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EPIDERMAL GROWTH FACTOR PLAYS A CRUCIAL ROLE IN MITOGENIC REGULATION OF HUMAN BRAIN TUMOR STEM CELLS

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Running head: EGF is essential for maintenance of brain tumor stem cells

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A cancer stem cell population in malignant brain tumors takes an essential part in brain tumor initiation, growth, and recurrence. Growth factors, such as epidermal growth factor, fibroblast growth factor-2, vascular endothelial growth factor, platelet-derived growth factor and hepatocyte growth factor are shown to support the proliferation of neural stem cells, and also may play key roles in gliomagenesis. However, the responsible growth factor(s), which controls maintenance of brain tumor stem cells, is not yet uncovered. We have established three cancer stem cell lines from human gliomas. These cells were immunoreactive with the neuronal progenitor markers, nestin and CD133, and established tumors that closely resembled the features of original tumor upon transplantation into mouse brain. Three cell lines retained their self-renewal ability and proliferation only in the presence of epidermal growth factor (>2.5 ng/ml). In sharp contrast, other growth factors, including fibroblast growth factor-2, failed to support maintenance of these cells. The tyrosine kinase inhibitors of epidermal growth factor signaling (AG1478 and gefitinib) suppressed the proliferation and self-renewal of these cells. Gefitinib inhibited phosphorylation of epidermal growth factor receptor as well as Akt kinase and extracellular signal-regulated kinase 1/2. Flow cytometric analysis revealed that epidermal growth factor concentration-dependently increased the population of CD133-positive cells. Gefitinib significantly reduced CD133-positive fractions and also induced their apoptosis. These results indicate that maintenance of human brain tumor stem cells absolutely requires epidermal growth factor, and that tyrosine kinase inhibitors of epidermal growth factor signaling potentially inhibit proliferation and induce apoptosis of these cells.

The biology of neural stem cells and their intrinsic properties are now recognized as integral to brain tumorigenesis (1-3). Evidence is presented that brain tumors contain a cancer stem cell (CSC)[3] population, capable of self-renewal and multi-lineage differentiation, which recapitulates the phenotype of the original tumor (4-12). The concept of CSCs is based on the similarities between stem cells and cancer cells with respect to their self-renewal capacity and multi-potential cell fate (13). Since CSCs is thought to play an important role in tumorigenesis and tumor recurrence, effective cancer treatments should be targeted at eliminating this population.

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The CD133 has been identified as a marker of neural stem cells in the adult central nervous system as well as of brain CSCs (7,8,14). Although the biological function of CD133 is not well understood, this molecule currently serves as a useful marker for the isolation of CSCs. The CD133-positive cells could be potential targets for cancer therapy. Therefore, the recent research is attempting to elucidate the nature and role in tumorigenesis of CSCs.

The proliferation and differentiation potential of neural stem cells can be modulated by endogenous and environmental factors (15). A combination of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) promotes their proliferation and differentiation (16-18). Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are also classified as neurotrophic factors, which play essential roles in the development, maintenance, and regeneration of the central nervous system (19,20). Moreover, hepatocyte growth factor (HGF) is present in the developing and mature central nervous system (21), implying its role in this system. These growth factors and their receptors should also be implicated in glioma tumorigenesis and the acquisition of malignancy (22-24). However, it remains unknown which of these growth factors play(s) a pivotal role in the proliferation and differentiation of CSCs.

In the present study, we have established CSC lines from three brain tumor patients and examined the growth factor dependence of these CSCs. The data obtained indicate that among the growth factors (EGF, FGF-2, PDGF, VEGF and HGF) tested, only EGF promoted sphere formation and enhanced the self-renewal capacities of CSCs including the CD133-positive subpopulations. The ability of tyrosine kinase inhibitors selective for EGF receptor (EGFR) kinase to block CSC self-renewal was also tested. The tyrosine kinase inhibitors gefitinib (ZD1839, Iressa; AstraZeneca) and AG1478 quite efficiently inhibited EGF-dependent sphere formation. Gefitinib also significantly decreased the CD133 populations by flow cytometric analysis. Autophosphorylation of EGFR was inhibited by gefitinib in a concentration-dependent manner. Among EGFR downstream signaling molecules (25,26), phosphorylation of Akt kinase and extracellular signal-regulated kinase (ERK) 1/2, but not STAT3, was suppressed by gefitinib. Blockage of both ERK and Akt pathways by selective inhibitors, PD98059 and LY294002, respectively, resulted in considerable inhibition of EGF-dependent sphere formation. These findings indicate that EGF signaling cascade is essential for the maintenance of brain tumor stem cells.

Experimental Procedures

Primary sphere formation-Prior informed consent was obtained from all 3 sample donors. Our study was approved by the Medical Review Board of Gifu University. Tumor-sphere cultures were performed as described previously with some modifications in medium containing DMEM-F12 (GIBCO-Invitrogen, La Jolla, CA), penicillin G, streptomycin sulfate, B-27 (GIBCO-Invitrogen), recombinant human FGF-2 (20 ng/ml; R&D Systems, Minneapolis, MN), recombinant human EGF (20 ng/ml; R&D Systems), and leukemia inhibitory factor (1000 U/ml) (6,7,11,12).

Limiting dilution assay-Sphere cells were dissociated and seeded in 96-well culture plates in 0.2 ml medium (7,12). The final cell dilutions ranged from 1 to 1000 cells/well. Cultures were fed 20 μl of medium every 2 days, until day 7. The percentage of wells not containing spheres for each cell plating density was calculated and plotted against the number of cells per well.

Immunofluorescent staining-Immunocytochemistry of tumor-spheres and

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differentiated spheres were performed as described (11,12). Antibodies used were as follows; anti-EGFR (mouse monoclonal antibody (mAb), 1:100; Upstate, Temecula, CA), anti-phospho-EGFR (mouse mAb, 1:200; Upstate), human anti-nestin (rabbit polyclonal antibody (pAb), 1:200; Chemicon, Temecula, CA) and CD133 (mouse mAb, 1:10; Miltenyi Biotec, Auburn, CA) for the detection of neural stem and progenitor cells, anti-βIII-tubulin (Tuj1) (mouse mAb, 1:200; Chemicon) for neurons, anti-glial fibrillary acidic protein (GFAP; rabbit pAb, 1:500; DAKO, Glostrup, Denmark) for astrocytes, anti-galactocerebroside (GalC; mouse mAb, 1:200; Chemicon) for oligodendrocytes, and anti-cleaved caspase-3 (rabbit pAb, 1:500; Cell Signaling Technology, Beverly, MA) for apoptosis assay. Visualization was performed with Alexa fluorophore-conjugated secondary antibodies (1:1,000; Molecular Probes, Eugene, OR). Cells were simultaneously stained with Hoechst 33342 for identifying nuclei.

Transplantation into immunodeficient mice-Our experimental procedures involving animals followed the guidelines of the Animal Experimental Committee of Gifu University. Tumorigenicity was determined by injecting brain tumor-derived CSCs orthotopically into non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice. After 8-12 weeks in primary culture, 2 μl of a cell suspension ($1x10^8$cells/ml) in proliferation medium was injected stereotactically into the right striatum (0.2 μl/min) of anesthetized NOD-SCID mice using a Hamilton syringe. The injection coordinates were 3 mm to the right of the midline and 2 mm anterior to the coronal suture at a depth of 3 mm. The mice were sacrificed at 4-14 weeks post-injection depending on the injected cell line (11,12).

Histochemical analysis of brain tissues-Tumor samples were fixed with 4% paraformaldehyde. 4-μm sections were cut from paraffinized tissue blocks. They were mounted on silanized slides, dried, deparaffinized with xylene, hydrated with decreasing concentrations of ethanol and washed with phosphate-buffered saline. For hematoxylin-eosin (HE) staining, slides were first stained with Harris hematoxylin (2 min) and then counterstained with alcoholic eosin. For immunohistochemical studies, endogenous peroxidase was neutralized with 3% H_2O_2 in methanol (15 min). The sections were stained with the Histofine mouse stain kit (Nichirei, Tokyo, Japan), and then with primary antibodies; anti-human nestin (mouse mAb, 5 μg/ml; R&D Systems) for neural stem cells, anti-human Ki-67 (mouse mAb, 1:50; DAKO) for proliferation indices, anti-GFAP (mouse mAb, 1:500; DAKO) for astrocytes, and anti-human βIII-tubulin (mouse mAb, 1:500; Chemicon) for neurons. After treatment with the secondary antibody and MAX-PO (Nichirei), color reactions were performed with peroxidase-substrate DAB (3,3'-diaminobenzidine; DAKO). All tissue sections were counterstained with Mayer's hematoxylin.

Growth factor stimulation-Cultures were initiated in medium containing a combination of EGF and FGF-2 (4-10). Then, the tumor spheres were washed and dissociated into single cells. They were transferred to medium containing human recombinant FGF-2, EGF, PDGF, VEGF, or HGF alone (all factors were added at 20 ng/ml), and cultured for 7-14 days. Upon sphere formation, we assessed the effects of the growth factors by plating 500 cells/well in 96-well plates and adding various concentrations of a growth factor. After sphere formation, the percentage of wells containing spheres was calculated.

Reverse transcription–polymerase chain reaction (PCR)-Total RNA was purified from cultures, using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's

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instructions. cDNA was prepared from RNA templates (5 μg) using oligo (dT) primers and Super Script II reverse transcriptase (GIBCO-Invitrogen). The cDNA product (1 μg) was subjected to PCR with *rTaq* polymerase (TaKaRa Shuzo Co., Ltd., Tokyo, Japan). Each PCR product was electrophoresed on 1% agarose gels and stained with ethidium bromide. Forward and reverse primer sequences for the specific amplification of EGFR and a constitutively active EGFR mutant (EGFRvIII) were 5'-CTT CGG GGA GCA GCG ATG CGA C-3' / 5'-ACC AAT ACC TAT TCC GTT ACA C-3'. These primers generate a 1044-bp PCR product for the wild-type EGFR transcript compared to a 243-bp PCR product for the EGFRvIII transcript (27). The sequences of the gene-specific primers (sense and antisense) were 5'-GCA CCA CAC CTT CTA CAA TGA GC-3' / 5'-TTG AAG GTA GTT TCG TGG ATG CC-3' for β-actin. The amplification conditions were 94°C for 2 min, followed by 42 cycles of denaturation at 95°C for 30 sec, annealing at 56.5°C for 30 sec, and extension at 68°C for 80 sec for (EGFR/EGFRvIII), and 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec for β-actin.

Antiproliferative assays-AG1478 (EMD Biosciences, San Diego, CA), a highly selective inhibitor of EGFR tyrosine kinase was dissolved in dimethysulfoxide (DMSO) to obtain final concentrations of 0.01, 0.1, 1.0, and 10.0 μM. The tyrosine kinase inhibitor gefitinib, a synthetic anilinoquinazoline that targets EGFR, was acquired by dissolving Iressa tablets (250 mg; AstraZeneca, Wilmington, DE) in the above concentrations of DMSO. For antiproliferative assays, the tumor spheres were washed and dissociated into single cells with phosphate-buffered saline. They were plated at 500 cells/well in 96-well plates in the medium containing DMSO, AG1478, or gefitinib (0-10.0 μM) in the presence of EGF (20 ng/ml). After sphere formation, the percentage of wells containing spheres was calculated. For functional analysis of EGFR downstream signals, a selective inhibitor of ERK pathway, PD98059 (28) (EMD Biosciences) and LY294002 (29) (EMD Biosciences) for phosphatidylinositol 3-kinase (PI3K)/Akt pathway were used. 1000 cells were plated in 24-well plates in the medium containing EGF (20 ng/ml) in the absence or presence of PD98059 (10 μM) and/or LY294002 (10 μM). The number of formed spheres was counted.

Western blotting-Western blot analysis was performed essentially as described previously (30). Inhibition of EGFR signaling by increasing concentrations of gefitinib was assessed by Western blotting for detecting EGFR autophosphorylation with antibodies against EGFR (BD Biosciences, San Jose, CA) and phospho-EGFR (P-Tyr1068, Cell Signaling Technology). The activation of EGFR downstream signaling molecules was determined by detecting their phosphorylation. The following antibodies were used: Akt (Cell Signaling Technology), phospho-Akt (P-Ser473, Cell Signaling Technology), STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-STAT3 (P-Ser727, Santa Cruz Biotechnology), ERK1/2 (Cell Signaling Technology) and phospho-ERK1/2 (P-Thr202/Tyr204, Cell Signaling Technology). Tumor spheres were lysed in lysis buffer consisting of 20 mM Tris-HCl (pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/ml leupetin, and 1 mM phenylmethylsulfonyl fluoride). After brief sonication, lysates were clarified by centrifugation at 12,000 x g for 10 min at 4°C, and protein content in the supernatant was measured according to the Bradford method. An aliquot (30-50μg of protein per lane) of total protein was

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separated by 7.5% SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose transfer membranes (0.2 μm; Amersham Biosciences, Buckinghamshire, UK). The membrane was blocked with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.01% Tween-20) for 1 h at room temperature, followed by incubation with the appropriate primary antibodies overnight at 4°C. After extensive washing with TBS-T, the membrane was further incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:1,000) in TBS-T containing 5% non-fat dry milk for 1 h at room temperature. Detection was performed using enhanced chemiluminescence reagent (Amersham Biosciences), according to the manufacture's protocol.

Flow cytometry-To assess the effects of the EGF and gefitinib on the population of the CD133-positive cells within tumor-spheres; $1x10^6$ cells were placed in proliferation medium containing growth factors. Different EGF and gefitinib concentrations were added each 2 days; control cells were grown in medium containing an equal concentration of DMSO. After 7 days, aliquots of CD133-positive and -negative cells were evaluated by flow cytometry with a fluorescence-activated cell sorter Aria (BD Biosciences), using anti-CD133/2 (293C3)-allophycocyanin (APC)-conjugated antibody (mouse mAb, Miltenyi Biotec) according to the manufacturer's recommendation. All experiments were performed in triplicate.

Cell colony formation assay-Overall survival of the CD133-positive cells in response to a tyrosine kinase inhibitor, gefitinib was assessed by colony formation. In colony formation assay, CD133-positive cells dissociated by a fluorescence-activated cell sorter were seeded ($5x10^4$) in 6-well dishes in medium containing 10% fetal bovine serum with or without gefitinib, and cultured for 5 days. Cell colonies were fixed and stained with 0.04% Crystal Violet in 4% paraformaldehyde solution.

Statistical analysis-Parametric comparisons used analysis of variance (ANOVA). The ANOVAs were followed by Tukey's honestly significant difference adjustment for multiple comparisons.

RESULTS

Isolation of tumorigenic CSCs from human gliomas. We analyzed fresh samples from 3 human brain tumors (2 glioblastomas: X01GB, X02GB, 1 anaplastic oligoastrocytoma: X03AOA) (12). Dissociated tumor cells, mostly in single-cell suspension, formed neuronal sphere-like aggregates within 7 days of culture. The sphere-forming cells resembled previously reported cells from tumor-derived spheres (Fig. 1A) (4-10). The spheres from 3 CSC lines continuously proliferated and all lines could be maintained for more than 100 weeks by refeeding fresh medium twice a week (Fig. 2A). X03AOA cells manifested higher proliferative capacity than X01GB and X02GB cells derived from high grade gliomas. The proliferative activity of these cells increased during the late subculture stages compared with earlier passages (data not shown). This phenomenon was previously reported by Galli et al (4). Limiting dilution analysis revealed that the number of cells required to form at least 1 tumor sphere/well was much lower X03AOA than X01GB and X02GB (Fig. 2B), indicating that X03AOA had the highest clonogenic sphere-forming capacity. X03AOA cells displayed higher proliferative and self-renewal capacity than X01GB and X02GB cells.

Cells grown in proliferation medium formed spheres. Cells in spheres were immunoreactive with the neural progenitor markers, nestin (31) and musashi-1 (12). They also expressed the

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tumor-derived stem cell surface marker CD133 (8,14). Flow cytometric analysis revealed that 1.02-12.5% of the cells in spheres from three CSC lines were positive for CD133 (12). These results collectively suggest the presence of undifferentiated stem cells in the spheres. In differentiation medium containing 10% fetal bovine serum, cells were attached to the culture dish. The outgrowth of cells and the extension of cell processes beyond the core of the spheres were observed. X01GB expressed markers of neuronal (Tuj1), glial (GFAP) and oligodendroglial (GalC) phenotypes in differentiation medium (Fig. 1B). X02GB and X03AOA also expressed markers of neuronal, glial and oligodendroglial phenotypes in differentiation medium (data not shown). To test whether tumor spheres established in culture retained the ability to form tumors after intracranial transplantation, we injected $1x10^5$ cells dissociated from tumor spheres into the neostriata of NOD-SCID mice. Transplanted spheres cells recapitulated the histopathological properties of the parental tumors (X01GB; 10/10, X02GB; 2/2, X03OA; 3/3). Typical tumor after transplantation of X01GB cells was shown in Fig. 1C. Additionally, 100-1000 CD133 positive cells were enough for tumor formations as previously described (12). Our findings indicate that small number of CD133-positive cells may have the potential for developing brain tumors. Immunohistochemical analysis (Fig. 1D) also showed that the xenograft of X01GB exhibited the same immunohistochemical profiles as the original human brain tumor; they expressed neuronal progenitor marker nestin, glial marker GFAP, and neuronal marker βIII-tubulin (Tuj1). MIB-1 (Ki-67) staining showed a high degree of proliferation. The phenotypes of X02GB and X03AOA xenografts matched well with those of the original human brain tumors (data not shown). These results indicate that our culture system allowed the isolation of clonogenic cells from the human brain tumors and that these tumors contained multipotent, long-term self-renewing, population-expanding cells that satisfy the defining criteria of CSCs (1-3).

EGF promotes sphere formation of CSCs. We investigated whether a certain growth factor is capable of controlling the self-renewal of tumor spheres in serum-free medium. Cells were first cultured in the medium containing EGF and FGF-2. Cell spheres were then subcultured onto uncoated dishes in serum-free medium containing 20 ng/ml of FGF-2, EGF, PDGF, VEGF, or HGF, if necessary, in the presence of FGF-2. In the presence of EGF alone, or EGF in combination with FGF-2, X01GB formed floating tumor spheres after 7 days (Fig. 3A). In sharp contrast, cells grown in medium containing the other growth factors failed to form spheres. Although colonies cultured in the presence of FGF-2 plus the other growth factor (PDGF, VEGF or HGF) grew significantly, the cells attached to the dishes and were most likely to be differentiated (data not shown). X02GB and X03AOA also formed floating spheres efficiently only in the presence of EGF (Fig. 3B). The sphere forming effect was observed at a concentration as low as 2.5 ng/ml EGF (Fig. 3C). The efficiency of sphere formation by EGF increased in a concentration-dependent manner up to 20 ng/ml. These findings indicate that EGF-signaling could promote sphere formation of the tumor cells irrespective of the presence or absence of FGF-2.

Inhibitory effects of tyrosine kinase inhibitors on CSCs proliferation. We next evaluated the expression of a wt-EGFR and EGFRvIII in our CSC lines. All lines were immunoreactive with wt-EGFR, as shown in the representative data of X03AOA in Fig. 4A. These spheres were also immnostained with anti-phospho-EGFR antibody, indicating the activation of EGF/EGFR signaling. EGFRvIII were detected in X01GB and X03OA, but not in

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X02GB by RT-PCR (Fig. 4B). Considering the fact that these CSC cells could not form tumor-spheres without EGF, these data suggest that sphere formations of brain CSCs are indispensably supported by EGF-EGFR signaling but not by the expression of EGFRvIII.

The strict dependence of our CSCs on EGF-signaling was further evaluated by means of tyrosine kinase inhibitors relatively selective for EGFR kinase, AG1478 (32) and the clinically used gefitinib (Iressa, ZD1839) (27,33) using modified limiting dilution assays (Fig. 5A). At 0.1 μM, AG1478 effectively inhibited sphere formation of these cell lines. Although gefitinib did not show a significant inhibitory effect at the same concentration, at 1.0 μM it almost completely suppressed the self-renewal of these spheres (Fig. 5B). Currently, we don't have sound explanation for the observed nearly 10-fold difference in effective concentration between AG1478 and gefitinib. This may be, however, due to the nearly 10-fold difference in reported IC_{50} for EGFR kinase (AG1478 0.003 μM (32) versus gefitinib 0.033 μM (33)). The effect of clinically used gefitinib was further examined in X01GB cells. Gefitinib concentration-dependently inhibited EGF-induced autophosphorylation of EGFR (Fig. 5C). At 0.01 μM, a concentration that did not inhibit EGF-induced sphere formation, the drug suppressed EGFR autophosphorylation. At 1.0 μM, EGFR autophosphorylation was completely inhibited. Intracellular EGFR downstream signaling molecules (25,26), Akt, STAT3 and ERK1/2 were activated in CSCs. Among them, phosphorylation of Akt and ERK1/2 was inhibited by gefitinib. In sharp contrast, STAT3 phosphorylation was never inhibited even at 10 μM. At 0.1 μM, the drug almost abolished ERK1/2 phosphorylation. However, at this concentration Akt phosphorylation was slightly affected. More than 1.0 μM gefitinib was required to completely cancel Akt activation. The concentration-dependent inhibitory profile of gefitinib for Akt phosphorylation corresponded well with that for EGF-induced sphere formation. A selective inhibitor of ERK pathway, PD98059 (10 μM) had no effect on EGF-induced sphere formation (Fig. 5D). LY294002 (10 μM), an inhibitor of PI3K/Akt pathway slightly but not significantly blocked sphere formation. When CSCs were treated with a combination of PD98059 and LY294002, sphere formation was significantly inhibited. These data also support the notion that EGF signaling plays a key role in CSC sphere formation and self-renewal.

Effects of EGF and gefitinib on CD133-positive cells. The effects of EGF and gefitinib on the CD133 (a marker of neural stem cells as well as CSCs)-positive sub-populations of X01GB cell line were further examined by flow cytometric analysis with a fluorescence-activated cell sorter. In the presence of EGF, the population of CD133-positive cells, indicated by green dots, increased in a concentration-dependent manner (Fig. 6A). EGF (more than 10 ng/ml) increased the number of CD133-positive cells almost 4-fold compared to that by 1 ng/ml EGF. On the other hand, the basal fraction of CD133-positive cells (6–10%) by EGF was decreased to 0-0.05% after gefitinib (1-10 μM) treatment (Fig. 6B). Furthermore, colony formations of the CD133-positive cells were suppressed by nearly 90% by the exposure to 10 μM gefitinib (Fig. 6C). In addition, CSC cells expressing cleaved caspase-3, a marker of apoptotic cell death, increased by 3–4-fold in the presence of 10 μM gefitinib (Fig. 6D).

DISCUSSION

The contribution of CSCs to several solid tumors has been established to date (1-3). CSCs with a capacity to self-renewal, undergo

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pluripotential differentiation, and form tumors after transplantation into immunodeficient mice have been isolated from human brain tumors (4,9). Therefore, the knowledge of brain CSCs is of great importance for our understanding of how brain tumors, including glioblastoma multiforme with poor prognosis, develop and expand at cellular and molecular levels. Moreover, the characterization of these cells will provide information useful for diagnostic and therapeutic purpose. Human brain CSCs reported to date were isolated using medium containing both FGF-2 (basic FGF as other name) and EGF, which was originally developed for the isolation and maintenance of neural stem cells (16-18). Lee et al. (10) have recently described that CSCs from gliomas maintain the phenotype and genotype of original human brain tumors in medium that contains both FGF-2 and EGF. However, CSCs cultured in medium containing 10% fetal calf serum, that is commonly used to maintain cancer cell lines, lose their self-renewal and differentiation capabilities. Although the possible implication of several growth factors has been proposed for the proliferation and differentiation of neural stem cells (16-21), other growth factors than FGF-2 and EGF has never been tested for the culture of brain CSCs. In order to gain further insight into brain CSCs, we examined their growth factor dependence. The data obtained in the present study indicate that among the growth factors (EGF, FGF-2, PDGF, VEGF and HGF) tested, only EGF promoted sphere formation and enhanced the self-renewal capacities of human glioma-derived CSCs, which we have established, including the CD133-positive subpopulations. The EGF signaling pathway plays an important role in gliomagenesis (34). Amplification of the EGFR gene is often associated with the formation of gliomas, and activation of EGFR promotes the growth of both astrocyte precursors and neural stem cells (34,35). EGFR gene amplification was detected in as many as 50% of high-grade astrocytomas, and EGFR activation was closely related with the transformation process in the course of brain tumor development (35). The survival of EGFR-expressing CSCs absolutely depends on the enhanced downstream signaling cascade triggered by EGF ligand-binding. Therefore, the blockage of EGF signaling by tyrosine kinase inhibitors lead to elimination of their proliferation capacity and then to apoptosis. In this study, tyrosine kinase inhibitors relatively selective for EGFR kinase (AG1478 and gefitinib) eradicated the self-renewal capacity of CSCs and the proliferation and differentiation of CD133 positive cells. Gefitinib also caused the apoptosis of CSCs *in vitro*. The EGFR gene is expected to be valuable molecular targets for therapeutic intervention (36). Taken together, EGF is essential to maintain the ability of human brain CSCs for their self-renewal and proliferation.

Many gliomas emerge either in periventricular regions or in areas contiguous with the subventricular zone, and they frequently express the progenitor-cell marker nestin (31,37,38). The hypothesis that neuronal stem cells may be susceptible to transformation is supported by observations that regions of the brain with a high proportion of proliferating cells expressing neural progenitor markers are more sensitive to oncogenic transformation than are areas with a low proportion of proliferating cells (3,39). In the subventricular zone, EGF-responsive astrocytes constitute a large population of migratory, rapidly dividing progenitor cells that give rise to neurospheres upon *in vitro* exposure to EGF (13,40,41). On the other hand, targeted inhibition of the pathways that regulate aberrant self-renewal represents an alternative approach to attacking CSCs (1,42). Effective treatment of the brain tumors might depend on the ability to develop drugs that selectively target CSCs while sparing their normal

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counterparts (42). Insight into the relationship between neural stem cells and CSCs, important for an understanding of the formation and growth of human brain tumors, may also help to identify novel tumor cell markers useful for the diagnosis and treatment of brain tumors.

Microarray analysis revealed that receptors for several growth factors (EGR, FGF-2, PDGF, VEGF, and HGF) were expressed at mRNA level in X01GB cells. Reverse-transcription-PCR analysis also revealed that receptors for several growth factors were expressed at mRNA level in all three CSCs that we have established. (Table S1 and S2 in the Supplementary data available). More carefully to say, these CSCs possess mRNA of the receptors for these growth factors. Although the role of FGF-2 in the proliferation and differentiation of neuronal stem cells in the literature (16-18), the growth factor alone seemed to stimulate the adhesion of CSCs to the culture dishes and possible differentiation in the present study. It remains unclear whether the receptor proteins of growth factors except EGF are insufficiently expressed. The data obtained in the present study indicate that growth factors except EGF, if any, mediate signals unrelated with the maintenance of CSCs.

EGF plays an important role in the maintenance of normal and malignant epithelial cells. In normal skin epithelial cells, EGFR activation drives cell cycle progression, supports migration, and affects differentiation (25,26). In cancer cells, EGF promotes cell proliferation and survival (26). At the down stream of EGFR activation, three major intracellular signaling pathways are activated (25,26). These include the Ras-Raf-ERK cascade (26,29,43,44), PI3K/Akt kinase pathway (26,29,44,45), and STAT3-dependent signaling events (25,46). The identification of signaling pathways necessary for the maintenance of CSCs phenotype may open new avenues for diagnostic and therapeutic purposes. In human brain CSCs, EGF activated ERK and PI3K/Akt pathways as shown in Fig. 5. Phosphorylation of STAT3 was also observed in these cells but was not inhibited by an EGFR tyrosine kinase inhibitor, gefitinib, suggesting constitutive phosphorylation. Similar differential inhibition of ERK, Akt and STAT3 phosphorylations was reported in normal human keratinocytes treated with an EGFR tyrosine kinase inhibitor, EKB-569 (47). ERK and PI3K/Akt pathways are involved in proliferation and cell survival (28,44,45). Their roles in the maintenance of human brain CSCs were evaluated by the use of selective inhibitors, PD98059 and LY294002. As a single agent, both inhibitors insufficiently blocked EGF-dependent sphere formation. However, a combination of both agents showed considerable inhibition of sphere formation. These results indicate that ERK and PI3K/Akt pathways are independently and closely involved in the maintenance of brain CSCs at downstream of EGFR activation.

There are some limitations in this study. First, it is possible that CSCs might adapt differently or incidentally to prevailing culture conditions; alternatively, they may reflect the intrinsic genetic fluctuations expected from tumor cells. Because we initially cultured tumor cells with EGF and FGF as described manners (4-10), these cells would be more PDGF, VEGF, HGF dependent if they were cultured in these growth factors immediately after the removal. Although we already tested their stem cell properties under several culture conditions (12), we did not reach the conclusion which factor might be importance to maintain these abilities expect of EGF. At least a part, EGF is the important factors in isolated CSCs from our patients, future analysis of other growth factors may be necessary to understand that factors involved with CSCs. Second, it is also possible that CSCs are re-programmed in culture, or they may have de-differentiated *in vitro* from a

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more differentiated cell type in response to some signal transduction cascade(s) (4,12,34). Recent papers might support the idea that stem cell characteristics could be imposed on more differentiated cell types. Okita et al. (48) developed induced pluripotent stem (iPS) cells engineered to expression of Klf4, Sox2, Oct3/4 and c-Myc. Other reports showed that CD133 negative fractions may have some tumorigenic or CSC properties (49,50). Although CD133-positive cells have stem cell properties and were enough for not only brain tumor but colon cancer formations (51), we could not completely exclude the differentiated tumor cells or CD133-negative cells might develop tumors. Future study of CD133-negative cells or mature tumor cells will be necessary to understand the tumorigenesis.

In summary, we have revealed that EGF is essential to maintain the ability of human glioma-derived CSCs for their self-renewal and proliferation. Further studies regarding the relationship between EGFR activation and other stem cell related signaling molecules (for example, Notch, Shh, Oct3/4 and Wnt) will provide useful information not only on the understanding of the brain CSC biology at molecular and cellular levels but also on the diagnostic and therapeutic approaches to brain tumors.

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FOOTNOTES

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[3]The abbreviations used are: CSC, cancer stem cell; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGFRvIII, constitutively active EGFR mutant; FGF-2, fibroblast growth factor-2; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; HGF, hepatocyte growth factor; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; GFAP, glial fibrillary acidic protein; GalC, galactocerebroside; mAb, monoclonal antibody; pAb, polyclonal antibody; HE, hematoxylin-eosin; PCR, polymerase chain reaction; DMSO, dimethylsulfoxide; TBS-T, Tris-buffered saline containing Tween-20; NOD-SCID, non-obesity diabetic-severe combined immunodeficient; APC, allophycocyanin.

FIGURE LEGENDS

FIGURE 1. **Characteristics of brain tumor stem cells.** A, Sphere formation of X01GB from a 68-year-old woman. B, Immunocytochemical analysis of the differentiated X01GB cells. They were positive for oligodendroglial (GalC), glial (GFAP) and neuronal (Tuj1) markers. C, Brain tumor of a NOD-SCID mouse xenografted with X01GB cells. D, Histological features of the original tumor (glioblastoma multiforme) and the intracranial xenograft of X01GB cells into NOD-SCID mouse. HE staining showed that the original tumor manifested the histological features of glioblastoma multiforme. The X01GB xenograft evidenced the cytoplasmic primitive intermediate filament nestin, the astrocyte marker GFAP, the neuronal marker βIII-tubulin (Tuj1), and a high proliferation index (MIB-1). The phenotype of the xenograft matched well with that of the original human tumor. Scale bars: 5 mm (C), 100 μm (D), 50 μm (A, B).

FIGURE 2. **Proliferation of cultured brain tumor stem cells.** A, Time-dependent sphere formation of three brain tumor stem cell lines, X01GB, X02GB and X03AOA. Cells were cultured in medium containing FGF-2 (20 ng/ml), EGF (20 ng/ml), and leukemia inhibitory factor (1000 U/ml). B, Limiting dilution analysis. Sphere cells were dissociated and plated in 96-well. The final cell dilutions ranged from 1 to 1000 cells/well. The percentage of wells not containing spheres for each cell plating density was calculated and plotted against the number of cells per well. Data are from representative experiments repeated at least five times.

FIGURE 3. **Growth factor dependence of established brain tumor stem cells.** A, X01GB was cultured in medium containing the indicated growth factor (20 ng/ml). Typical sphere formation obtained in X01GB at day 7. Scale bar = 100 μm. Data are from representative experiments repeated at least seven times. B, The numbers of tumor spheres formed in X01GB, X02GB, and X03AOA cultured in the presence of the indicated growth factor. The spheres of the three CSC lines maintained in proliferation medium were transferred to medium containing a single growth factor; FGF-2, EGF, PDGF, VEGF, or HGF (20 ng/ml). The results shown are means ± S.D. from three experiments. $^{*}p<0.05$ versus the control (in the absence of a growth

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factor). C, Concentration-dependent effect of EGF. The percent of wells that contained at least one tumor sphere at the indicated EGF concentration is plotted. The results shown in the graph are means ± S.D. from three experiments.

FIGURE 4. **Expression of EGFR in brain tumor stem cells.** A, X03AOA spheres were immunostained with anti-EGFR (red) or anti-phospho-EGFR (green) antibodies. Scale bars = 50 μm. B, Expression of wt-EGFR and EGFRvIII were analyzed by reverse transcription-PCR. Data are from representative experiments repeated at least three times.

FIGURE 5. **Inhibition of EGF-dependent sphere formation by tyrosine kinase inhibitors relatively selective for EGFR and inhibitors for ERK and PI3K/Akt pathways.** CSCs were essentially cultured in the medium containing 20 ng/ml EGF. A, Concentration-dependent inhibition of sphere formation by AG1478 and gefitinib. Sphere-forming capacity was determined by the modified limiting dilution assay in the absence or presence of various concentrations of AG1478 or gefitinib. The results shown are means from three independent experiments. *$p<0.05$ versus the control (in the absence of an inhibitor). B, X03AOA spheres in the presence of 0.1 or 1.0 μM of gefitinib. Scale bars = 100 μm. C, Concentration-dependent effects of gefitinib on EGFR, Akt, STAT3 and ERK1/2 phosphorylation. X01GB CSCs were incubated with the indicated concentrations of gefitinib for 3 h. Phospho-EGFR, -Akt, -STAT3 and -ERK1/2 protein levels were analyzed by Western blotting. As loading controls, the levels of EGFR, Akt, STAT3 and ERK1/2 are also shown. D, Inhibition of sphere formation by PD98059 and/or LY294002. X01GB CSCs were cultured in the absence or presence of PD98059 (10 μM) and/or LY294002 (10 μM) and the number of formed spheres was counted. The results shown are means from three independent experiments. *$p<0.05$ versus the control (in the absence of an inhibitor). B,C, Data are from representative experiments repeated at least three times.

FIGURE 6. **EGF-dependent increase of the CD133-positive population in brain tumor stem cells.** A, Changes of CD133-positive cells (green dots) in response to EGF (1 – 80 ng/ml) by flow cytometric analysis. Data are from representative experiments repeated at least three times. The results shown in the graph are means ± S.D. from three experiments. *$p<0.05$ versus the control (in the absence of EGF). B, Inhibition of EGF-dependent increase of CD133-positive cells by gefitinib. Data are from representative experiments repeated at least three times. The results shown in the graph are means ± S.D. from three experiments. *$p<0.05$ versus the control (in the absence of gefitinib). C, Inhibition of colony formation of CD133-positive cells by gefitinib. CD133-positive cells were culture for 5 days in the presence or absence of 10 μM gefitinib. D, Increase in cleaved caspase-3-positive cells after gefitinib treatment. CSC cells cultured in medium containing 10% fetal bovine serum were incubated with 10 μM gefitinib for 0 and 48 hrs. Scale bar = 50 μm. C,D, Data are from representative experiments repeated at least three times.

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Figure 1

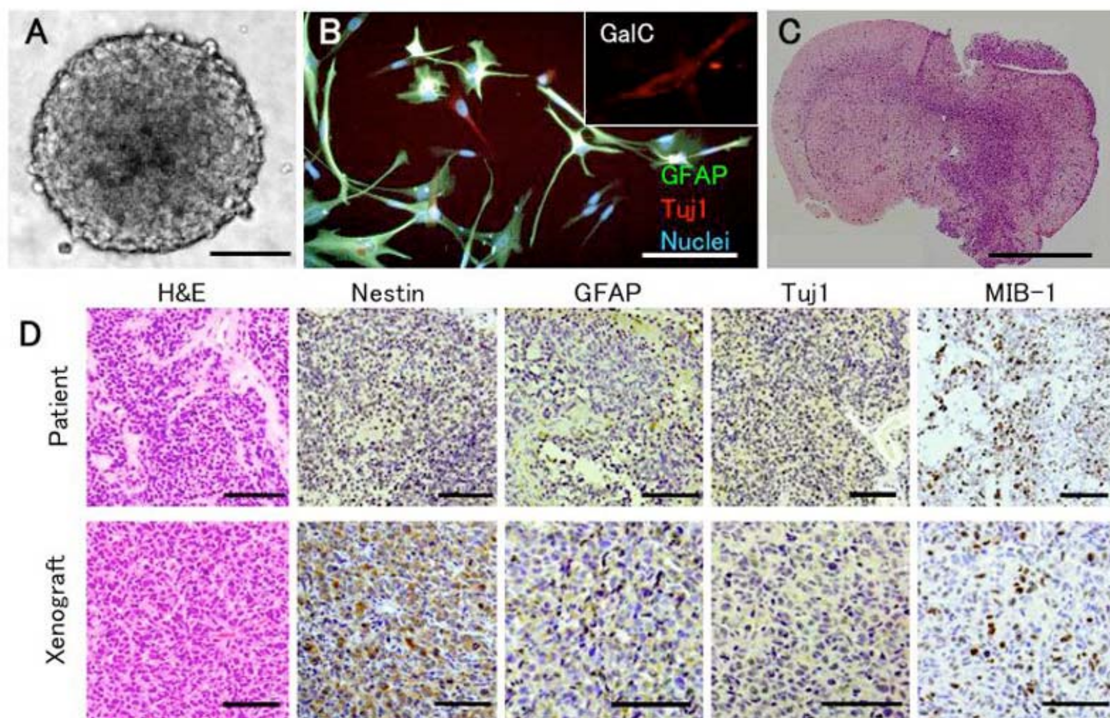

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Figure 2

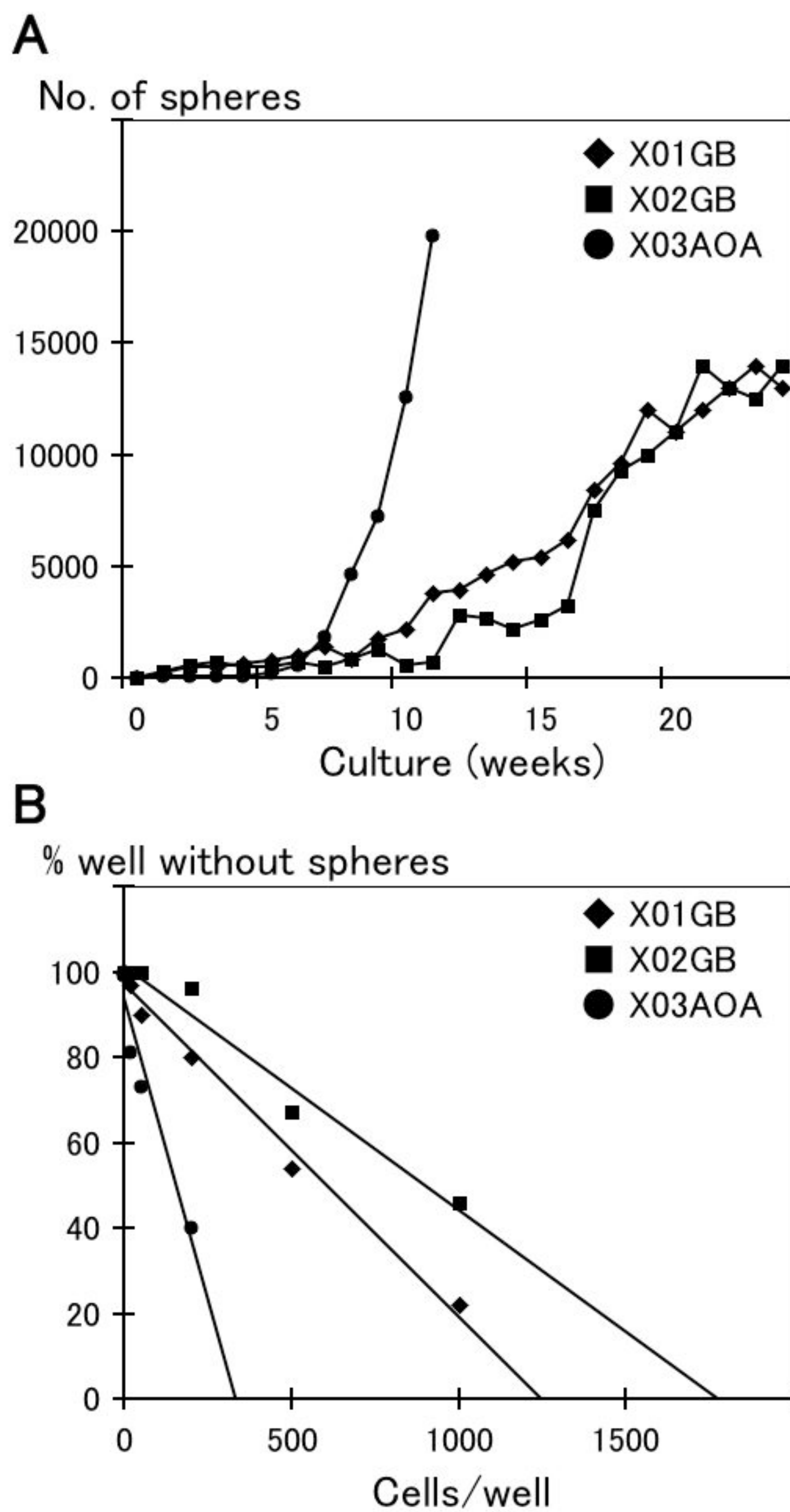

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Figure 3

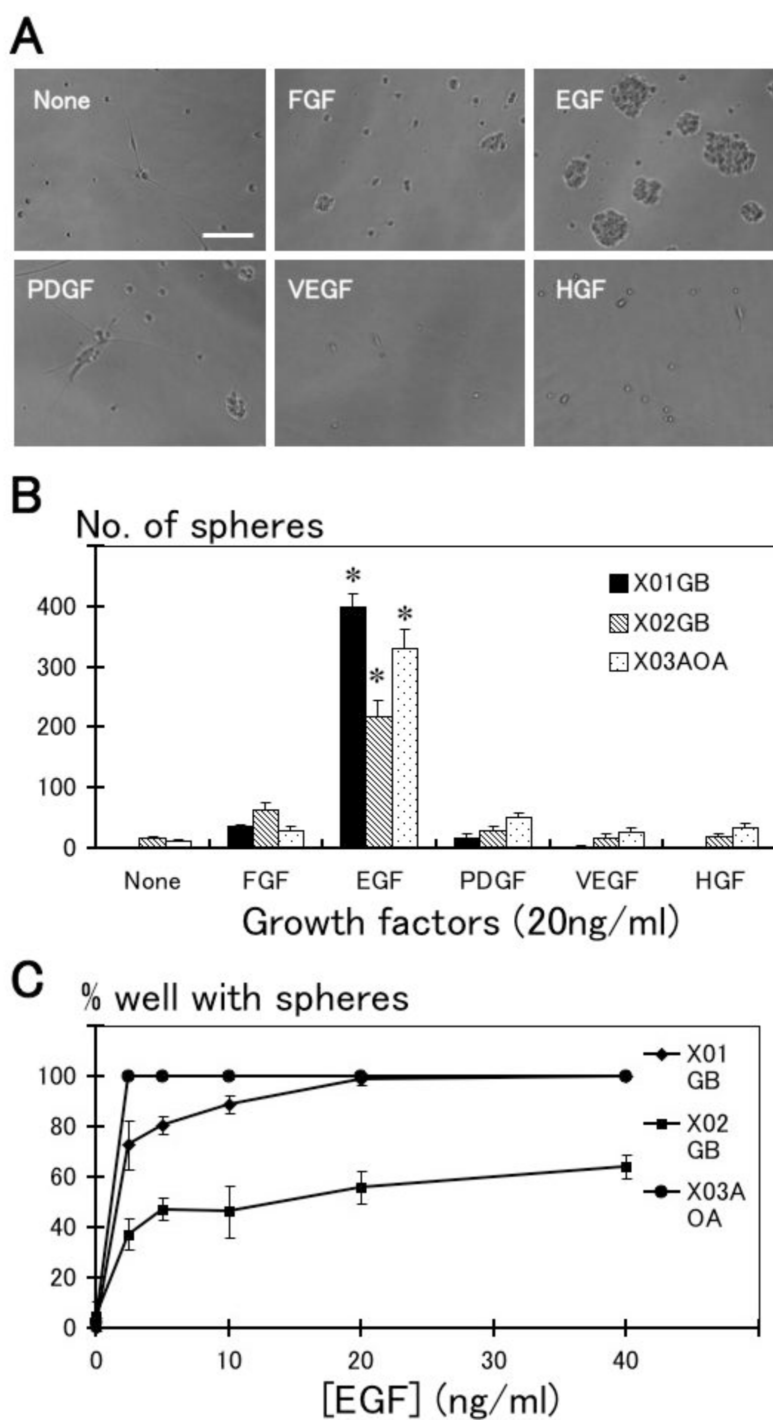

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Figure 4

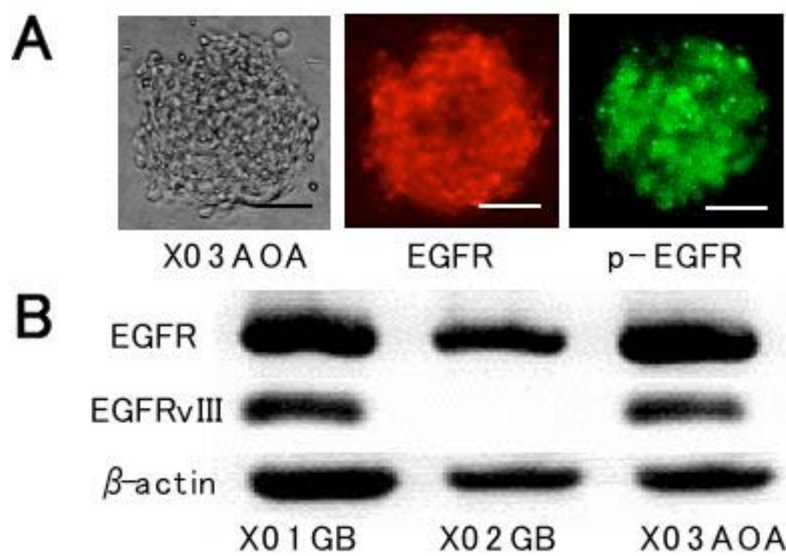

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Figure 5

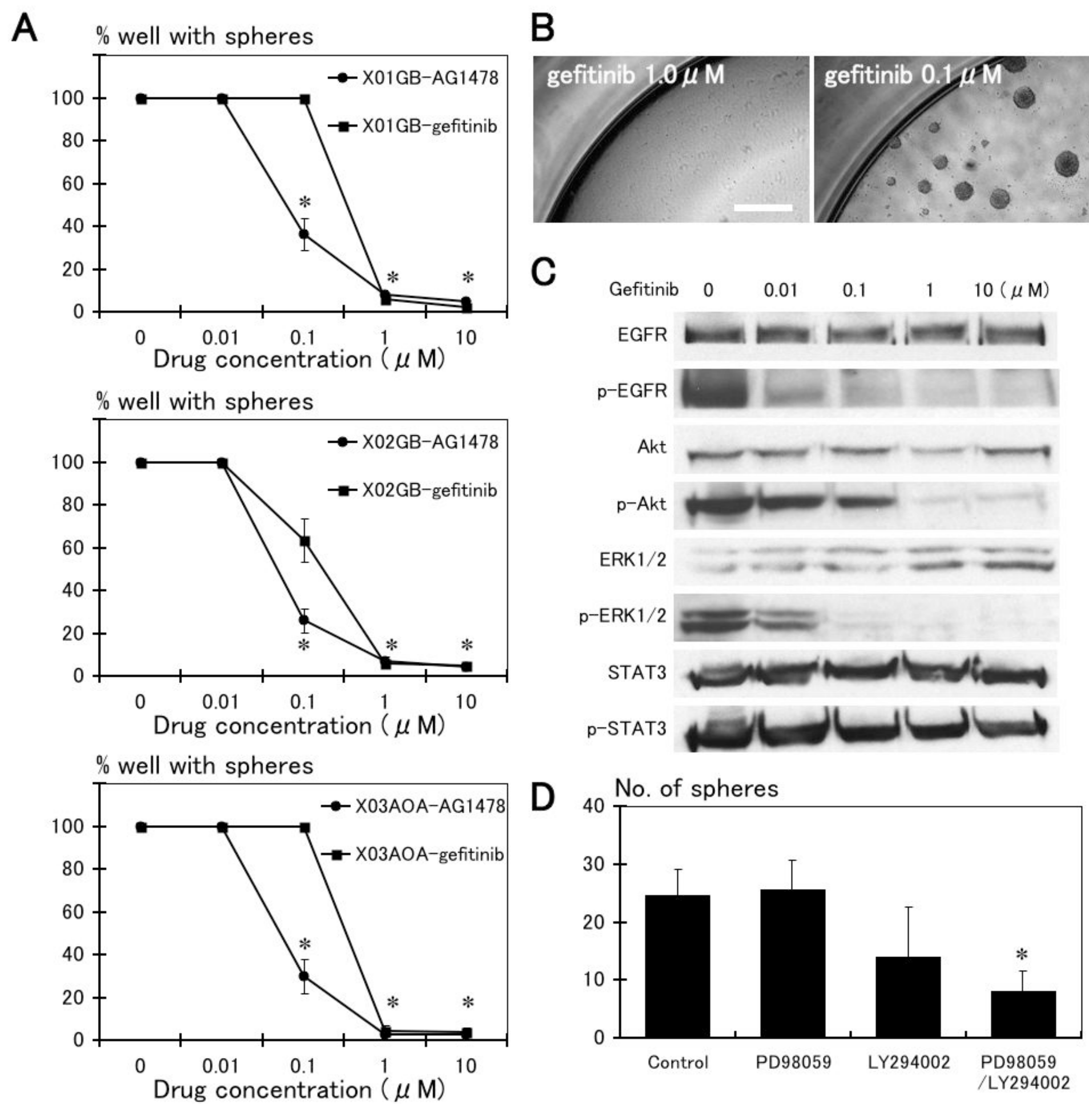

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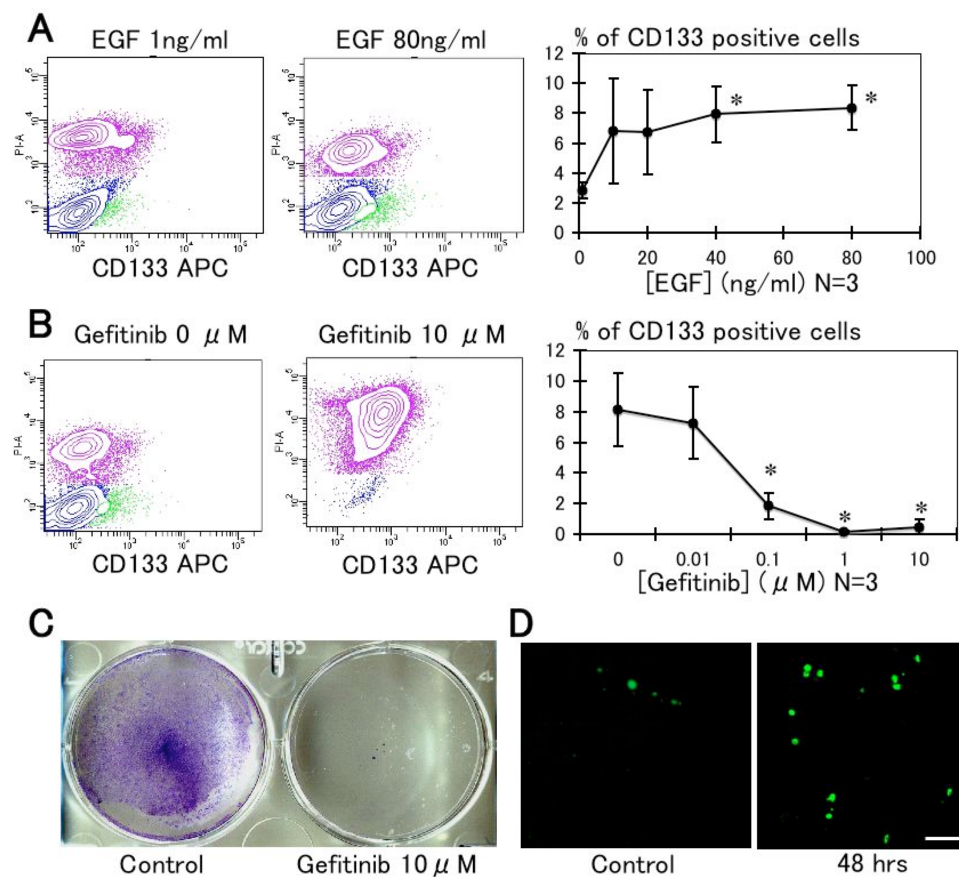
Figure 6



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