Supporting Material & Methods

Immunocytochemistry (ICC)

One primary culture from a human GBM biopsy and U251 glioma cells were grown on cover slips to approximately 70-80 % confluence. Prior to fixation, 10µM BrdU was added to the media of the U251 glioma cells that were further incubated for 45 minutes. Cells were fixed with 4 % paraformaldehyde for 10 minutes and permeabilized in 0.5 % Triton X-100 in PBS for 4 minutes, both at room temperature. Blocking was done using 0.5 % BSA in PBS for 15 minutes at room temperature. Anti-BrdU (RPN202, Amersham, GE Healthcare, Uppsala, Sweden) working solution was diluted 1:2 in blocking buffer, and cells were incubated for 45 minutes at 37 ^{o}C. Following washing with PBS, primary antibodies Nestin (AB5922, Chemicon, Billerica, MA), GFAP (AB5804, Chemicon) and NSE (SC-132009, Santa Cruz Biotechnology Inc, Santa Cruz, CA) were diluted 1:500, 1:200 and 1:200 respectively in blocking buffer, and cells were incubated for 45 minutes at 37 ^{o}C. Cells were washed in PBS and incubated with secondary antibodies (diluted 1:100 in blocking buffer) for 45 minutes at 37 ^{o}C. The secondary antibodies used were goat anti-mouse IgG_{2a} conjugated to FITC (Southern Biotechnologies Associates Inc., Birmingham, AL), goat anti-rabbit conjugated to TexasRed (Southern Biotechnologies Associates Inc) or rabbit anti-goat conjugated to TRITC (Invitrogen, Paisley, UK). After washing with PBS cells were mounted with Vectashield mounting medium with Dapi (Vector Laboratories, Burlingame, CA). Fluorescent images were obtained with a Zeiss LSM 510 Meta confocal microscope, using a 63x oil immersion objective. Images were obtained through several optical slices (Z-stack) throughout the cells to prevent loss of information.

Flow cytometry (FCM) and cell sorting

Cell cycle analyses

Sub-confluent cells were harvested by trypsination and fixed by drop wise addition of 5ml ice-cold 100 % EtOH, while vortexing. The samples were incubated in 0.5 % Pepsin, pH 1.5 at 37 ºC for 15 min and the isolated nuclei were washed once in PBS. The pellet was resuspended in 100µl 1mg/ml RNase and 500 µg/ ml propidium iodode, and incubated for 30 min at room temperature prior to data acquisition with CellQuest in pulse processing mode on a FACS Calibur (BD Biosciences). DNA analysis was performed using ModFit *LT* Flow Cytometry Modeling Software (Verity Software House, Topsham, ME).

FACS

Cells were stained by PE-conjugated CD133 according according to the protocol used for FCM, and CD133 positive cells were removed by cell sorting. Lentivirally transduced cells harboring high eGFP expression were sorted using a FACS Aria Special Order Cell Sorting Instrument (Beckton Dickinson, Franklin Lakes, NJ) on the basis of single cell viability and eGFP expression. The FACS experiments were performed at the Molecular Imaging Center (Fuge, Norwegian Research Council), University of Bergen.

Animal experiments

The experiments were conducted with male and female homozygous SCID mice bred and maintained in an isolation facility in a pathogen free environment on a standard 12/ 12 h day and night cycle. In total, 53 SCID mice were implanted with glioma cells intracerebrally. For comparison of tumorgenicity of CD133 positive and negative cells we implanted 80.000 cells in each animal. For the implantation of the various CD133 negative subpopulations isolated from the U373 glioma cell line and the patient biopsy, we implanted 10.000 cells in each animal. Animals were fed a standard sterilized pellet diet and provided sterile tap water *ad libitum*. The animals were anaesthetized with Hypnorm-Dormicum (0.4 ml/ kg) s.c., the head

secured in a stereotactic frame (Benchmark; Neurolab, St Louis, MO) and a short longitudinal incision was made in the scalp exposing the calvarium. A burr-hole was made 0.5 mm posterior to the bregma and 1 mm right lateral to the sagittal suture with a micro-drill with a bit diameter of 2,9 mm. A Hamilton syringe was introduced to a depth of 1.5 mm below the brain surface, and the spheroids were slowly injected and the syringe left in place for 3 min before withdrawal. The skin was closed with an Ethilon 3-0 suture. Animals were sacrificed at the onset of symptoms using CO_2 and the brains were removed. Brains were immersion fixed in 4 % formaldehyde in Dulbecco's Phosphate-Buffered Saline for 24 h and then embedded in paraffin. 5 µm sections were prepared and every 20th section was collected for further histological analysis. These sections were stained with haematoxylin and eosin, and examined under a light microscope.

Statistics

The log rank test was used for testing significance survival data analysed with the Kaplan-Meier test. Tumor take rates expressed as proportions were compared using the Fisher's exact test. A p-value of <0.05 was considered significant.

Supporting figure legend

Supporting Figure 1. U251 glioma cell subpopulations proliferate in vitro. *A) Co-staining for BrdU (green) and the respective cell type markers (red) in the U251 glioma cell line. DAPI counterstaining (blue). Scale bars = 50 µm. B) Bar graph presentation of the fraction of BrdU positive cells in the different subgroups of the U251 glioma cell line.*

Supporting Figure 2. CD133$^-$ and CD133$^+$ are equally tumorigenic in vivo. *Survival curves for scid mice xenografted intracerebrally with CD133$^-$ (n=5) and CD133$^+$ (n=4) glioma cells.*