

CD133 Is Not Present on Neurogenic Astrocytes in the Adult Subventricular Zone, but on Embryonic Neural Stem Cells, Ependymal Cells, and Glioblastoma Cells

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Abstract

Human brain tumor stem cells have been enriched using antibodies against the surface protein CD133. An antibody recognizing CD133 also served to isolate normal neural stem cells from fetal human brain, suggesting a possible lineage relationship between normal neural and brain tumor stem cells. Whether CD133-positive brain tumor stem cells can be derived from CD133-positive neural stem or progenitor cells still requires direct experimental evidence, and an important step toward such investigations is the identification and characterization of normal CD133-presenting cells in neurogenic regions of the embryonic and adult brain. Here, we present evidence that CD133 is a marker for embryonic neural stem cells, an intermediate radial glial/ependymal cell type in the early postnatal stage, and for ependymal cells in the adult brain, but not for neurogenic astrocytes in the adult subventricular zone. Our findings suggest two principal possibilities for the origin of brain tumor stem cells: a derivation from CD133-expressing cells, which are normally not present in the adult brain (embryonic neural stem cells and an early postnatal intermediate radial glial/ependymal cell type), or from CD133-positive ependymal cells in the adult brain, which are, however, generally regarded as postmitotic. Alternatively, brain tumor stem cells could be derived from proliferative but CD133-negative neurogenic astrocytes in the adult brain. In the latter case, brain tumor development would involve the production of CD133. [Cancer Res 2007;67(12):5727–36]

Introduction

CD133 has emerged as an important surface marker in stem cell research. The protein was originally described as “Prominin” in murine embryonic neural stem cells (later termed Prominin-1; refs. 1, 2) and as an antigen on human fetal and adult hematopoietic stem and progenitor cells (3). Its role remains unclear, although mutations in the human *PROMININ-1* gene can cause retinal degeneration, indicating a function for photoreceptor cells (4). An involvement in murine neuroepithelial cell division has also been suggested (5).

CD133 antibodies were used to isolate fetal and adult human hematopoietic stem and progenitor cells (6), human neural stem

cells from fetal brain (7, 8), and human endothelial precursor cells (9). Recently, CD133 has gained increased attention as a surface marker to isolate a subpopulation of cells from glioblastoma, medulloblastoma, and ependymoma, which initiates and sustains tumor growth (also called cancer or tumor stem cells; refs. 10–13). CD133 antibodies furthermore allowed the enrichment of tumor stem cells from prostate and colon cancer (14–16).

Brain tumor stem cells might be derived from normal neural stem or progenitor cells, in particular those present in the major neurogenic region of the adult mammalian brain, the subventricular zone at the lateral ventricle wall (LVW; refs. 17, 18). This idea is supported by phenotypic and functional similarities (17–20), the development of brain tumors upon gene transfer to immature, Nestin-positive cells (21), and the occurrence of premalignant or malignant lesions at or close to the postnatal subventricular zone (17, 18, 22, 23). Moreover, the presence of CD133 on fetal neural stem cells and the enrichment of brain tumor stem cells in the CD133-positive fraction has led investigators to postulate a stem cell origin of brain tumor stem cells, and in this context CD133 is called a “neural stem cell marker” based on ref. 7. However, such considerations do not take into account the difference in ontogeny between normal neural stem cells from fetal brain and stem cells in tumors, which arise late in adulthood. CD133 antibodies had been used to isolate neural stem cells exclusively from human fetal brain, but not from later developmental stages (7, 8, 24). Likewise, a CD133 antibody stained murine neural stem cells in very early (E11.5 and E12) embryos (1, 25). Lee et al. (26) reported the isolation of a CD133-positive cell population with neural stem cell properties from postnatal murine cerebellum. Whether it persists in the adult cerebellum has not been reported thus far.

If CD133 protein production indicates a lineage relationship between brain tumor stem cells and a normal brain cell, the exact identity of the latter needs to be established. We studied the localization of CD133 in mouse brain of different developmental stages, as well as in the human adult LVW. Furthermore, we isolated CD133-positive and CD133-negative cells, investigated them morphologically, and tested their proliferative (neurosphere-forming) and differentiation capacity. Our study revealed that CD133 is present on neural stem cells in the embryonic brain, on an intermediate radial glial/ependymal cell type in the early postnatal stage, and on ependymal cells in the adult brain. Based on these results and previously published findings, we suggest two different scenarios for the origin of brain tumor stem cells.

Materials and Methods

Human tissue. A postmortem LVW specimen was obtained from a patient (36 years) without brain pathology and fixed with formaldehyde.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Glioblastoma samples (WHO grade 4) from three patients were obtained during surgery and were snap frozen. Collection of specimens was in accordance with the regional ethical review board in Lund.

Animals. Embryonic forebrain, postnatal day 4 (P4), and adult brain tissue were obtained from wild-type (wt) C57Bl/6 mice (Jackson Laboratory). In addition, transgenic C57Bl/6 mice, expressing green fluorescent protein (GFP) under a chicken β -actin promoter, were used. All procedures were done with consent from the ethical committee at Lund University.

Cell isolation and culture. Whole forebrain (E9.5, E14.5) or P4 and adult LVW tissue was dissected from 5 to 10 mice per experiment. For magnetic affinity cell sorting (MACS), E9.5, E14.5, and P4 tissue was digested with TrypLE Express (TLE; Invitrogen) for 10 min at 37°C, triturated, and filtrated (cell strainer 40 μ m; BD Biosciences). For fluorescence-activated cell sorting (FACS), tissue digestion was done with Trypsin/EDTA (0.05%, Invitrogen) for 20 min at 37°C and stopped with DMEM/F12 (Invitrogen), containing 10% FCS (Biochrom). Adult LVW tissue was treated with trypsin/EDTA.

After sorting, cells were either attached onto coverslips by centrifugation or identical cell numbers of the CD133-positive and CD133-negative fraction were plated out at 100,000 (FACS) or 100,000 to 300,000 cells (MACS) per milliliter in neurosphere medium, consisting of DMEM/F12 (1:1) with Glutamax, B27, HEPES (10 mmol/L), insulin (20 μ g/mL), penicillin (100 units/mL), streptomycin (100 μ g/mL; all from Invitrogen), partricine (Biochrom), recombinant human epidermal growth factor (EGF; 20 ng/mL), and rhGFbasic (20 ng/mL; PAN Biotech), and kept at 37°C and 5% CO₂. Neurospheres were counted after 4 to 7 days.

For the cocultivation of cells from adult GFP-expressing and wt mice, a larger tissue subsection including the LVW was dissected and digested with TLE. Fifteen thousand MACS-isolated cells per milliliter were plated out.

For further cultivation, spheres were dissociated after 7 to 10 days by incubation with TLE and replated at 100,000/mL. For differentiation experiments, spheres were plated onto poly-L-lysine-coated coverslips (Sigma-Aldrich) in neurosphere medium, without EGF and FGF, but with NT4 (20 ng/mL; Promega) or 1% FCS, and kept for 3 days.

Pictures of cultured cells were taken with an Olympus IX70 microscope and a Nikon Coolpix 4500 camera.

Immunofluorescence. Cells were fixed with 4% formaldehyde/PBS (10 min, room temperature). Mouse brains were either fixed in 4% formaldehyde/PBS and 25% sucrose/PBS or, for stainings with platelet-derived growth factor receptor α (PDGFR α) antibody, frozen in 2-methylbutane, and postfixed with ice-cold methanol (5 min) and acetone (30 s) after cryosectioning. Glioblastoma tissue was postfixed in 4% formaldehyde/PBS after sectioning. Except for human LVW, tissue cryosections (10–12 μ m) and cells on coverslips were stained as described previously (27). Human LVW cryosections were blocked with 10% donkey serum in 0.1% Triton X-100 for 1 h at room temperature, and primary antibodies were applied in 0.1% Triton X-100 with 10% donkey serum for 24 h at 4°C and secondary antibodies in PBS for 24 h at 4°C. Antibodies against CD133 (1:100, clone 13A4, recognizing mouse CD133, eBioscience), CD133/2 (1:100, clone 293C3, recognizing human CD133, Miltenyi Biotec), CD146 (1:100, Chemicon), glial fibrillary acidic protein (GFAP; 1:800, DakoCytomation), GFAP (1:500, Chemicon), GLAST (1:300, Abcam), Nestin (1:200, BD Bioscience), O4 (1:100, Chemicon), PDGFR α (1:100, Santa Cruz Biotechnology), S100 (1:200, DakoCytomation), SOX2 (1:100, Santa Cruz Biotechnology), tubulin- β -III (1:100, Chemicon), and tubulin- β -IV (1:100, Abcam) were used. PBS served as negative control. Immunofluorescence was documented with an Axiovert 200M microscope using AxioVision 4.5 software (Zeiss).

Magnetic and fluorescence-activated cell sorting. Isolated cells were incubated with anti-CD133-phycoerythrin (1:100, clone 13A4, eBioscience) for 30 min on ice, then with anti-phycoerythrin magnetic microbeads (Miltenyi Biotec), and applied to a LS/MS column (Miltenyi Biotec) according to the manufacturer's protocol. Effluent was collected as negative, magnetically labeled cells as positive fraction. To increase the purity, both fractions were passed separately over new columns. Aliquots of CD133-positive and CD133-negative fractions were evaluated by a FACSCalibur flow cytometer (BD Bioscience).

For flow cytometry, CD133-phycoerythrin-labeled cells were washed thrice and 7-amino-actinomycin D (eBioscience) was added. Rat IgG1-phycoerythrin (1:100, eBioscience) served as isotype control. Cells were sorted with a FACSDiva (BD Bioscience) excluding doublets and dead cells. Data analysis was done using FlowJo software (Tree Star).

Live cell microscopy. CD133-positive sorted cells were monitored at 37°C and 5% CO₂ with a POC chamber (Zeiss) immediately after sorting. Differential interference contrast microscopy images were taken for up to 30 s and compiled using AxioVision (Zeiss).

Western blot analysis. Cells or tissue were dissolved in lysis buffer [25 mmol/L HEPES (pH 7.9), 300 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1% Triton X-100, 0.1 mmol/L DTT] supplemented with Complete protease inhibitors (Roche) and separated on a 10% SDS-polyacrylamide gel. Western blotting followed standard protocols with anti-CD133 antibody (1:500, clone 13A4, Chemicon) and anti-HSP105 antibody (1:200, Santa Cruz Biotechnology).

Results

CD133 is expressed by neuroepithelial and radial glial cells in the embryonic mouse brain. The first neural stem cells appearing in the developing mammalian central nervous system are neuroepithelial cells in the ventricular zone, and CD133 is localized on the apical surface of these cells in mouse brain as shown previously (1, 25). In the mouse midbrain and forebrain, neuroepithelial cells are starting to be replaced by radial glial cells (RGC) at E10.5 (28). We found CD133 colocalized with the RGC markers GLAST and Nestin on ventricular zone cells in E14.5 mouse brain (Fig. 1A). CD133 staining was very faint to absent at the ventral LVW lining the striatum. The heterogenous distribution of CD133 could be explained by regional developmental differences, as reported for other RGC markers (28). We conclude that in addition to neuroepithelial cells, certain RGCs express *CD133*.

CD133 localization in the LVW of the early postnatal mouse brain. RGCs disappear from the murine brain during the first 14 postnatal days, but a proliferative and neurogenic subventricular zone persists in adulthood at the LVW. According to refs. 29 and 30, a lineage relationship between RGCs and cell types in the adult brain exists. RGCs give rise to mature astrocytes, but also to ependymal and type B cells (neurogenic astrocytes) in the adult subventricular zone. The identity of the neural stem cell at the LVW has been a matter of debate. Ependymal cells were earlier proposed as neural stem cells (31), but strong evidence has later been provided for type B cells to represent neural stem cells in this region (30).

We investigated which cells express *CD133* in the murine LVW at P4, during the phase of RGC disappearance, by immunostaining of brain sections. CD133 antibodies were combined with antibodies against ependymal cell proteins [tubulin- β -IV, a marker for ciliated cells (32), and S100 (30)] and against the RGC marker GLAST. In addition, a SOX2 antibody was applied. This transcription factor is expressed by embryonic neural stem cells in the ventricular zone, mature ependymal cells, and the vast majority of dividing cells in the adult subventricular zone (33).

At P4, CD133 was enriched at the apical surface of GLAST-positive cells at the ventral wall, whereas almost all GLAST-positive cells at the dorsal wall were CD133 negative (Fig. 1B). GLAST-positive cells were all GFAP negative at this stage (not shown). Interestingly, the CD133 staining pattern of some cells resembled cilia and we found a coexpression with tubulin- β -IV (Fig. 1B). CD133-positive cells at the ventral wall also synthesized S100 and SOX2. In conclusion, there are two types of GLAST-positive cells, which are distinguished by CD133. At the ventral side, the majority of them are CD133-positive cells expressing *tubulin- β -IV*, *S100*, and

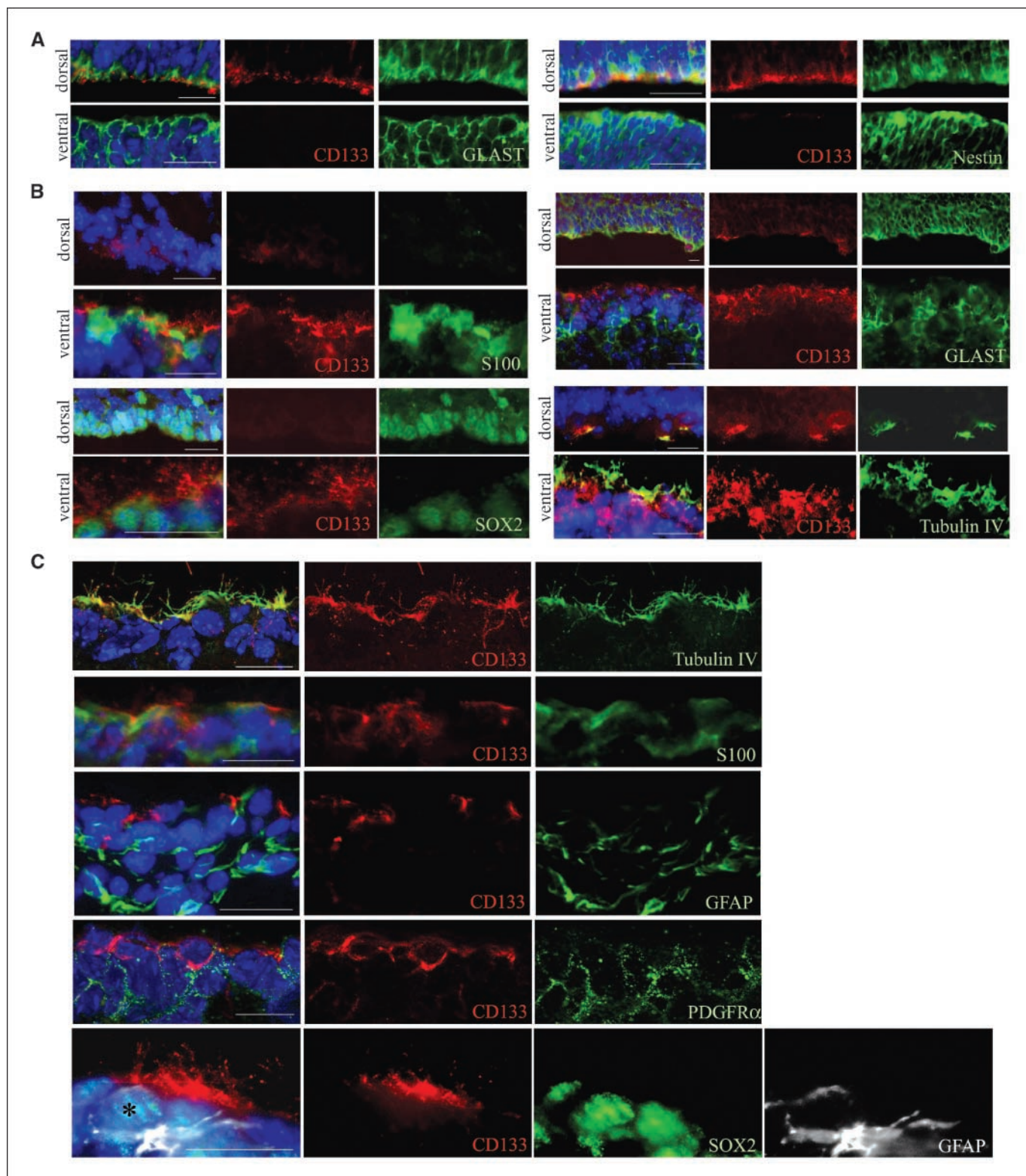


Figure 1. Immunolocalization of CD133 at the embryonic, early postnatal, and adult mouse lateral ventricle. Sagittal sections through the LVW of E14.5 (A), P4 (B), and adult (C) mouse brain. Images of the dorsal (top images) and ventral ventricular wall (bottom images) are depicted in A and B with the ventricular surfacing facing down (top images) or up (bottom images). Nuclei are shown in blue [4',6-diamidino-2-phenylindole (DAPI) staining]. Bar, 20 μ m. A, CD133 (red) localization at the ventricular surface of the dorsal wall. CD133-positive cells are also stained by GLAST (green, left images) and Nestin (green, right images). CD133 staining was faint to absent at a region of the ventral wall lining the striatum. B, CD133 (red) is present at the ventral side (bottom images) of the P4 LVW. Very few CD133-positive cells are found at the dorsal side (top images). The dorsal ventricle wall still consists of a thicker cell layer than the ventral wall at this stage. Note that CD133-positive cells are also synthesizing S100, GLAST, SOX2, and tubulin- β -IV (green). C, top two panels, CD133 (red signals) colocalizes with tubulin- β -IV and S100 (green) at the surface of the adult LVW. Two middle panels, the vast majority of cells synthesizing GFAP and PDGFR α (green) do not present CD133 (red). Lowest panel, a single cell (asterisk) positive for CD133 (red), SOX2 (green), and GFAP (white).

SOX2, thus representing an intermediate radial glial/ependymal cell type. At the dorsal side, almost all GLAST-positive cells are CD133 negative and these cells are furthermore characterized by *SOX2* expression, but an absence of tubulin- β -IV and S100. The expression pattern of these cells is compatible with RGCs, which are CD133-negative at this localization and developmental stage. The different ventral/dorsal distribution of CD133 resembles spatiotemporal differences in ependymal cell maturation described earlier (30).

CD133 is present on ependymal cells, but not on the majority of type B neurogenic astrocytes in the adult LVW. In the adult LVW, the CD133 antibody stained cells, which were also positive for tubulin- β -IV and S100 (Fig. 1C). Costainings with antibodies against proteins characteristic for type B subventricular zone astrocytes (GFAP, PDGFR α ; refs. 23, 34) revealed that almost all GFAP-positive and PDGFR α -positive cells were CD133 negative, indicating that the CD133 epitope is not present on the majority of type B cells in the adult mouse subventricular zone. Some *CD133*-expressing cells were also recognized by the *SOX2* antibody and triple stainings revealed that 1.6% of the CD133-positive cells ($n = 182$) at the ventricular surface also expressed *SOX2* and *GFAP*. A small population of type B cells has been described, which directly contacts the ventricle and carries a single cilium (35). These few triple-positive cells could therefore be superficially located B cells, which present CD133 on their surface. An alternative cell type would be tanyocytes, rare cells at the adult lateral ventricle surface, which are unciliated and contain microvilli (36). Unfortunately, sufficient markers for these cells are lacking. No CD133 signals were detected in the hippocampal dentate gyrus and only very faint vessel-like structures in the adult cerebellum (not shown).

Because the anatomy of the adult human subventricular zone differs from the one in mouse (37), and surface markers are not always shared between species, we also investigated the CD133 localization in the adult human LVW (intermediate zone of the anterior horn). The CD133 epitope was detected at the surface of cells lining the LVW (Fig. 2A). Cells at the same localization were also positive for tubulin- β -IV, but negative for GFAP, indicating that as in the adult mouse brain, human ependymal cells present CD133.

CD133 localization in human glioblastoma. To test whether the CD133 epitope found on adult human ependymal cells is also present on putative tumor stem cells in human glioblastomas, the same antibody (CD133/2) was applied to stain human glioblastoma samples. We found CD133 localized at the surface of tumor cells (Fig. 2B and C), consistent with ref. 38. The majority of CD133-positive cells were weakly stained, but some cell clusters showed higher signals, indicating the presence of *CD133* high and *CD133* low expressing cells. To further characterize *CD133*-expressing tumor cells and to rule out CD133-positive endothelial progenitors, stainings with antibodies against GFAP, PDGFR α , and CD146 were done. No coexpression of *CD133* and *PDGFR α* could be detected, and PDGFR α was only found on CD146-positive blood vessel cells. A subpopulation of the *CD133*-high expressing cells showed *SOX2* staining, and some of these cells also synthesized GFAP (Fig. 2B and C). From these results, we conclude that adult human ependymal cells and glioblastoma cells share CD133 epitopes.

Cells with long-term neurosphere-forming capacity and multilineage potential are enriched in the CD133-positive fraction from mouse embryonic brain and P4 LVW, but in the CD133-negative fraction from the adult LVW. CD133-positive and CD133-negative cells were isolated from brains of different

mouse developmental stages and adult animals. The number of formed neurospheres per total number of plated cells was determined and we refer to it as neurosphere frequency (Fig. 3A). The MACS-isolated CD133-positive cells from E9.5 brain showed an average neurosphere frequency of 1 in 3,268 cells, whereas no neurosphere formation was observed for the CD133-negative fraction. CD133-positive derived neurospheres gave rise to astrocytes and neuronal cells (Fig. 4A). In case of E14.5 brain tissue, neurospheres were formed in both the CD133-positive (average frequency 1 of 323) and CD133-negative cell fractions (average frequency 1 of 809). Similarly, high neurosphere numbers were found for CD133-positive and CD133-negative cells isolated from P4 LVW tissue (Fig. 3A). These results with MACS-isolated cells from E14.5 and P4 were each confirmed by one FACS experiment (Fig. 3A). E14.5 and P4 neurospheres of both fractions could be kept in culture for more than eight passages and differentiated into glial and neuronal cells (Fig. 4B and C). In contrast, neurosphere frequencies of MACS-isolated adult LVW cells were higher in the CD133-negative (1 of 121) compared with the CD133-positive (1 of 1,389) fraction. Because adult brain preparations contained a high amount of debris and subcellular particles, to which CD133 antibodies bound unspecifically, we further concentrated on FACS isolations for adult LVW cells (Fig. 3B and C). Three independent FACS experiments confirmed the large difference in neurosphere frequency between CD133-positive and CD133-negative cells seen with MACS, with even lower neurosphere frequency in the CD133-positive (1 of 8,850) compared with the CD133-negative fraction (1 of 192; Fig. 3A). Neurospheres of the CD133-negative fraction gave rise to all three neural lineages (Fig. 4D), even at passage 6. In contrast, neurospheres of the CD133-positive sorted fraction only gave rise to astrocytes and the majority of spheres were very small. These spheres could either represent a contaminating CD133-negative progenitor cell fraction or they could be derived from CD133-positive ependymal cells, as cells from a separated ependymal layer of the adult mouse brain have been shown to generate spheres with very similar characteristics (39). Because CD133 surface protein is lost on normal cells cultured as neurospheres outside their normal tissue context (not shown), we cannot distinguish these two possibilities. Importantly, the CD133-negative-derived spheres could be kept for more than nine passages, whereas the CD133-positive-derived cells only generated secondary spheres, which could not be passaged further.

In conclusion, under these culture conditions, cells with long-term neurosphere-forming capacity and multilineage potential are enriched in the CD133-positive fraction from mouse embryonic brain and P4 LVW. However, in the adult LVW, long-term neurosphere formation and cells with multilineage potential are found in the CD133-negative cell fraction.

Detection of CD133 protein in positive and negative sorted cell fractions. To exclude the possibility that the CD133 epitope is changed or removed during the tissue digestion and cell isolation, we did a Western blot analysis with CD133-positive and CD133-negative MACS-isolated cells obtained from E14.5 brain (Fig. 5). This stage was chosen because we obtained high cell numbers in both fractions. Bands of 105 to 128 kDa were detected in the positive fraction, in accordance with ref. 1. Thus, the CD133 epitope is still present and the MACS isolation enriched CD133-positive cells. A faint signal was seen in the CD133-negative fraction, indicating a small amount of contaminating CD133-positive cells, which can be expected in case of MACS. The purity

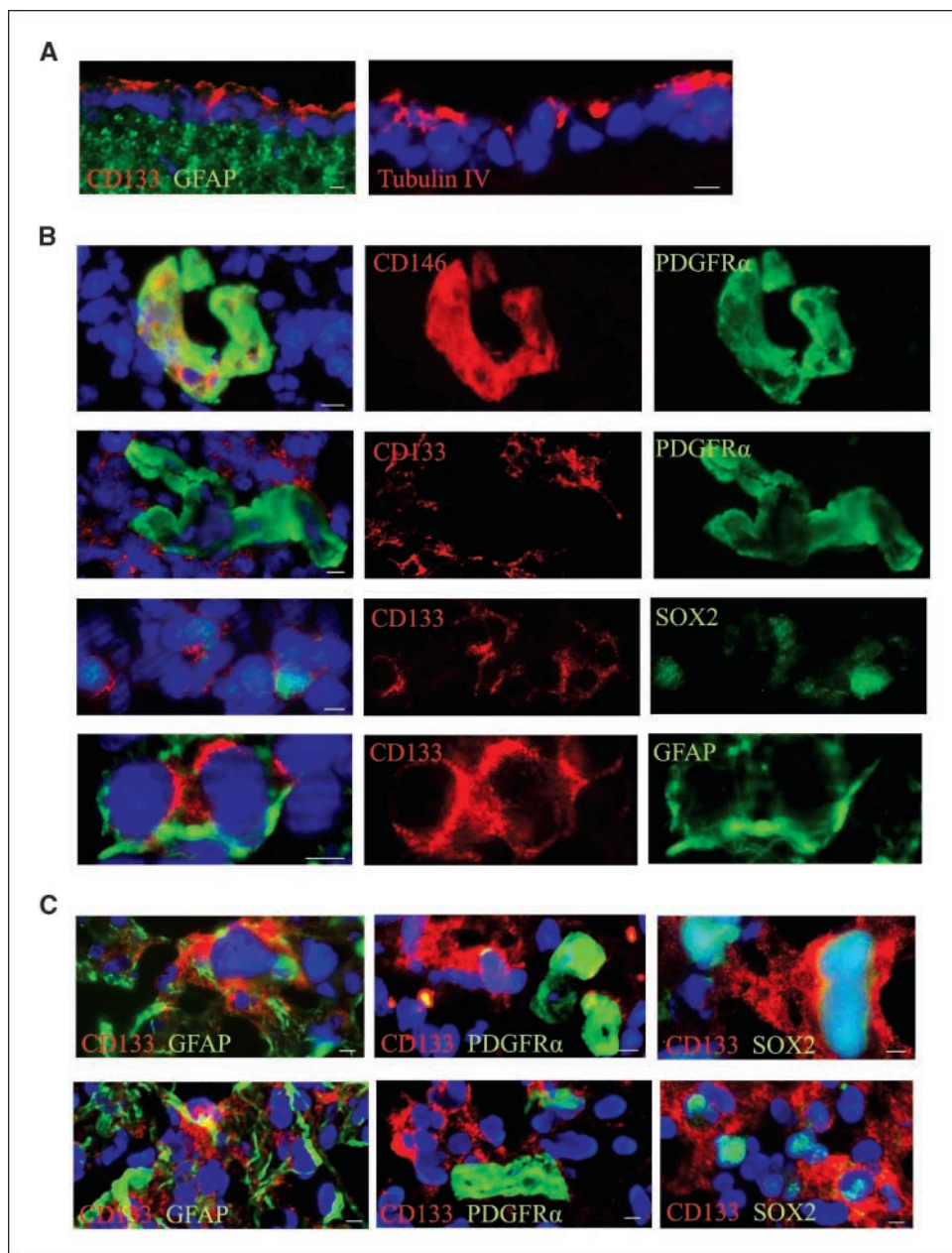
of the MACS-isolated CD133-positive and CD133-negative fraction was on average 92% and 85%, respectively, as determined by FACS reanalysis (not shown).

The low neurosphere-forming capacity of CD133-positive cells from the adult mouse LVW is not due to a lack of permissive factors. To test whether secreted factors present in the medium of adult CD133-negative, but absent in CD133-positive cell cultures, could explain the difference in neurosphere-forming capacity, we did coculture experiments with CD133-negative and CD133-positive MACS-sorted cell fractions. Cells from the adult LVW of C57Bl/6 and of transgenic mice expressing *GFP* were isolated. CD133-positive *GFP*-expressing cells were plated out together with the same number of CD133-negative cells from wt mice and vice versa. The neurosphere frequency derived from the CD133-negative sorted fraction (*GFP*-negative in the first and *GFP*-positive in the reverse experiment) was higher in both cases,

namely 1 of 936 and 1 of 750 compared with CD133-positive-derived cells (1 of 3,750 in both experiments). In these experiments, done early in our study, cells were isolated from larger tissue subsections, including the LVW, than in all other experiments, which might explain the lower neurosphere frequencies of the CD133-negative fraction. In conclusion, the low neurosphere-forming capacity of CD133-positive cells from the adult mouse LVW is not due to a lack of permissive factors.

CD133-positive cells from the adult LVW show morphologic features of ependymal cells. After FACS isolation and cultivation, the majority of cells within the CD133-positive fraction from adult LVW attached to the bottom of tissue culture flasks within 2 to 3 days and showed a flat morphology and tubulin- β -IV staining (Fig. 4D). Immediately after sorting, cells carried multiple CD133- and tubulin- β -IV-positive cilia (Fig. 4D) and their motility could be monitored through live cell imaging (Supplementary Data). We

Figure 2. Localization of CD133 at the adult human LVW and in human glioblastoma. **A**, sections through the human LVW are stained with antibodies against CD133/2 (red, left) and against tubulin- β -IV (red, right). Blue signals, DAPI-stained nuclei; green signals, staining with an anti-GFAP antibody. Bar, 5 μ m. Note that antibodies against both CD133 and tubulin- β -IV stain apical membranes of cells at the superficial cell layer. Because both antibodies are derived from the same species, costainings could not be done. GFAP-synthesizing cells are arranged in an astrocyte ribbon, previously described by Quinones-Hinojosa et al. (37). No overlapping staining of CD133/2 and GFAP antibodies was detected. **B**, glioblastoma sections from one patient stained with different antibodies. Blue signals, DAPI-stained nuclei. Bar, 5 μ m. Top images, PDGFR α antibody (green) stains cells, which are also recognized by the CD146 antibody detecting endothelial cells and pericytes (red). Bottom three panels, CD133/2 (red) and PDGFR α , SOX2, GFAP (green). Cells recognized by the CD133/2 antibody are localized outside the endothelial/pericyte cell layer of blood vessels, which are stained with the PDGFR α antibody. Some cells within the tumor, which are stained by the CD133/2 antibody, are also positive for GFAP and SOX2. **C**, glioblastoma sections from two additional patients stained with different antibodies. Blue signals indicate DAPI-stained nuclei. Bar, 5 μ m. CD133/2 (red) and PDGFR α , SOX2, GFAP (green).



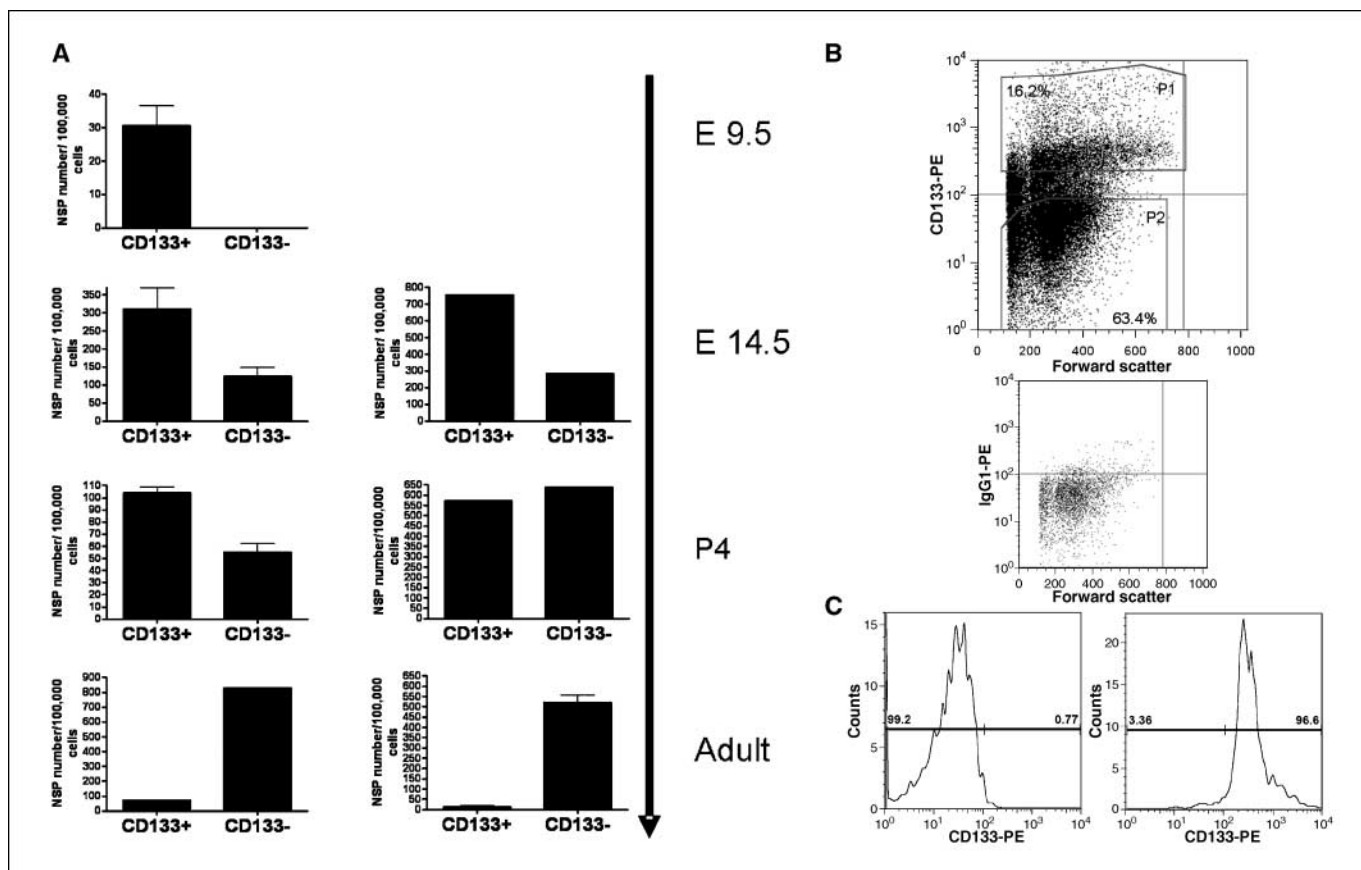


Figure 3. Neurosphere frequencies of CD133-positive and CD133-negative populations at different stages of brain development and flow cytometry of CD133-labeled adult LVW cells. **A**, columns, neurosphere (NSP) numbers per 100,000 plated cells. *Left*, neurosphere frequencies of MACS-isolated cell populations; *right*, results of FACS-isolated cells. CD133-positive and CD133-negative cells were isolated from E9.5 forebrain, E14.5 forebrain, and P4 and adult mouse LVW. Three independent experiments were done in case of MACS-isolated cells from E9.5 forebrain, E14.5 forebrain, P4 lateral ventricles, and in case of FACS-isolated cells from the adult murine lateral ventricle. Note that the neurosphere number of TLE-digested cell populations (MACS-isolated cells from E9.5, E14.5, and P4) is generally lower compared with trypsin-treated cells (see Materials and Methods). Bars, SD. The other results represent single experiments. **B**, top dot plot, distribution and sorting gates of CD133-labeled cells of a representative experiment (*X axis*, forward scatter; *Y axis*, CD133 intensity). Two regions (P1:CD133 positive and P2:CD133 negative) and as control all live cells were sorted. Sort regions were set based on the isotype control (*bottom dot plot*). Numbers, percentages of CD133-positive and CD133-negative sorted events of all 7-amino-actinomycin D-negative single events. PE, phycoerythrin. **C**, histogram of CD133-negative (*left*) and CD133-positive (*right*) subsets shows a high purity of sorted populations. Reanalysis shows a purity of 96.6% of CD133-positive and 99.2% of CD133-negative sorted events according to the isotype control. *X axis*, CD133-phycoerythrin intensity; *Y axis*, number of event counts.

conclude that the majority of CD133-positive cells isolated from the adult LVW show features of ependymal cells.

Discussion

CD133 is in the current literature commonly called a neural stem cell marker. Because tumorigenic cells from human glioblastoma, medulloblastoma, and ependymoma were successfully enriched with a CD133 antibody (10–13), the presence of this protein was used as one argument for the origination of tumor stem cells from normal neural stem cells. Our study shows that CD133 is a marker for embryonic neural stem cells; however, in the adult brain, ependymal cells and not the vast majority of neurogenic astrocytes are recognized by the CD133 antibody.

In addition to previously published immunostainings showing CD133 on murine neuroepithelial cells (1, 25), we show its localization on RGCs. In P4 mice, a time point at which RGCs turn into ependymal cells, we detect a CD133-positive intermediate radial glial/ependymal cell type mainly at the ventral side of the LVW, consistent with findings published by Spassky et al. (30). These are GFAP-negative cells

expressing the RGC marker *GLAST* and postnatal ependymal cell markers (*SI00*, *tubulin- β -IV*) in addition to *SOX2*. Although neurogenic astrocytes in the adult subventricular zone share certain markers with RGCs they are derived from (27, 36), we do not find the CD133 epitope on PDGFR α - and GFAP-positive astrocytes underneath the ependymal layer in the adult mouse brain. We cannot exclude that few neurogenic astrocytes in the adult mouse subventricular zone, which infrequently directly contact the ventricular surface (35), also express CD133, because very few cells at the adult LVW surface appear to be positive for GFAP, *SOX2*, and CD133. However, *SOX2* is also expressed by ependymal cells (33), and GFAP-positive processes can be tightly wound around ependymal cells, making it difficult at the light microscopic level to assign GFAP immunoreactivity.

Importantly, our immunostainings show that CD133 is present on multiciliated ependymal cells in adult mouse and human brain. The same monoclonal antibody (CD133/2) recognizing normal human ependymal cells also detects cells within human glioblastoma, which are not CD146-positive blood vessel cells, confirming shared CD133 epitopes between these normal and malignant cell types.

In addition to immunolocalization studies, we evaluated CD133-positive and CD133-negative cells from different developmental stages for their neurosphere forming and differentiation capacity. Certain caveats need to be considered when applying and interpreting this assay (40, 41). For our study, it is important to discuss potential biases to the number of neurospheres counted and whether the assay allows conclusions about the cell of origin

leading to neurosphere formation. Although neurospheres can be derived from single cells, the neurosphere-forming frequency is decreased at low cell densities (42). We plated out relatively high numbers of cells to rule out that an absence of neurosphere formation is because of too low cell numbers. Moreover, single neurospheres can fuse to larger aggregates, especially at higher neurosphere densities and with larger neurosphere size (41).

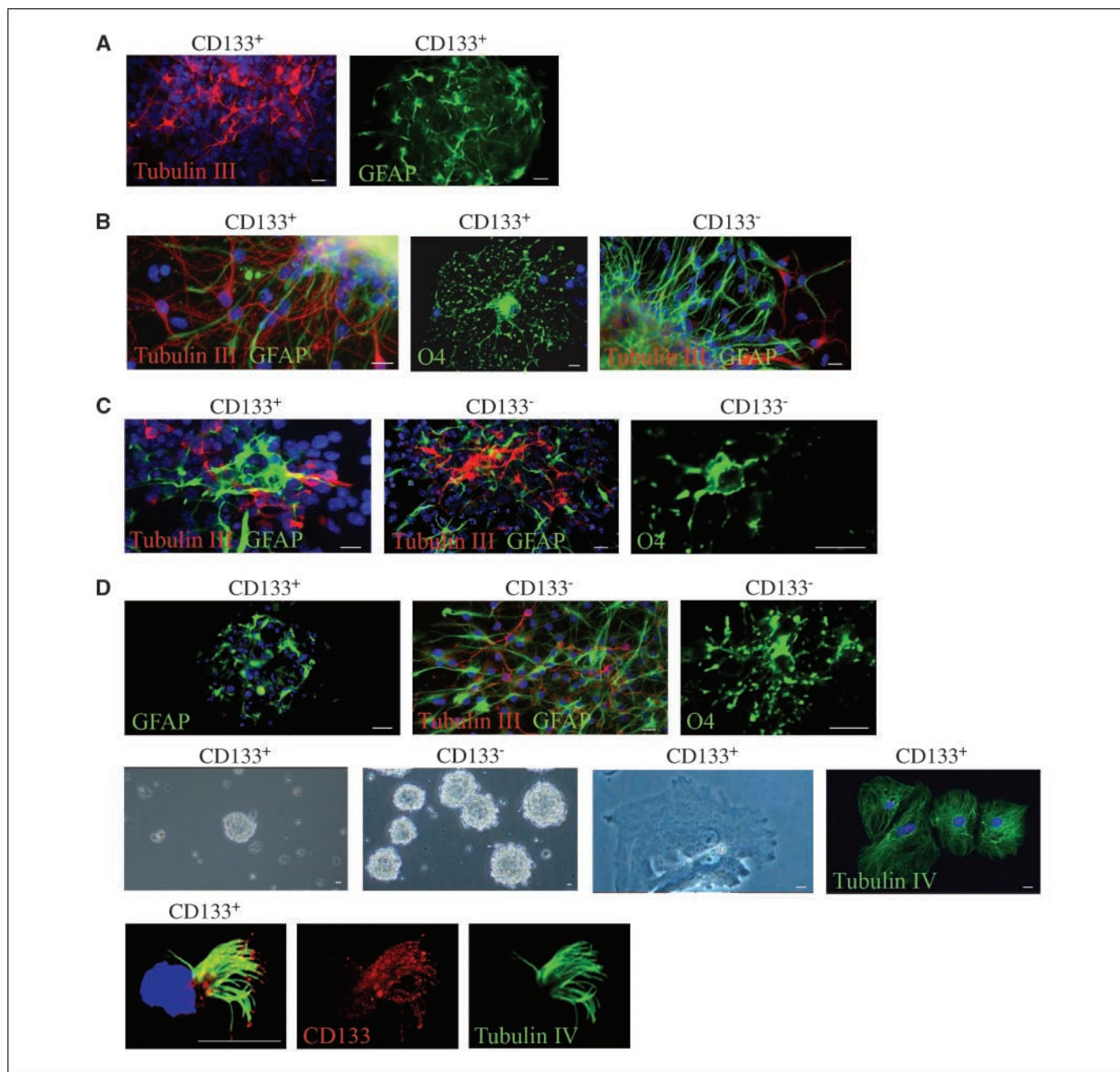


Figure 4. Immunofluorescence microscopy of cultured and uncultured cells from murine CD133-positive and CD133-negative populations. Neurospheres are derived from CD133-sorted cells of E9.5 forebrain (A), E14.5 forebrain (B), P4 LVW (C), and adult LVW (D, top). Tubulin- β -III-positive neuronal cells (red), GFAP-positive astrocytes (green), and O4-positive oligodendrocytes (green) are shown. Cell nuclei were stained with DAPI (blue). Bar, 15 μ m. Note that at E14.5 (B), neurosphere cells derived from the CD133-positive fraction gave rise to cells of all three neural lineages, whereas CD133-negative derived spheres only differentiated into astrocytes and neurons. At P4 (C), CD133-negative-, but not CD133-positive-derived spheres differentiated into oligodendrocytes. D, top, a differentiating sphere from the CD133-positive adult LVW fraction expresses only GFAP, whereas differentiating spheres from the CD133-negative fraction are positive for GFAP, tubulin- β -III, and O4. The vast majority of neurospheres derived from the adult LVW were formed by the CD133-negative fraction (D, middle). A low number of small spheres was found in the CD133-positive fraction, but the majority of cells attached to the cell culture dish and synthesized tubulin- β -IV (D, middle). D, bottom, CD133-positive uncultured cell isolated from the adult LVW, attached to coverslips with a cytospin centrifuge directly after sorting, showing multiple cilia, stained with antibodies against CD133 (red) and tubulin- β -IV (green).

Neurosphere numbers were therefore counted at early time points and we are aware of the fact that the true number of initially formed neurospheres can be higher considering that some of the counted aggregates could have been derived from separate spheres. However, such a bias would be more relevant for the cultures with higher neurosphere numbers, and therefore this aspect does not change our conclusions.

With regards to the cell of origin forming a neurosphere, the potential of a cell to form a primary sphere and the number of possible passages need to be considered. Cells from the hippocampal dentate gyrus can form neurospheres, but dissociation and replating does not lead to secondary spheres or only generates spheres until the third passage (43, 44). Neurospheres from cells of the adult LVW can be passaged up to 10 times, indicating a higher self-renewal capacity *in vitro* (43–45). Furthermore, single adult dentate gyrus spheres do not differentiate into the three neural lineages (neurons, oligodendrocytes, and astrocytes; refs. 43, 46). Based on these differences, adult neurogenic dentate gyrus cells are called neural progenitor cells and long-term passagable neurosphere-forming cells with multilineage potential from the adult subventricular zone as neural stem cells (42). It remains controversial which cells of the adult subventricular zone have neurosphere-forming capacity (42). A study by Morshead et al. (47) indicates that GFAP-positive (type B cells) represent the origin of neurospheres. Doetsch et al. (34) conclude from their experiments on Dlx2+ cells that also type C cells, which are derived from type B cells, can form neurospheres. However, these spheres were passaged only once to form secondary spheres. Here, we show that isolated CD133-positive RGCs from E14.5 mouse brain give rise to passagable neurospheres (>7 passages), which retain multilineage potential, indicating that these embryonic neural stem cells can be cells of origin for neurospheres. A large number of spheres passaged at least eight times was also generated from CD133-negative cells of the E14.5 mouse brain. This population could represent cells from the embryonic subventricular zone, being derived from RGCs and whose

cell bodies do not contact the ventricular surface (48, 49) or from CD133-negative RGCs.

In contrast to the high neurosphere frequencies achieved with CD133-positive cells from embryonic and early postnatal tissue, our study shows a strongly reduced or almost absent neurosphere formation for CD133-positive cells isolated from the adult LVW. We have ruled out the absence of permissive factors by cocultures with CD133-negative cells. Furthermore, spheres derived from CD133-positive sorted cells could only be passaged once, whereas spheres from CD133-negative sorted cells were kept for at least nine passages. Together with our finding that neurosphere cells from the adult CD133-positive population do not show multilineage differentiation potential as is the case for CD133-negative-derived spheres even at high passages, we conclude that in the adult brain, neural stem cells are contained in the CD133-negative, but not in the CD133-positive fraction. As discussed above, CD133 is present on ependymal cells of the adult LVW and live cell microscopy revealed that cells within this isolated CD133-positive fraction carry multiple beating cilia, characteristic for ependymal cells.

If brain tumor stem cells in glioblastoma, medulloblastoma, and ependymoma are indeed derived from a CD133-positive cell in the ventricular wall of the embryonic, perinatal, or adult brain, our study in principle reveals neuroepithelial cells, RGCs, an intermediate radial glial/ependymal cell and ependymal cells as possible sources (Fig. 6).

Ependymal cells in the adult brain are regarded primarily as postmitotic (30). Thus far, strong evidence has only been provided for dividing progenitor or stem cells as origin for tumor stem cells, and all these findings were made in the hematopoietic field (50). Although it is conceivable that postmitotic cells acquire tumor stem cell features, this is less likely than for dividing cells. At the other end of the spectrum are neuroepithelial cells, which occur very early in brain development, but disappear long before birth (28). If these cells should be the origin of CD133-positive brain tumor stem cells, they would abnormally have to remain in the brain with their number being held under tight control until the final transforming event. Although not impossible, such a scenario would be less likely for tumors arising very late in adulthood. Singh et al. (10), for example, enriched tumor stem cells from glioblastoma samples derived from patients 65 to 70 years of age. The same arguments apply to RGCs or intermediate radial glial/ependymal cells, which are normally not found in the adult brain. However, these two cell types can persist in the brain until the early postnatal period and are therefore more likely to represent a source for tumor stem cells, in particular in pediatric brain tumors. RGCs were recently suggested as origin for ependymoma (11).

In addition to a possible lineage relationship between CD133-enriched brain tumor stem cells and CD133-positive cells of the normal brain, CD133 could be newly produced by tumor stem cells, which are derived from a CD133-negative cell. This idea is supported by animal models, in which gene targeting to GFAP-positive astrocytes leads to the formation of brain tumors with features of human astrocytoma and oligodendroglioma (21). Except for Bergmann glia cells in the cerebellum, which are similar to and can originate from RGCs, rodent RGCs do not express *GFAP* (27), and our study shows that the vast majority of GFAP-positive cells in the adult subventricular zone do not carry the CD133 epitope. Outside the subventricular zone, we only detected CD133 at ependymal cells of the third and fourth ventricle and very weakly at vessel-like structures. Moreover, some of the animal models cited

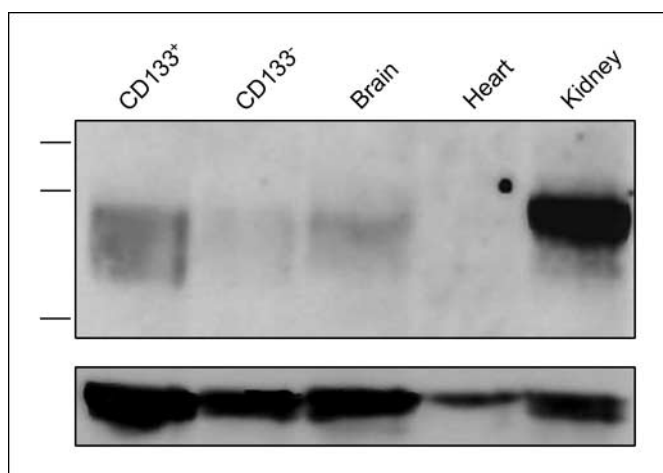
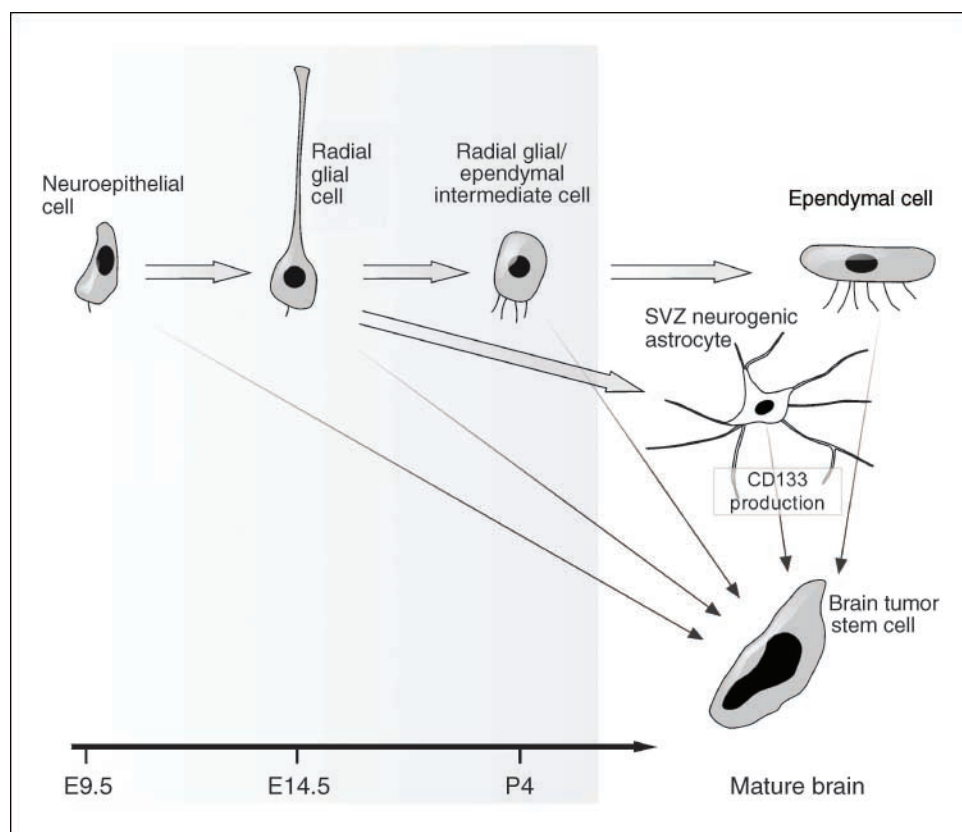


Figure 5. Detection of mouse CD133 protein in MACS-isolated populations from E14.5 forebrain. Protein extract (19 μ g) of CD133-positive and CD133-negative cells, isolated from E14.5 forebrain, were analyzed by Western blotting. Bands of 105 to 128 kDa were detected, indicating different glycosylation forms in the protein extract enriched for CD133-positive cells. This size is in line with previous reports on the mouse CD133 protein (1). Faint bands of the same size are seen in the CD133-negative fraction. CD133 is detected in E14.5 brain and adult kidney tissue (positive controls), but not in adult heart tissue (negative control). Twenty micrograms protein extract per tissue were loaded. HSP105 was used as loading control (bottom). Size marker (left), 170, 130, and 100 kDa.

Figure 6. Illustration of CD133-positive (gray) and CD133-negative (white) cell types throughout brain development. The neuroepithelial, radial glial, intermediate radial glial/ependymal, and ependymal cell are depicted with their ventricular surface facing down. Developmental relationships between normal brain cells based on published findings (thick, light arrows). Note that in the mature brain, postmitotic ependymal cells express CD133, whereas proliferating neurogenic astrocytes in the subventricular zone are CD133 negative. Thin black arrows, two possible scenarios for the origin of brain tumor stem cells enriched in the CD133-positive cell population: The derivation from CD133-positive cells (immature cell types, which are normally not present in the mature brain, or postmitotic ependymal cells) or CD133-negative neurogenic astrocytes in the adult brain. In the latter case, brain tumor development would involve production of CD133.



above were generated through somatic gene transfer into frontal brain regions of postnatal animals, thus excluding the cerebellum. Altogether, our findings in combination with published data indicate that the genetically altered GFAP-positive cells in several brain tumor mouse models was initially CD133 negative. It remains unknown whether some of the targeted GFAP-positive cells were neurogenic astrocytes (type B cells) in the postnatal subventricular zone. To tackle the precise cellular origin of stem cells in brain tumors and to answer the question whether a CD133-positive cell can be a source, further experimental data is needed. It will be important to target CD133-expressing neural cell types in the brain, which we have described in detail here. In addition, it will be crucial to investigate whether a CD133-positive tumor stem cell population is present in mouse models, in which tumors arise from CD133-negative cells, for example, GFAP-

expressing neurogenic astrocytes in the adult subventricular zone. In the latter case, it is tempting to speculate that a production of CD133 could be of functional significance for brain tumor stem cells.

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