Ceacam1L Modulates STAT3 Signaling to Control the Proliferation of Glioblastoma-Initiating Cells

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Abstract

Glioblastoma-initiating cells (GIC) are a tumorigenic cell subpopulation resistant to radiotherapy and chemotherapy, and are a likely source of recurrence. However, the basis through which GICs are maintained has yet to be elucidated in detail. We herein demonstrated that the carcinoembryonic antigen–related cell adhesion molecule Ceacam1L acts as a crucial factor in GIC maintenance and tumorigenesis by activating c-Src/STAT3 signaling. Furthermore, we showed that monomers of the cytoplasmic domain of Ceacam1L bound to c-Src and STAT3 and induced their phosphorylation, whereas oligomerization of this domain ablated this function. Our results suggest that Ceacam1L-dependent adhesion between GIC and surrounding cells play an essential role in GIC maintenance and proliferation, as mediated by signals transmitted by monomeric forms of the Ceacam1L cytoplasmic domain.

Introduction

Gliomas are the most common brain tumor, possess the characteristics of gliomas, astrocytes, and oligodendrocytes, and have been classified into four grades (WHO grade 1–4) based on their pathologic features. Glioblastoma multiforme (GBM) is the most malignant glioma (WHO grade 4), and patients with GBM have a median survival of approximately 1 year. In spite of tremendous efforts to effectively treat GBM, the overall survival rates of patients with GBM have remained unchanged over the past few decades.

The discovery of GBM-initiating cells (GIC) has had a significant impact on GBM research (1). GICs have a strong self-renewal capability, express neural stem cell (NSC) markers, such as CD133 (also known as Prominin1), CD15 (also known as stage-specific embryonic antigen 1 and Lewis X), and the intermediate filament protein Nestin, and are more resistant to radio- and chemotherapies than non-GICs (2–4). GICs have also been shown to exploit the signaling pathways involved in maintaining NSCs (2–4).

Among the candidate genes evaluated, we focused on carcinoembryonic antigen–related cell adhesion molecule 1 (Ceacam1, also known as CD66a and BGPI). Human Ceacam1 consists of 11 splicing variants, seven of which are transmembrane proteins with either a short (Ceacam1S) or long cytoplasmic tail (Ceacam1L) whereas the others are secretion forms (10–12). Ceacam1L was also identified as a substrate of the insulin growth factor receptor and EGFR, both of which are frequently activated in malignant gliomas (13), whereas modified...
Ceacam1 acted as either an amplifier or attenuator of these receptors in a cell-dependent manner. These findings prompted us to investigate the role of Ceacam1 in GICs.

Materials and Methods

Animals and chemicals

Animals were obtained from the Laboratory for Animal Resources and Genetic Engineering at the RIKEN Center for Developmental Biology (CDB) and from Charles River Japan, Inc. All mouse experiments were performed following protocols approved by the Animal Care and Use Committees of RIKEN CDB, Ehime University, and Hokkaido University. Chemicals and growth factors were purchased from Sigma and PeproTech, respectively, except where indicated.

Cell culture

Mouse primary neural cells (NSCs and OPCs), NSCL61, OPC161, human NSCs (hNSC; Invitrogen), and GICs (hGICs, E3 and E6) were cultured as described previously (7–9, 14). Cells were cultured in chamber slides [Nunc] precoated with fibronectin and poly-β-lysine for immunostaining, as described previously (14).

Immunostaining

Immunostaining of paraffin-embedded human brain tumor sections (6-μm thick) and mouse or human brain sections was performed as described previously (7, 8). Ceacam1 was retrieved by HistoVT One according to the supplier’s instructions (Nacalai Tesque). The sections were permeabilized with 0.3% TritonX-100 in PBS for penetration, treated with a blocking solution (2% skim milk, 0.3% Triton X-100, and PBS) for 1 hour, and incubated with primary antibodies for 16 hours at 4°C. Cells were fixed and immunostained as described previously (14). The following antibodies were used to detect antigens: mouse anti-Ceacam1 (1:50; R&D Systems), anti-GFP (1:500; Chemicon, 1:400; Sigma for human cells), rat monoclonal anti-GFP (1:500; Nacalai Tesque), mouse monoclonal anti-Nestin (1:200; BD Biosciences), mouse monoclonal anti-CD15 (1:200; BD Pharmingen), rabbit polyclonal anti-STAT3 (1:100; Santa Cruz Biotechnology), rabbit polyclonal anti-EGFR (1:50; Cell Signaling Technology), rabbit polyclonal anti-Caspase 3 (1:1,000, Cell Signaling Technology), and rabbit monoclonal anti-Ki67 (1:200, Thermo Scientific). Antibodies were detected with Alexa568-conjugated goat anti-rabbit IgG (1:500; Molecular Probe), Alexa488-conjugated goat anti-mouse, rabbit, or rat IgG (1:500; Molecular Probe) and goat anti-mouse IgG-Cy3 (1:500; Jackson ImmunoResearch). Cells were counterstained with DAPI (1 μg/mL) to visualize the nuclei.

Human brain tumors

hGICs were used according to the research guidelines of the Ehime University Graduate School of Medical Science and the Hokkaido University Institute for Genetic Medicine. The detailed characterization of hGICs has been reported in ref. 9. Poly(A)+ RNA was prepared using a QuickPrep mRNA Purification Kit (GE Healthcare). Control human brain total mRNA (CB) was purchased from Invitrogen. CBNA was synthesized using a Transcription First Strand cDNA Synthesis Kit (Roche).

RT-PCR

RT-PCR was carried out as described previously (14), with the cycle parameters of 94°C for 30 seconds at 94°C, 30 seconds at 53°C, and 60 seconds at 72°C for 35 cycles (hGICs) or 40 cycles (GBM tissues). Cycles for gapdh were 15 seconds at 94°C, 30 seconds at 53°C, and 90 seconds at 72°C for 22 cycles. The following oligonucleotide DNA primers were synthesized: for mouse Ceacam1, the 5′ primer was 5′-ATCCCTCCCAAGGCCCTTTATC-3′, and the 3′ primer was 5′-TTTTGGCTCATGAGAATGGAAACTTG-3′; for human Ceacam1, the 5′ primer was 5′-ACACACCTGGGCAACCTCTCA-3′, and the 3′ primer was 5′-GATGCTGTAGCTGTGTTGCCT-3′; for sox1, the 5′ primer was 5′-AGGGCTACATGAGCGCGTCG-3′, and the 3′ primer was 5′-CTTGAGGCCCTTGGGCCT-3′; for dll1, the 5′ primer was 5′-TGGTGTCTGCCTGGCCCTG-3′, and the 3′ primer was 5′-ACGCCAGGAAATGGAAACCTG-3′; for notch3, the 5′ primer was 5′-ATGGTGCAAGACGCTACTGC-3′, and the 3′ primer was 5′-TGGCCTCTGCCTCTTCTTG-3′; for hey1, the 5′ primer was 5′-CCGAGGCAATGGAAACCTG-3′, and the 3′ primer was 5′-ATGCCTCTAACATGCTCAGAT-3′; for egrf, the 5′ primer was 5′-GATGAAAGATGCATTTGCCAAC-3′, and the 3′ primer was 5′-GGGGCTATTGTGATAGAGAGG-3′; for stat3, the 5′ primer was 5′-GTGTCAGATCATGAGGCCCTA-3′, and the 3′ primer was 5′-TGCCCTCCCTCTGGAATGCT-3′; for ppp5r6, the 5′ primer was 5′-GTGTCATCTGTCCTGCTC-3′, and the 3′ primer was 5′-GTGTGTCATCTGTCCTGATC-3′; for ptpn11, the 5′ primer was 5′-AAAGGAGGAGCAATGAGGCG-3′, and the 3′ primer was 5′-ATCCACCGTGTTGATGAGGC-3′; for c-Src, the 5′ primer was 5′-ACATCCCCAGCAGAATACGTG-3′, and the 3′ primer was 5′-AGCTTTCCTCAGATCCGGCCG-3′. The primers for gapdh were described previously (14).

Flow cytometry

hGICs were immunolabeled with rabbit polyclonal anti-Ceacam1 (5 μg/mL; LSBio) and mouse monoclonal anti-CD15 (5 μg/mL; BD Pharmingen), following with Alexa 488-conjugated goat anti-mouse IgG (1:400; Molecular Probe) and Cy5-conjugated goat anti-rabbit IgG (Molecular Probe; diluted 1:400). The cells were analyzed in an Aria II (Becton Dickinson) using a dual-wavelength analysis (488-nm solid-state laser and 638-nm semiconductor laser). Propidium iodide–positive (i.e., dead) cells were excluded from the analysis.

The side population (SP) was analyzed as shown previously (15). Reserpine (10 μM/L), an inhibitor of some ABC transporters, was used to identify SP.

Vector construction

Complementary DNAs (cDNA) were cloned as described previously (7). Human ceacam1L cDNA was inserted into pcDNA3.1-hyg (Invitrogen), pcDNA3-2xFLAG-c and pMY-EGFP vectors to produce pcDNA3.1-hyg-hceacam1L, pcDNA3-h-ceacam1L-2xFLAG-c and pMY-EGFP-hceacam1L. The following oligonucleotide DNA primers were synthesized: For the full-length human ceacam1L, the 5′ primer was 5′-ACCTAGCCGCGGACATGAGGAAATGCT-3′, and the 3′ primer was 5′-ACFCGAATTCACACTTACCCATCCCCACCAGG-3′; for the FLAG-tagged human ceacam1L, the 5′ primer was 5′-GCTTTGTCCTGACACACTACCCATCCCACGAGGAAATGCT-3′.
ceacam1L described previously (7, 8). The GFP-expressing hGICs (hereafter, infected with pMY-EGFP or pMY-EGFP-hCeacam1L vector as from the sagittal suture, and 5 mm deep. In injection site were 2 mm forward from the lambda, 2 mm lateral with 10% pentobarbital. The stereotactic coordinates of the week-old female NOD/SCID mice that had been anesthetized brains.

Intracranial cell transplantation into immunodeficient mouse brains

To mark the transplanted hGICs in vivo, the cells were transfected with pMY-EGFP or pMY-EGFP-hCeacam1L vector as described previously (7, 8). The GFP-expressing hGICs (hereafter, hGICs) and ceacam1L-overexpressing hGICs were suspended in 5 μL of culture medium and injected into the brains of 5- to 8-week-old female NOD/SCID mice that had been anesthetized with 10% pentobarbital. The stereotactic coordinates of the injection site were 2 mm forward from the lambda, 2 mm lateral from the sagittal suture, and 5 mm deep.

Mouse brains were dissected, fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, processed on Tissue-Tek VIP (Sakura Finetek Japan), and embedded in paraffin. Coronal sections (6-μm thick) from the cerebral cortex were prepared on a microtome and stained with hematoxylin–eosin (H&E).

Proliferation assay

Two thousand cells were cultured in 100 μL of the culture medium in each well of a 96-well plate. To examine cell proliferation, the MIT assay was performed as follows. Ten microliters of MTT (5 mg/mL; Nacalai Tesque) was added to each well on days 0, 2 or 3, and 4 in vitro. The cells were incubated for 4 hours, the medium was replaced with 100 μL of DM50, the cells were dissociated, and cell proliferation was quantified on a Benchmark microplate reader (Bio-Rad) with the absorption spectrum at 570 nm.

Soft-agar assay

We performed a soft-agar assay to determine whether transfected cells proliferated in an anchorage-independent manner. The transfected cells were suspended in 0.3% top agar made with the optimized medium and layered onto 0.6% bottom agar made with the same medium. After the top agar had polymerized, culture medium was added and the cells were cultured for 20 days with medium changes every 3 days.

Microarray hybridization and data processing

Total RNA was extracted from mouse NSCs and NSCL61 using the TRIzol Plus RNA Purification System (Invitrogen). Purified RNA was then amplified and labeled with Cyanine 3 (Cy3) using the one-color Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer’s instructions. Labeled cRNAs were fragmented and hybridized to the Agilent mouse GE 8 × 60 K Microarray. After washing, microarrays were scanned with an Agilent DNA microarray scanner. Intensity values for each scanned feature were quantified using Agilent feature extraction software, which performed background subtraction.

Normalization was achieved using Agilent GeneSpring GX version 11.0.2. After normalization, hierarchical sample clustering of the expressed genes (DEGs) was performed with the Euclidean distance and average linkage methods (Agilent GeneSpring GX).


Brain fixation and histopathology

Dissected mouse brains were fixed in 4% paraformaldehyde at 4°C overnight. After fixation, the brains were cryoprotected with 12% to 18% sucrose in PBS and embedded in Tissue-Tek OCT compound (Miles). Coronal sections (6-μm thick) were prepared from the cerebral cortex and stained with H&E using a standard technique or immunolabeled for GFP and either the active form of caspase-3 to detect dying cells or Ki67, a marker for proliferating cells.

Immunoprecipitation and Western blotting

Immunoprecipitation was performed as previously described (17). Cell lysates were incubated with Protein G sepharose (GE Healthcare) and the anti-FLAG (10 μm/mL) antibody for 4 hours at 4°C. The mixtures were centrifuged, and the precipitants were triple-washed and analyzed by Western blotting.
Western blotting was performed as previously described (17). The blotted membranes were probed with anti-Nestin (1:1000; BD Pharmingen, 1:200; Chemicon for human cells), anti-GFAP (1:1000; BD Pharmingen, 1:200; Chemicon for human cells), rabbit anti-STAT3 (1:1000; Santa Cruz Biotechnology), rabbit anti-phospho-STAT3 (1:1000; Cell Signaling Technology), rabbit anti-PTPN6 (1:1000; Cell Signaling Technology), rabbit anti-PTPN11 (1:1000; Cell Signaling Technology), rabbit anti-phospho-PTPN11 (1:1000; Cell Signaling Technology), rabbit anti-c-Src (1:1000; Cell Signaling Technology), rabbit anti-phospho-c-Src (1:1,000; Cell Signaling Technology), or a mouse anti-GAPDH antibody (1:1,000; Chemicon). An ECL system (Amersham) was used for detection.

Statistical analysis
Survival data were analyzed for significance by Kaplan–Meier methods using GraphPad Prism version 4 software (P values were calculated with the log-rank test). All experiments were conducted more than three times with similar results.

Results
Ceacam1L was identified as a novel GIC marker
We confirmed that the expression of ceacam1L was higher in NSCL61, OPCL61, and hGICs, E3 and E6 that were prepared from human GBM tissues, than in mouse parental cells (p53-deficient mNSCs and mOPCs) or normal human NSCs (hNSC; Fig. 1A; refs. 7, 9). An immunocytochemical analysis revealed that more than 70% of cultured human and mouse GICs were positive for Ceacam1 (Fig. 1B). Ceacam1L was more prominently expressed in GBM than in other malignant gliomas, anaplastic astrocytoma (AA), anaplastic oligoastrocytoma (AOA), or anaplastic oligodendroglioma (AO; Supplementary Fig. S1A). Although ceacam1S was expressed in mouse and human GICs, the expression level of ceacam1S was significantly lower than that of ceacam1L in hGICs (Supplementary Fig. S1B and S1C). Furthermore, more than 70% of Ceacam1-positive cells were coimmunolabeled for the well-known GIC marker CD15 in the periphery of human GBM (Fig. 1C; ref. 18), whereas all Ceacam1+ cells were positive for EGFR, which is frequently amplified in human GBM (Fig. 1D; refs. 1, 13).

Figure 1.
Ceacam1 was predominantly expressed in GICs. A, Ceacam1 expression in mNSCs, NSCL61, mOPCs, OPCL61, hNSC, and hGICs, E3 and E6, examined by RT-PCR. The expression of gapdh was used as an internal control. B, representative data of NSCL61, OPCL61, and hGICs immunostained for Ceacam1 (green). C, representative images of immunoreactivity for Ceacam1 (green) and CD15 (red) in one of three primary human GBM specimens. D, representative images of immunoreactivity for Ceacam1 (green) and EGFR (red) in one of three primary human GBM specimens. E, representative data from an expression analysis of Ceacam1 and CD15 in one of three human GBMs by flow cytometry. Nuclei were counterstained with DAPI (blue); scale bar, 100 μm.
Ceacam1L was involved in GIC proliferation and angiogenesis

We analyzed the function of Ceacam1L in GICs. Using ceacam1L-specific shRNA (ceacam1sh) expression vectors (Supplementary Fig. S2), we found that the depletion of Ceacam1L inhibited the proliferation of human and mouse GICs (Fig. 2A) as well as their colony-forming ability in soft agar (Fig. 2B). In contrast, ceacam1L-overexpressing hGICs enhanced their colony-forming activity in soft agar (Fig. 2C) and killed mice more quickly than their parental cells (Fig. 2D). H&E staining revealed that ceacam1L-overexpressing hGICs formed larger tumors than parental hGICs at 40 days after injection and induced massive hemorrhages in tumors (Fig. 2E), consistent with the previous finding in which Ceacam1 acted as an angiogenesis-inducing extracellular factor (19). We further found that the tumors formed by ceacam1L-overexpressing hGICs contained more Ki67+ proliferating cells, but less activated caspase-3+ dying cells than control tumors (Fig. 2F and G). These results indicated that Ceacam1L regulated GIC tumorigenesis intracellularly and tumor angiogenesis extracellularly.

Ceacam1L regulated the expression of stemness-related genes and the side population through STAT3 activation in GICs

To identify the molecular mechanism regulated by Ceacam1L, we compared the gene expression profile of ceacam1L-overexpressing NSCs with that of NSCs, in which an endogenous expression of ceacam1L was undetectable, and found that 1,347 genes were upregulated whereas 1,286 were downregulated in ceacam1L-overexpressing NSCs (Supplementary Fig. S3A). Of these, we noted that the overexpression of ceacam1L strongly induced the expression of STAT3 target genes, including glial fibrillary acidic protein (GFAP), suppressor of cytokine signaling 3 (SOCS3), S100β, angiopoietin 1 (ANGPT1), and angiotensinogen (AGT), in NSCs (Fig. 3A). We confirmed that the overexpression of Ceacam1L induced the nuclear translocation of the phosphorylated form of STAT3 (p-STAT3) and GFAP expression in NSCs (Fig. 3B–D). These results indicated that Ceacam1L activated the STAT3 signaling pathway in NSCs.

We also compared the gene-expression profile of NSCL61 with that of ceacam1sh-overexpressing NSCL61, and found that 4,864 genes were upregulated whereas 3,984 were downregulated in ceacam1L-overexpressing NSCL61 (Supplementary Fig. S3A). We noted a significant downregulation in the expression of stemness-related genes, including Notch factors (notch3, notch4, hey1, and dll1), aldehyde dehydrogenases (aldoh1a1, aldoa1, aldol1, -1a2, -1a3, -1a4), -1a7, -1a10, and -1a12), SRY-box transcription factors (sox1, -2, -3, -4, -9), ATP-Binding Cassette (ABC) transporters (abc-a1, abc-a2, abc-a3, abc-b1), chemokine receptor 4 (cxcr4), patched 1, and stat3 in ceacam1sh-overexpressing NSCL61 (Supplementary Fig. S3B). Because STAT3 is a well-known important factor for both stemness and tumorigenesis (20–23), we determined which genes were potential Ceacam1L/STAT3 targets using the Champion Chip Transcription Factor Search Portal based on the SABiosciences’ proprietary database (http://www.sabiosciences.com/chipqpcrsearch.php), and found that dll1, hey1, notch

Figure 2. Ceacam1L was involved in GIC malignant phenotypes. A, the decreased proliferation of ceacam1sh-overexpressing NSCL61, OPCL61, and hGICs, E3 and E6. B, the colony formation ability of ceacam1L-overexpressing NSCL61, OPCL61, and hGICs in soft agar was lower than that of controlsh (contsh)-expressing cells. C, the colony formation ability of ceacam1L-overexpressing hGICs (Ceacam1L) in soft agar was greater than that of control hGICs (Cont). D, survival curves for mice injected with cont (black dotted line) or ceacam1L (red solid line). Data are displayed as the mean ± SEM with n = 5 mice per group. E, representative images of H&E staining of tumors formed by cont and ceacam1L at 40 days after transplantation. White dotted area, tumor. Left, the high-magnification images of black dashed squares. F, increased proliferation of ceacam1L-overexpressing hGICs, compared with their parental cells. G, decreased number of the activated caspase-3 (Casp3)-positive cells in ceacam1L-overexpressing hGICs, compared with their parental cells; scale bar, 0.5 mm.

Flow cytometric analysis showed that 3.2% of freshly prepared GBM cells were Ceacam1L+ and more than 90% of Ceacam1L+ cells (2.9% in total) were also positive for CD15 (Fig. 1E). Taken together, these results suggested that Ceacam1 was a novel GIC marker.
Ceacam1L as a New GIC Regulator

We confirmed that the overexpression of dnSTAT3 as well as that of ceacam1Lsh decreased the expression of sox1, aldha1a1, cxcr4, dll1, notch3, and hey1 in hGICs (Fig. 4A and B). We verified that the overexpression of dnSTAT3 inhibited GFAP expression in E3 cells, even when cultured in differentiation medium with 10% FCS (Fig. 4C), and E3 proliferation in NSC medium (Fig. 4D).

We then examined whether Ceacam1L was indeed involved in the stemness maintenance in GICs. We found that the overexpression of Ceacam1L strongly increased the SP (9.0% ≥ 30%; Fig. 5A), whereas that of ceacam1Lsh abolished the population in hGICs (9.3% ≥ 1.2%; Fig. 5B). The overexpression of Ceacam1L upregulated the expression of GFAP in NSC medium and that of Nestin in 1% FCS medium, whereas the knockdown of Ceacam1L downregulated the expression of Nestin in NSC medium (Fig. 5C and D). The expression level of neuronal (Tuj1) and oligodendrocyte (GC) markers did not change by either Ceacam1L overexpression or knockdown. We verified that ceacam1L-overexpressing hGICs kept the neurosphere formation activity even when cultured sparsely, whereas the parental hGICs lost their self-renewal activity in the same condition (Supplementary Fig. S4). Furthermore, we found that ceacam1L overexpression made hGICs to be resistant to the temozolomide, which is a standard anti-GBM medicine (Supplementary Fig. S4). The expression of gapdh was used as an internal control. C, dnSTAT3-expressing E3 cells (arrows, green) were negative for GFAP (red); scale bar, 20 μm. D, overexpression of dnSTAT3 inhibited the proliferation of E3 cells (*, P < 0.01).

Figure 3.

Ceacam1L overexpression activated STAT3 signaling in NSCs. A, microarray data of STAT3-target genes that were significantly upregulated in NSCs by the overexpression of Ceacam1L. B, control NSCs (Cont) and Ceacam1L-overexpressing NSCs (Ceacam1L) were immunostained for phosphorylated STAT3 (p-STAT3, green) and Ceacam1L (red). C, cont and ceacam1L were immunostained for GFAP (green) and Ceacam1L (red). D, the enforced expression of ceacam1L increased p-STAT3 and GFAP in NSCs; scale bar, 50 and 20 μm (insets).

Figure 4.

The dominant negative form of STAT3 decreased the expression of stemness-related genes and GFAP in hGICs and inhibited GIC proliferation. A and B, RT-PCR analysis of the expression of sox1, aldha1a1, cxcr4, dll1, notch3, and hey1 in ceacam1Lsh (A)- and dnSTAT3 (B)-overexpressing E3 cells. The expression of gapdh was used as an internal control. C, p-STAT3-expressing E3 cells (arrows, green) were negative for GFAP (red), even when cultured in the differentiation medium. Nuclei were counterstained with DAPI (blue); scale bar, 20 μm. D, overexpression of dnSTAT3 inhibited the proliferation of E3 cells (*, P < 0.01).

The monomeric Ceacam1L cytoplasmic tail activated c-Src-dependent STAT3 signaling, whereas its oligomerization abolished this activity.

Ceacam1L was previously shown to activate the signaling factors, c-Src and two protein tyrosine phosphatases, nonreceptor type 6 and 11 (PTPN6 and 11, also known as SHP-1 and -2, respectively), upon phosphorylation of the Ceacam1L cytoplasmic tail by various types of tyrosine kinase receptors (10, 11, 25). In turn, c-Src and PTPN6 then activated and inactivated STAT3, respectively, while PTPN11 modulated the Ras, NF-kB, and EGFR signaling pathways positively, and the JAK–STAT pathway negatively (26–29). We found that hGICs expressed ptpn11 and c-Src, but not ptpn6 (Fig. 6A), and that the phosphorylated forms of PTPN11 (p-PTPN11) and c-Src (p-c-Src) bound to Ceacam1L in hGICs (Fig. 6B), indicating that the Ceacam1L-dependent negative feedback signal, which blocks STAT3 activation, was abolished in hGICs. We evaluated whether c-Src activated STAT3 in hGICs. The overexpression of a constitutive active form of c-Src (caSrc) not only increased STAT3 phosphorylation and GFAP expression, but also enhanced cell proliferation, whereas the overexpression of a dominant negative form of c-Src (dnSrc) inhibited these inductions (Fig. 6C and D). These results suggested that Ceacam1L regulated GIC proliferation through the activation of c-Src/STAT3 signaling.
Ceacam1L exists as a monomer, cis-/trans-homodimer, or cis-/trans-heterodimer with Ceacam1 splicing variants or other Ceacam family members; therefore, the intracellular domain of Ceacam1L is a monomer or homodimer, depending on the circumstances (10, 11). To determine which form of the Ceacam1L cytoplasmic tail activated c-Src/STAT3 signaling, we constructed two chimera proteins, FGC1L and FGC1S, consisting of FLAG, a part of the extracellular domain of the granulocyte colony-stimulating factor receptor, the Ceacam1-transmembrane domain, and either the cytoplasmic tail of Ceacam1L or Ceacam1S, respectively (Fig. 7A). We established FGC1L- and FGC1S-expressing hGIC and NSC lines, cultured them in the presence or absence of an anti-FLAG antibody, and then examined the phosphorylation of c-Src and STAT3 as well as the expression of GFAP. We found that p-c-Src, p-STAT3, and GFAP levels were increased in FGC1L-expressing hGICs in the absence of the antibody, whereas these inductions were not detected in FGC1L-expressing hGICs treated with the antibody or FGC1S-expressing cells (Fig. 7B). We confirmed these results using FGC1L-expressing NSC lines (Fig. 7C). We demonstrated that FGC1L associated with p-c-Src and p-PTPN11 in hGICs in the absence of the antibody (Aggregation/C0), whereas these associations were abolished in antibody-treated FGC1L-expressing cells (Fig. 7D). We also confirmed these results using FGC1L-expressing NSC lines (Fig. 7E). Thus, these results revealed that the monomeric Ceacam1L cytoplasmic tail activated c-Src/STAT3 signaling in GICs (Fig. 7A).

Discussion

Ceacam 1 is known to be involved in various biologic functions, including proliferation, angiogenesis, tumorigenesis, and

Figure 5. Ceacam1L levels affected SP and Nestin expression in hGICs. A, representative data of SP in control E3 and ceacam1L-overexpressing cells. The SP, which disappeared in the presence of 10 μmol/L reserpine (right), is outlined and shown as a percentage of the total cell population. B, representative data of SP in control E3 and ceacam1sh-overexpressing cells, as in A. C, the ratio of the neural differentiation marker-positive control, ceacam1L- and ceacam1sh-overexpressing E3 cells, when cultured under the indicated conditions; *, P < 0.05; **, P < 0.01.
inhibition of both cytokine production and cytotoxic activity of immune cells, as an intracellular and intercellular factor (19, 30). We showed that Ceacam1L-overexpressing hGICs formed larger colonies in soft agar and tumors with massive hemorrhaging in the brains of immunodeficient mice, whereas the knockdown of Ceacam1 blocked GIC proliferation. In addition to previous findings in which Ceacam1 acted as a major effector of VEGF-induced angiogenesis (19, 31), we revealed that Ceacam1L regulated the expression of Angpt1, IL18, and secretogranin II, all of which play an important role in vascular development and angiogenesis in GICs (32–34). Because neurovascularization is not only a common characteristic of GBM, but has also been correlated with poor outcomes, these findings indicate that Ceacam1L is an indispensable therapeutic target for GBM.

Ceacam1L is a highly glycosylated protein that carries a Lewis X (LeX) structure, also known as CD15 and SSEA1 (35, 36), which is a well-known stem cell marker expressed on many types of normal stem cells, including embryonic stem cells, bone marrow stem cells, NSCs (37–39), and GICs (18). We herein confirmed that more than 90% of CD15high GICs were positive for Ceacam1L when GICs were immunolabeled in the presence of TritonX-100, suggesting that CD15-carrying Ceacam1L mainly existed in the cytoplasm. This may also explain why CD15-negative glioma cells self-renewed and formed tumorspheres similar to CD15-positive glioma cells (40). Because Ceacam1L was previously shown to be expressed at low levels on the surface of resting T cells and was quickly mobilized from the intracellular compartment to the cell surface following T-cell activation (41), the molecular mechanisms regulating Ceacam1L mobilization in GICs need to be elucidated in more detail.

We found that Ceacam1L positively regulated the expression of a number of stemness-related genes in GICs both directly and indirectly. Ceacam1L controlled the expression of NSC factors (dll1, notch3, hey1, and Sox1-4, 9) and astrocyte markers (GFAP and S100) (42, 43). We also found that ceacam1L overexpression kept self-renewal capability in hGICs. Because Ceacam1L regulated the expression of many ABC transporters and ALDH family members in GICs, Ceacam1L appears to widely govern drug resistance in GICs. Indeed, we found that the ceacam1L-overexpressing hGICs were more resistant to temozolomide, the standard medicine for GBM, than the parental hGICs. This is consistent with a finding that Aldh1a1, one of downstream factors of Ceacam1L, mediated temozolomide resistance in GBM, although the detail mechanism remained elusive (25). Furthermore, FGC1L-overexpressing NSCs formed larger spheres in cultures and continued to express Nestin even in differentiation medium with 1% FCS. These results suggest that Ceacam1L contributed to the maintenance of stemness in GICs. It is unlikely that STAT3, a crucial Ceacam1L signaling factor, predominated the expression of all Ceacam1L-downstream genes; therefore, the transcription factor, other than STAT3, that controls the expression of stemness-related genes needs to be identified.

When phosphorylated by EGFR, Ceacam1L was shown to activate c-Src, PTPN6, and PTPN11 and also enhance or prevent the proliferation signals of the receptor in a cell type–dependent manner (11, 44, 45). We unexpectedly found that the expression of PTPN6, which prevents the JAK-dependent STAT3 signaling pathway (46), was silenced in hGICs, suggesting that a Ceacam1L-dependent negative feedback signal to EGFR was abolished in hGICs. Taken together with the finding that PTPN11 and c-Src activated EGFR and STAT3 signals, respectively (21, 27, this
manuscript), our results suggest that Ceacam1L acted as an amplifier of both EGFR and STAT3 signaling in hGICs.

We demonstrated that the monomeric Ceacam1L-cytoplasmic tail activated c-Src/STAT3 signaling, whereas its oligomerization abolished this activity. The state of Ceacam1L (monomer or oligomer) in GICs may be dependent on their circumstances, in which GIC-associating (niche) cells, such as immune cells and microglia, may express Ceacam1L-binding partners, including Ceacam1L, 1S, soluble forms of Ceacam1, and Ceacam5 (10–12). The finding that lung metastasis by Ceacam1L+/EGFR+ cells and CD68+ activated microglia in human GBM tissues (Supplementary Fig. S6). It has been shown that CD68+ cells increased in high-grade gliomas (48), and that CD68+ infiltrating microglia/macrophages were involved in gliomagenesis (49). Together, these suggest that CD68+ microglia/macrophages play a crucial part to maintain/activate GICs in the niche. Therefore, next crucial challenge is to investigate how Ceacam1L-dependent intercellular

![Diagram](image_url)

**Figure 7.** The Ceacam1L monomer activated c-Src/STAT3 signaling. A, a model of FGC1L monomer-dependent activation of the c-Src-STAT3 signaling pathway. B and C, a Western blotting analysis of c-Src/STAT3 phosphorylation and GFAP expression in FGC1S- and FGC1L-expressing hGICs (B) and mNSCs (C) in the presence (+) or absence (−) of a FLAG antibody. D and E, a binding analysis of the phosphorylated forms of both c-Src and PTPN11 with FGC1L in hGICs (D) and mNSCs (E), in the presence (aggregation +) and absence (aggregation −) of a FLAG antibody (left). Cell lysates were analyzed for these factors by Western blotting (right).
association between GICs and their surrounding cells regulates GIC maintenance and tumor-niche formation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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