Graphical abstract

Conversion of glioma cells to glioma stem-like cells in vivo

GCs-Oct4-Promoter-GFP-Negative

Tumorigenesis

GSCs-Oct4-Promoter-GFP-Positive

GSC reprogramming by angiocrine factors

Tumor vasculature

Angiocrine facotrs (e.g., Nitric Oxide)

sGC
NO signaling
PKG
ID4
JAG/NOTCH
OCT4
OCT4p-GFP

Glioma stem-like cells
Conversion of glioma cells to glioma stem-like cells by
angiocrine factors

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Abstract

Glioma stem-like cells (GSCs) contribute to tumor initiation, progression, and therapeutic resistance, but their cellular origin remains largely unknown. Here, using a stem/progenitor cell-fate tracking reporter system in which eGFP is expressed by promoter of OCT4 that is activated in stem/progenitor cells, we demonstrate that eGFP-negative glioma cells (GCs) became eGFP-positive-GCs in both in vitro cultures and in vivo xenografts. These eGFP-positive-GCs exhibited GSC features and primarily localized to the perivascular region in tumor xenografts, similar to the existence of OCT4-expressing GCs in the perivascular region of human glioblastoma specimens. Angiocrine factors, including nitric oxide (NO), converted eGFP-negative-GCs into eGFP-positive-GCs. Mechanistically, NO signaling conferred GSC features to GCs by increasing OCT4 and NOTCH signaling via ID4. NO signaling blockade and a suicide gene induction prevented tumorigenicity with a decrease in eGFP-positive-GCs in the perivascular region. Taken together, our results reveal the molecular mechanism underlying GSCs generation by cancer cell dedifferentiation.

Keywords: Angiocrine factors, glioma cells, glioma stem-like cells, ID4, OCT4
Introduction

Cancer stem-like cells (CSCs) exist in many types of cancers including glioblastoma, which is the most aggressive and lethal of brain tumors [1-3]. Because glioma stem-like cells (GSCs) are responsible for tumor initiation and progression, they are considered a therapeutic target for cancer eradication [4]. Thus, understanding the mechanisms underlying the generation and cellular origin of GSCs provide a novel opportunity to develop a targeted therapeutic strategy for the treatment of glioblastoma.

The tumor microenvironment plays an important role in regulating the cellular state and characteristics of GSCs by providing critical maintenance cues [5, 6]. The perivascular niche regulates GSC maintenance by coordinated communication between endothelial cells and GSCs through platelet-derived growth factor (PDGF), nitric oxide (NO), and the Notch signaling node [7, 8]. Although many studies have explored the role of the tumor microenvironment in maintaining GSCs, its function in giving rise to GSCs remains largely undetermined.

In the present study, we have established an experimental model to elucidate the origin of GSCs using an enhanced green fluorescence protein (eGFP) reporter gene expressed under the control of the promoter of human OCT4 (hOCT4-p-eGFP), a stem/progenitor cell marker. We found that endothelial cells and angiocrine factors convert hOCT4-p-eGFP-negative-GCs to hOCT4-p-eGFP-positive-GCs that display the features of GSC.
Materials and Methods

Cells and culture conditions

Glioma cell (GC) lines, A1207, LN229, and U87MG, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). GSC-8 is a primary differentiated GC that was established from a glioblastoma patient-derived GSC by serum treatment. All established GC lines (maintained for fewer than 5 passages and used within 6 months after purchase from ATCC) and GSC-8 were maintained in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12; Lonza, Basel, Switzerland) supplemented with 0.2% B27 (Invitrogen, Carlsbad, CA, USA), 20 ng/mL bFGF (R&D Systems, Minneapolis, MN, USA), and 20 ng/mL EGF (R&D Systems). Human umbilical vein endothelial cells (HUVECs) were grown in endothelial cell growth medium (EGM-2, Lonza). Co-culture of HUVECs with hOCT4-p-eGFP-negative-GCs and GSC-8 was performed in DMEM/F12 medium supplemented with B27, bFGF, and EGF.

Mouse xenograft

For subcutaneous cell implantation, $2 \times 10^6$ PN-GC and PN-GSC-8 were injected into nude mice (BALB/c nu/nu). After the tumors reached a mean volume of $10^3$ mm$^3$, the mice received intraperitoneal injections of L-NAME (20 mg/kg per day) or PBS. For intracranial implantation, $10^5$ PN-GCs vs. $10^3$ PN-GCs vs. TP-GCs, $5 \times 10^3$ PN-GSC-8, and $10^5$ LN229-puro cells were stereotactically injected into the brain of nude mice (coordinates: 2 mm right and 1 mm anterior of the bregma). The mice received intraperitoneal injections of
L-NAME (45 mg/kg per day) at 7 days post tumor cell implantation. All mouse experiments were approved by the Animal Care Committee of Korea University (Seoul, Republic of Korea), and were performed in accordance with government and institutional guidelines and regulations.

Statistical analysis

Statistical analyses were performed using a two-tailed Student’s t-test. A p-value less than 0.05 or 0.01 was considered significant or very significant, respectively.

Detailed experimental procedures are provided in the Supplemental information.
Results and Discussion

*hOCT4*-p-eGFP-positive-GCs derived from *hOCT4*-p-eGFP-negative-GCs display GSC features

To investigate whether the tumor microenvironment is involved in giving rise to CSCs from cancer cells by dedifferentiation, we utilized a cell-fate tracking reporter system, in which eGFP gene expression is under the control of the human *OCT4* promoter that is activated in stem/progenitor cells [9]. We established three human GC lines (A1207, LN229, and U87MG) and one primary GSC-derived differentiated GC (GSC-8) transfected with the *hOCT4*-p-eGFP vector. Unexpectedly, the fluorescence-activated cell sorting (FACS) analysis revealed that 10-20% of *hOCT4*-p-eGFP-transduced cells spontaneously expressed eGFP in serum-free medium supplemented with EGF and bFGF (Figure 1A). These parental-eGFP-positive-GCs (PP-GCs) showed higher tumorsphere-forming ability and drug resistance than parental-eGFP-negative-GCs (PN-GCs) (Figures S1A and S1B). The depletion of *OCT4* expression by an *OCT4*-specific siRNA reduced tumorsphere-forming ability of the PP-GCs (Figures S1C and S1D).

To elucidate a possible conversion of PP-GCs from PN-GCs *in vivo*, we first sorted PN-GCs from *hOCT4*-p-eGFP-transduced cells by FACS and generated tumors by subcutaneously inoculating PN-GCs into immunocompromised mice. Then we sorted tumor-derived eGFP-negative-GCs (TN-GCs) and tumor-derived eGFP-positive-GCs (TP-GCs) from dissociated tumors. The results showed that 10-25% of PN-GCs-derived tumor cells expressed eGFP (TP-GCs; Figures 1A and 1B). OCT4 mRNA and protein levels were markedly elevated in all TP-GCs compared to their PN-GCs and TN-GCs counterparts.
(Figure 1C). OCT4 is highly expressed in human embryonic stem cells and acts as a crucial regulator in maintaining undifferentiated stem/progenitor cells [10]. Thus, we examined several hallmarks of GSCs to elucidate whether TP-GCs acquire undifferentiated GSC features. Stem cell marker (NESTIN, CD133, and CD15) expression, tumorsphere-forming ability, side population, and drug resistance were all markedly increased in all four TP-GCs compared to PN-GCs and TN-GCs (Figures 1D, S1E, S1F, and S1G). Furthermore, the survival period of mice injected intracranially with four TP-GCs was significantly shorter than that of mice injected with the corresponding PN-GCs cells (Figure S1H). As increased stem cell marker expression, self-renewal ability, side population, drug resistance, and tumor-initiating capacity are considered GSC properties [11], we conclude that TP-GCs are GSCs.

Taken together, these results suggest that the in vivo tumor microenvironment is capable of converting GCs negative for OCT4-promoter activity to GCs positive for OCT4-promoter activity and which show the features of GSCs.

**Angiocrine factors convert GCs to GSCs**

GSCs are enriched in the perivascular niche and communicate with the vasculature to maintain the stem cell state and its proliferative potential [12]. Therefore, we examined whether TP-GCs also exist in the perivascular niche of intracranial tumor xenografts. Immunofluorescence analysis revealed that the majority of all four TP-GCs were located near von Willebrand factor (vWF)-positive endothelial cells (within 60 µm of vWF-positive vessels; Figure 2A). Similar to the results from our intracranial xenograft models, OCT4-positive-GCs were predominantly detected in the perivascular region of human glioblastoma specimens (Figure S2A).
In order to elucidate whether communication between GCs and endothelial cells is important for the conversion of GCs to GSCs, we next examined, *in vitro*, the generation of eGFP-positive-GCs by co-culturing PN-GCs with human umbilical vein endothelial cells (HUVECs). FACS analysis revealed that the number of eGFP-positive-GCs gradually increased during co-culturing, and reached 22–38% in all four PN-GCs at day 5 (Figure 2B). Because vascular endothelial cells sustain the self-renewal ability of GSCs through the action of various angiocrine factors [7], we examined whether angiocrine factors have the potential to generate eGFP-positive-GCs from PN-GCs. Using a multi-label plate fluorescence reader, we found that most angiocrine factors only slightly increased the eGFP fluorescence intensity in PN-GCs, whereas S-nitrosoglutathione (GSNO), an NO donor, dramatically increased eGFP fluorescence intensity in all four PN-GCs (Figure 2C). FACS analysis also revealed that 70–80% of GSNO-treated PN-GCs expressed eGFP (Figure S2B). We found that HUVECs generated higher levels of NO compared to GCs, and that L-NG-nitroarginine methyl ester (L-NAME; an NO synthase inhibitor) suppressed NO secretion in HUVECs and GCs (Figure S2C). When co-cultures of PN-GCs and HUVECs were treated with L-NAME and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; a soluble guanylate cyclase (sGC) inhibitor) [8, 13], the number of eGFP-positive-GCs from PN-GCs dramatically decreased (Figure S2D). Furthermore, PN-GCs, cultured in serum-free medium supplemented with EGF and bFGF and spontaneously expressing eGFP (Figure 1A), were also dramatically reduced after ODQ treatment (Figure S2E). Therefore, among angiocrine factors, NO signaling should play an important role in not only CSC maintenance but also in CSC genesis via the dedifferentiation of non-CSCs.
NO and ID4 activates OCT4 and Jagged1/Notch1 signals

Among the numerous developmental signaling factors, the inhibitor of differentiation (ID) family plays a crucial role in GSC generation and maintenance [14]. Thus, we compared the expression of ID family in PN-GCs, TN-GCs, and TP-GCs, and found that ID4 mRNA and protein levels were significantly increased in TP-GCs (Figures 3A and 3B). GSNO treatment and ectopic ID4 expression augmented ID4, JAGGED1, NOTCH intracellular domain (NICD), OCT4, SOX2, and NANOG expression in all four parental GCs (Figures 3C and 3D). To further examine whether NO directly enhances NOTCH signaling, we treated GSNO-pretreated GCs with either ODQ (soluble guanylyl cyclase inhibitor), KT5823 (protein kinase G inhibitor), or DAPT (γ-secretase inhibitor). All of these inhibitors significantly suppressed both GSNO-induced NICD expression in GCs and tumorsphere-forming abilities in TP-GCs (Figures 3E and 3F). Thus, these results suggest that endothelial cell-driven NO should increase embryonic factors and NOTCH signaling via the sGC-PKG-ID4 axis and thereby induce stem cell properties in GCs.

NO signaling inhibition prevents tumorigenicity by decreasing the hOCT4-p-eGFP-positive-GC population

To understand the significance of NO secreted by endothelial cells to tumorigenicity in vivo, we first investigated whether L-NAME reduces the eGFP-positive-GC population and tumorigenicity in mouse subcutaneous xenografts. L-NAME treatment significantly suppressed tumorigenicity in all four PN-GCs (Figure 4A), along with marked decreases in the eGFP-positive-GC population in tumor tissues (Figure 4B). L-NAME also significantly increased the survival of mice intracranially injected with LN229 or PN-GSC-8 (Figures 4C
and S3A), and markedly reduced stem cell markers and OCT4 expression (Figures S3B, S3C, S3D, and S3E).

To investigate further the significance of TP-GCs on tumorigenicity, we used an hOCT4-p-DT-A vector that enabled us to directly eliminate TP-GCs generated during tumorigenesis in vivo. We first tested the in vitro cytotoxicity of DT-A by examining the luciferase activity in GCs following transient co-transfection of vectors expressing the luciferase and DT-A genes [15, 16]. DT-A dramatically inhibits luciferase activity by decreasing the co-transfected GC population (Figure S3F). We injected 10^5 PN-LN229-OCT4-p-DT-A cells and 10^5 PN-LN229-OCT4-p-LacZ cells into brains of mice. When the PN-LN229-OCT4-p-LacZ-injected mice began to exhibit neurological symptoms, they were sacrificed along with the PN-LN229-OCT4-p-DT-A-injected mice, which did not demonstrate any neurological symptoms. In sharp contrast to PN-LN229-OCT4-p-LacZ cells, PN-LN229-OCT4-p-DT-A cells give rise to very few tumors (Figure 4D). OCT4-positive-GSCs were markedly decreased in tumor tissue derived from PN-LN229-OCT4-p-DT-A-injected mice compared to OCT4-positive-GSCs in tumor tissue derived from PN-LN229-OCT4-p-LacZ-injected mice (Figure S3G).

Taken together, our findings indicate that inhibition of angiocrine factors restrains tumorigenicity through the suppression of the crosstalk between endothelial cells and cancer cells, the dedifferentiation of cancer cells into CSCs, and the expansion of CSCs. Therefore, it is plausible that investigating the mechanisms governing the angiocrine factor-driven generation of GSCs may be an alternative way to develop more effective cancer therapies, and furthermore, that the combination of a treatment targeting angiocrine factors with cytotoxic drugs may provide a better outcome in cancer treatment.
Author Contributions

JKK, HMJ, HYJ, SYO, and HK designed experiments and wrote the main manuscript; JKK, HMJ, HYJ, and SYO performed most of the experiments and analyzed data; EJK and XJ (Xiong) helped in in vivo work; SHK (Sung) and XJ (Xun) were involved with in vitro experiments; SHK (Se) provided samples and materials; SHK (Sung), and XJ (Xun) provided key experimental advice; HK directed this study.

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Conflict of interest

The authors declared that they have no conflict of interest.
References


Figure Legends

Fig 1. hOCT4-p-eGFP-positive-GCs derived from hOCT4-p-eGFP-negative-GCs display GSC features

(A) Percentage of eGFP-negative and -positive cells present in the four hOCT4-p-eGFP-GCs (GCs were A1207, LN229, U87MG and GSC-8 respectively) before cell sorting (left), and in the primary tumor cells dissociated from tumor xenografts of hOCT4-p-eGFP-negative-GCs (right). Data are expressed as mean ± the standard error of the mean (SEM).

(B) Schematic diagram describing the relationship between parental eGFP-negative-GCs (PN-GCs), tumor eGFP-positive-GCs (TP-GCs), and tumor eGFP-negative-GCs (TN-GCs).

(C) OCT4 protein levels (upper panel) and OCT4 mRNA levels (lower panel) in PN-GCs, TN-GCs, and TP-GCs derived from the four GCs. The mRNA level data are expressed as mean ± SEM. **p < 0.01 (n = 3).

(D) Expression levels of the stem cell markers, NESTIN, CD133, and CD15 determined by FACS analysis (upper left panel); tumorsphere-forming ability (upper right panel); side population percentage (lower left panel); and the % of trypan blue-positive cells following treatment with the anticancer drug BCNU (lower right panel) in PN-GCs, TN-GCs, and TP-GCs. Data are expressed as mean ± SEM. **p < 0.01 (n = 3).

Fig 2. Endothelial cells and angiocrine factors convert GCs to GSCs.

(A) Immunofluorescent staining showing the distribution of hOCT4-p-eGFP-positive-GCs and vWF-positive endothelial cells (vessels) in intracranial tumor xenografts of four PN-GCs
A (upper panel, 200x magnification). Quantification of eGFP fluorescence intensity depicting the distance between GCs and vWF-positive vessels (bottom panel).

(B) Percentage of hOCT4-p-eGFP-positive cells in four PN-GCs after co-culturing with HUVECs over a culture period spanning five days. Data are expressed as mean ± SEM.

(C) OCT4 promoter activity measured by eGFP fluorescence intensity in PN-GCs after treatment with angiocrine factors. Data are expressed as mean ± SEM. **p < 0.01 (n = 3).

Fig 3. NO regulates ID4, embryonic factors and tumorsphere-forming ability.

(A) The expression levels of ID1, ID3, and ID4 mRNA in PN-GCs, TN-GCs, and TP-GCs were determined by q-RT-PCR analysis. Data are expressed as mean ± SEM. *p < 0.05 and **p < 0.01 (n = 3).

(B) The expression levels of ID1, ID3, and ID4 proteins in PN-GCs, TN-GCs, and TP-GCs assessed by Western blot analysis.

(C) ID4, OCT4, JAG1, NICD, SOX2, and NANOG protein levels in GCs following GSNO treatment.

(D) ID4, OCT4, JAG1, NICD, SOX2, and NANOG protein levels in GCs following ID4 overexpression.

(E) NICD protein levels in GSNO-pretreated GCs upon treatment with ODQ (A1207, U87MG, and GSC-8 cells, 2 µM; LN229 cells, 4 µM), KT5823 (3 µM), and DAPT (A1207 and GSC-8 cells, 1 µM; U87MG and LN229 cells, 5 µM).

(F) In vitro limiting dilution assays showing tumorsphere-forming ability of eGFP-negative-
GCs (control) and eGFP-positive-GCs treated with or without ODQ, DAPT, and KT5823. *p < 0.05 and **p < 0.01 (n = 24).

Fig 4. Blockade of NO signaling inhibits tumorigenicity of PN-GCs in vivo.

(A) The tumorigenicity of four PN-GCs treated with or without L-NAME. Data are expressed as mean ± SEM. (p < 0.05, n = 6). Arrows indicate the start time for PBS or L-NAME treatment.

(B) Representative photographs showing tumor xenograft sizes and general morphology at the end-point of the experiments described in (A). Fluorescent images of the same tumor samples showing hOCT4-p-eGFP-positive-GCs (green) and vWF-positive vessels (red). (200× magnification).

(C) Percent survival of mice injected with PN-GSC-8 treated with or without L-NAME. (p < 0.01).

(D) Hematoxylin-eosin staining showing brain tumor masses in mice injected with PN-LN229-OCT4-p-DT-A or PN-LN229-OCT4-p-LacZ cells (n = 3).
Figure 1
Figure 2

A

PN-GCsa-derived tumors

A1207 LN229 U87MG GSC-8

Distance from VWF-positive vessel (µm)

Relative aPDF intensity

hOCT4pr VWF DAPI

B

HUVEC co-culture

Day 1 Day 2 Day 3 Day 4 Day 5

Relative aPDF intensity

A1207 LN229 U87MG GSC-8

C

Normalized of gRNA intensity

Mock BDNF BMP4 BFGF GSNQ IL8 IL12 JAG1 PDGF TGFS VEGF EGF TNFe

**
Figure 4

A

B

C

D

PN-A1207
PN-LN229
PN-U87MG
PN-GSC-8

PBS
L-NAME

PBS
L-NAME

PBS
L-NAME

PBS
L-NAME

Time (days)

Tumor volume (mm³)

Tumor volume (mm³)

Tumor volume (mm³)

Tumor volume (mm³)

0 3 6 9 12 15 18 21 24 27 30 33

0 3 6 9 12 15 18 21 24 27 30 33

0 3 6 9 12 15 18 21 24 27 30 33

0 3 6 9 12 15 18 21 24 27 30 33

hOCT4-p-eGFP(+)

A1207
LN229
U87MG
GSC-8

PN-LN229

hOCT4-p-LacZ
hOCT4-p-DTA

PN-GSC-8

Vehicle
L-NAME (5mg/kg)

p < 0.05

p < 0.05

p < 0.01

p < 0.05
Highlights

- Glioma cells are converted to glioma stem-like cells in \textit{in vivo} xenografts
- Angiocrine factors convert glioma cells to glioma stem-like cells
- Nitric oxide regulates ID4 and OCT4 expression in glioma stem-like cells
- Blockade of nitric oxide signaling prevents tumor progression