transcriptional expression of SNAI1, Wnt signaling may increase EMT by increasing SNAI1 protein stability by not only inhibiting GSK3β-dependent phosphorylation and subsequent polyubiquitination-mediated proteosomal degradation, but also nucleocytoplasmic trafficking of GSK3β by Axin2, a target gene of the β-catenin/TCF complex (11, 13). Therefore, our findings that ectopic expression of FZD4 in U87L4 cells led to a mesenchymal-like morphological change along with increased vimentin and SNAI1 expression, and depletion of SNAI1 in U87R4 cells resulted in decreases in vimentin and other mesenchymal marker expression indicate that an EMT-like event in U87R4 cells likely occurs via FZD4-dependent induction of SNAI1.

Taken together, our results suggest that FZD4 promotes induction of glioma stem cell-like cells with a highly invasive potential by activating canonical Wnt signaling, and may be a novel therapeutic target for suppressing tumor invasion and recurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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