

## The Stem Cell Marker Prominin-1/CD133 on Membrane Particles in Human Cerebrospinal Fluid Offers Novel Approaches for Studying Central Nervous System Disease

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### ABSTRACT

Cerebrospinal fluid (CSF) is routinely used for diagnosing and monitoring neurological diseases. The CSF proteins used so far for diagnostic purposes (except for those associated with whole cells) are soluble. Here, we show that human CSF contains specific membrane particles that carry prominin-1/CD133, a neural stem cell marker implicated in brain tumors, notably glioblastoma. Differential and equilibrium centrifugation and detergent solubility analyses showed that these membrane particles were similar in physical properties and microdomain organization to small membrane vesicles previously shown to be released from neu-

ral stem cells in the mouse embryo. The levels of membrane particle-associated prominin-1/CD133 declined during childhood and remained constant thereafter, with a remarkably narrow range in healthy adults. Glioblastoma patients showed elevated levels of membrane particle-associated prominin-1/CD133, which decreased dramatically in the final stage of the disease. Hence, analysis of CSF for membrane particles carrying the somatic stem cell marker prominin-1/CD133 offers a novel approach for studying human central nervous system disease. *STEM CELLS* 2008;26:698–705

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Cerebrospinal fluid (CSF) is routinely used in the diagnosis of a wide variety of human diseases affecting the central nervous system (CNS) [1, 2]. Although CSF is an internal body fluid with a relatively low protein concentration, the advanced technology of current protein analysis has revealed that CSF contains a large number of specific proteins [2, 3]. There are two principal sources of CSF proteins, the blood plasma and the CNS tissue itself [1, 2]. Irrespective of their source, the vast majority of CSF proteins are soluble (the notable exception being proteins associated with the few cells that are found in normal CSF) [1–3]. This also holds true for integral membrane proteins, as it is typically their proteolytically derived ectodomain fragments that are found in CSF [4]. Remarkably, even when integral membrane proteins of CNS tissue have been detected in CSF as apparently full-length polypeptides, as is the case for the synaptic vesicle membrane protein synaptotagmin,

they have been reported to be present in CSF exclusively in soluble form [5].

However, in the case of other body fluids, the occurrence of membrane particles has received increasing attention [6–11]. Such extracellular membrane particles may provide information about the membrane dynamics of the cells from which they are derived and may serve as carriers and means of signaling between cells. Exosomes, the most extensively studied extracellular membrane particles that are formed inside cells as the internal vesicles of multivesicular bodies and that are released by exocytosis, occur in both external and internal body fluids, such as urine and blood, respectively [6–8, 10, 12]. In contrast, extracellular membrane particles distinct from exosomes that are generated by a budding process from the cell surface have been found in various external, but not internal, body fluids [9].

A characteristic constituent of the latter type of extracellular membrane particles is the pentaspan membrane protein prominin-1 (CD133) [9]. Prominin-1 is the first characterized member of a novel family of cholesterol-binding membrane proteins and the defining component of a specific cholesterol-based mem-

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**Table 1.** Clinical parameters of the included healthy subjects

Parameters	Values for healthy subjects ( <i>n</i> = 73)	
	Adults ( <i>n</i> = 61)	Children ( <i>n</i> = 12)
Age (years), median (range)	49 (17–85)	5 (1.5–12)
Sex (female/male)	33/28	5/7
Reason for lumbar puncture ( <i>n</i> )		
Exclusion of subarachnoid hemorrhage	18	2
Exclusion of inflammatory disease	40	7
Other	3	3

brane microdomain [13–17]. Importantly, prominin-1 has emerged as a major somatic stem cell and malignant tumor marker [13, 16, 18–31].

One of the exciting recent discoveries with obvious medical relevance is that the adult CNS, like other tissues, contains stem cells [32–38]. Moreover, cancer stem cells have been implicated in brain tumors, such as glioblastoma [24, 25, 29, 30]. Despite these observations, the CSF has not been reported to contain stem cell markers derived from brain tissue. Here, we report on the occurrence, in CSF, of membrane particles carrying the stem cell marker prominin-1 and describe the potential of these particles as a novel tool for studying neurological diseases.

## MATERIALS AND METHODS

### Patient Selection

This triple-center study was approved by the institutional review boards of the Universities of Heidelberg, Erlangen, and Dresden, Germany. All patients analyzed gave informed consent. CSF samples were obtained from patients who underwent a lumbar puncture for CSF analysis (a) at the Department of Neurology of Heidelberg University between October and December 2005, (b) at the Departments of Neurology and Neurosurgery of Erlangen University since September 2006, and (c) at the Departments of Pediatrics, Neurology, and Neurosurgery of Dresden University since October 2006.

Adults (*n* = 61) and children (*n* = 12) who received a lumbar puncture for exclusion of subarachnoid hemorrhage or inflammatory diseases and whose CSF analyses were normal were considered healthy subjects (*n* = 73). In addition, CSF samples of (a) 14 patients with a histopathologically proven glioblastoma (i.e., astrocytoma of WHO grade IV; for analysis of prominin-1 in lower-grade astrocytomas; [39]), (b) 10 patients with a neurographically proven polyneuropathy, and (c) 8 patients with a remitting-relapsing multiple sclerosis were analyzed. The clinical parameters of the subjects analyzed are listed in Tables 1 and 2.

The CSF samples were routinely analyzed for the following basic parameters. In healthy subjects, the total cell count was  $\leq 4/\mu\text{l}$  (with a proportion of white blood cells to red blood cells of  $\sim 1:2$ ), and the total protein was  $\leq 450$  mg/l (of which albumin accounted for  $\sim 60\%$ ). Control experiments (data not shown) indicated that blood plasma did not contain detectable levels of prominin-1 and that blood contamination did not influence the prominin-1 values obtained for the various CSF samples. First, no prominin-1 was detected upon immunoblot analysis of a P4 pellet obtained from 1 ml of blood plasma by differential centrifugation (described below). Second, addition to clear CSF of blood to a level (absorbance value at 540 nm of  $\sim 1.5$ ) corresponding to that of the heavily blood-contaminated CSF samples (those obtained intraoperatively) did not increase the amount of prominin-1 immunoreactivity as detected after differential centrifugation and immunoblotting. Third, comparison of the prominin-1 levels between blood-contaminated CSF obtained intraoperatively and clear CSF obtained by lumbar puncture, both obtained from individual glioblastoma patients, revealed no obvious difference.

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**Table 2.** Clinical parameters of the included patients

Parameters	Values
Glioblastoma patients	<i>n</i> = 14
Age (years), median (range)	59 (35–73)
Sex (female/male)	4/10
Site of tumor (frontal/temporal/parietal/multiple lobes)	1/5/1/7
Distance to ventricular system (mm), mean $\pm$ SD	16 $\pm$ 10
Tumor volume (mm <sup>3</sup> ), median (range)	242 (129–735)
Collection of CSF at:	
First diagnosis/surgery ( <i>n</i> )	4
Relapse surgery ( <i>n</i> )	7
Other ( <i>n</i> )	3
History of radiochemotherapy ( <i>n</i> )	8
Subanalysis of patients with a disease duration >30 months ( <i>n</i> = 4):	
History of surgery	<i>n</i> = 4
History of radiochemotherapy	<i>n</i> = 4
Tumor volume (mm <sup>3</sup> ), median (range)	260 (185–418)
Polyneuropathy patients	<i>n</i> = 10
Age (years), median (range)	65 (54–76)
Sex (female/male)	3/7
Multiple sclerosis patients	<i>n</i> = 8
Age (years), median (range)	36 (25–44)
Sex (female/male)	5/3
EDSS (first manifestation)	2 (1.0–3.5)
EDSS (time-point of CSF analysis)	2.25 (1.0–3.5)
Annual relapse rate (mean $\pm$ SD)	0.82 $\pm$ 0.32
Disease duration (years), mean $\pm$ SD	4.3 $\pm$ 2.2

Abbreviations: CSF, cerebrospinal fluid; EDSS, expanded disability status scale.

### Isolation of Prominin-1-Containing Membrane Particles

**CSF.** After lumbar puncture, CSF was directly (<20 minutes) frozen at  $-80^{\circ}\text{C}$ . Samples were defrosted on ice, and protease inhibitor was added according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany, <http://www.roche-applied-science.com>). Control experiments showed that freezing-thawing of CSF (either clear or blood-contaminated CSF) did not influence the amount of prominin-1 detected upon immunoblotting of the P4 pellet (described below) (data not shown).

**Differential Centrifugation.** CSF was centrifuged at  $4^{\circ}\text{C}$  for 5 minutes at 300g, and the supernatant was centrifuged for 10 minutes at 1,200g, for 30 minutes at 10,000g, and for 1 hour at 200,000g. The resulting pellets (P1–P4) and the final S4 supernatant (after precipitation using a methanol/chloroform protocol) were resuspended in Laemmli buffer and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

**Sucrose Gradient Centrifugation.** CSF (3 ml) was centrifuged at 10,000g, the supernatant was concentrated using Centricon Plus-30 (Millipore, Billerica, MA, <http://www.millipore.com>), and the concentrated sample was subjected to equilibrium sucrose gradient centrifugation (0.1–1.2 M) as described previously [9, 40]. The sample was loaded on top of the gradient and centrifuged at  $4^{\circ}\text{C}$  for 4 hours at 335,000g using a SW60 swinging bucket rotor. Fractions (500  $\mu\text{l}$ ) were collected, and a 50- $\mu\text{l}$  aliquot of each fraction was used to determine the sucrose concentration by measuring the refractive index [40]. Proteins of the residual 450  $\mu\text{l}$  were precipitated using a methanol/chloroform protocol, resuspended in Laemmli buffer, and subjected to SDS-PAGE and immunoblotting.

**Standard Protocol for Patient Analysis.** CSF (1 ml) was centrifuged at  $4^{\circ}\text{C}$  for 30 minutes at 10,000g, and the supernatant was centrifuged for 1 hour at 200,000g. The resulting P4 pellet was resuspended in Laemmli buffer and subjected to SDS-PAGE and

immunoblotting. In the case of CSF samples that were obtained during surgery and that contained blood ( $n = 5$ ), sodium carbonate was added to the 10,000g supernatant prior to the 200,000g centrifugation to a final concentration of 0.1 M (pH 11). The resulting P4 pellet was neutralized by addition of 1 ml of H<sub>2</sub>O and centrifuged again for 30 minutes at 200,000g. This protocol effectively prevented adsorption of serum proteins to the membrane particles but did not affect the level of prominin-1 immunoreactivity in the P4 pellet (data not shown).

**Caco-2 Cells.** Caco-2 cells were used as positive control [9, 16, 41]. Caco-2 cells were grown at 37°C in a 5% CO<sub>2</sub> atmosphere in minimal essential medium supplemented with 20% fetal calf serum, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown to confluence and received fresh medium every second day until 10 days post-confluence [9, 40]. The cells were lysed in RIPA buffer, incubated for 30 minutes on ice, and then centrifuged for 10 minutes at 13,000 rpm. Protein concentration of the resulting supernatant, referred to as extract, was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL, <http://www.piercenet.com>). The extract used here had a protein concentration of 10 µg/µl and was diluted 1:100 to establish the standard curve.

### Endoglycosidase Digestion and Detergent Treatment

**Endoglycosidase Digestion.** The 200,000g (P4) pellet obtained from human CSF after differential centrifugation was incubated in the absence or presence of 1 U of peptide-*N*-glycosidase F (PNGase F) according to the manufacturer's instructions (Roche Molecular Biochemicals).

**Detergent Treatment.** CSF (six 1-ml samples) was centrifuged as described above. The resulting 200,000g pellets were resuspended in 100 µl of phosphate-buffered saline (PBS) and incubated for 30 minutes at 37°C. Subsequently 100 µl of PBS containing either 2% Triton X-100 or 2% Lubrol WX (Lubrol 17A17; Serva, Heidelberg, Germany, <http://www.serva.de>) was added to reach a final concentration of detergent of 1%. Samples were incubated for 30 minutes at 4°C. Samples were centrifuged at 4°C for 60 minutes at 100,000g [40], and pellets and supernatants were analyzed by SDS-PAGE and immunoblotting.

### Quantitative Immunoblotting

Immunoblotting was performed as described [27], with the following modifications. Proteins were subjected to SDS-PAGE (7.5%) and transferred to poly(vinylidene difluoride) membranes (Millipore; pore size, 0.45 µm) by standard procedures [42], followed by staining with Red Ponceau S solution (Serva). Membranes were blocked for at least 1 hour in PBS containing 0.2% Tween-20 and 5% low-fat milk powder (blocking medium); incubated for 1 hour in 20–40 ml of blocking medium containing 1 µg/ml of mouse monoclonal antibody (mAb) 80B258 (IgG), which recognizes an epitope in the first extracellular loop of human prominin-1 (E. Missol-Kolka, C.A. Fargeas, D. Corbeil, manuscript in preparation) [27]; washed three times for 15 minutes in PBS containing 0.2% Tween-20; and incubated for 1 hour in 100 ml of blocking medium containing 1.5 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, <http://www.jacksonimmuno.com>). Antigen-antibody complexes were detected using enhanced chemiluminescence (SuperSignal West Femto Substrate; Pierce) and visualized using an LAS 3000 image reader (Raytest GmbH, Straubenhardt, Germany, <http://www.raytest.com>). The quantification was performed using AIDA-software (Raytest) software for Macintosh. Only absorbance values indicated as being within the linear range by the image reader were used. Exposure times for Caco-2 membrane extract varied from 2 to 12 minutes, with 6 minutes being the standard time (Fig. 2A legend). Exposure times for human CSF were usually the same, except when the prominin-1 immunoreactivity was markedly below or above that of the Caco-2 standards, in which case exposure times were chosen that yielded absorbance values within the linear range of the Caco-2 standard curve (Fig. 2A).

To determine how much mAb 80B258 was bound to prominin-1 upon immunoblotting of a defined amount of Caco-2 membrane extract, which served as internal standard, 14 µl of Caco-2 membrane extract (in triplicate) and 10, 20, 30, and 40 ng of purified mAb 80B258 (in duplicate) were subjected to SDS-PAGE and prominin-1 immunoblotting on the same gel and membrane as above, followed by densitometry. The absorbance values obtained for the 10–40 ng of mAb 80B258 were used to construct a standard curve ( $R^2 = 0.9892$ ), which was used to determine the amount of bound mAb 80B258 (in nanograms) that yielded the absorbance value obtained for the 14 µl of Caco-2 membrane extract.

### Immunohistochemistry

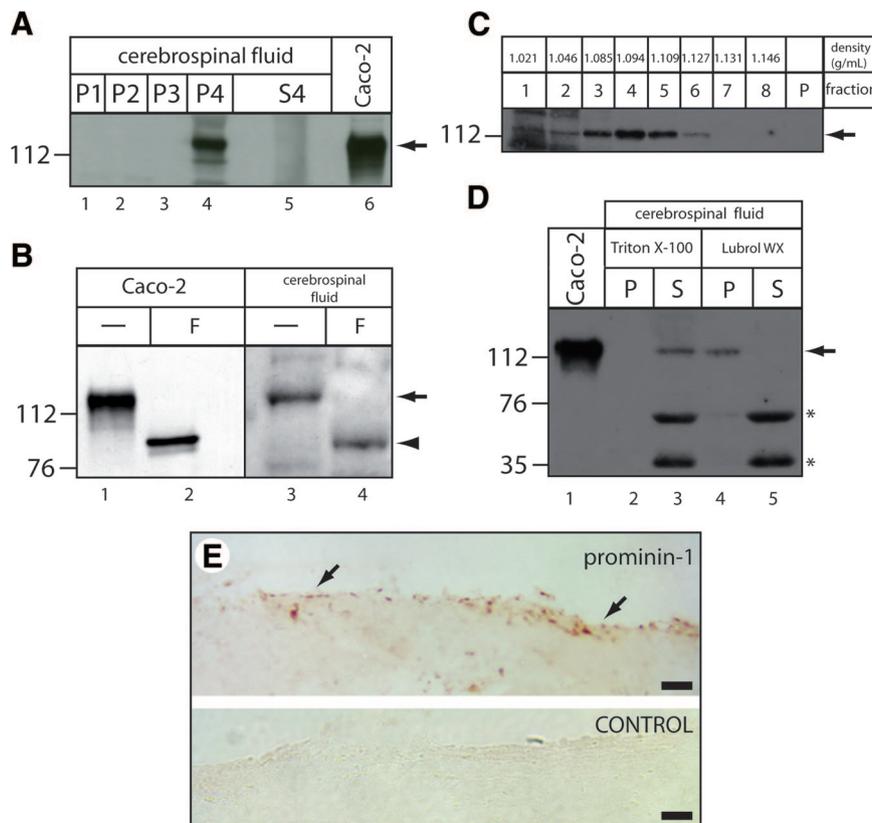
Adult human post-mortem tissue of the ventricular ependyma (temporal horn of the lateral ventricle) was cryofixed using evaporating liquid nitrogen. After embedding in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA, <http://www.sakura.com>), samples were cryosectioned (12 µm) on a Microm HM560 cryostat (Adamas Instrumenten, Rheden, Netherlands, <http://www.adamas-instrumenten.com>). The sections were air-dried for several hours and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The fixative was removed by three 5-minute washes with PBS. Endogenous peroxidase was neutralized with 2% H<sub>2</sub>O<sub>2</sub>-PBS for 15 minutes. To unmask the prominin-1 epitope, sections were incubated for 30 minutes in 0.2% gelatin-PBS containing 0.005% SDS [27]. Afterward, the samples were permeabilized three times for 30 minutes each time with 0.15% saponin/0.2% gelatin-PBS. Sections were incubated overnight at 4°C with antiserum αhE2 [27] (1:500) diluted in 0.15% saponin/0.2% gelatin-PBS. The negative control did not receive the primary antibody. All samples were washed three times for 10 minutes each time in 0.15% saponin-PBS and once in 0.15% saponin/0.2% gelatin-PBS for 30 minutes. Sections were incubated for 1 hour the HRP-conjugated goat anti-rabbit secondary antibody (1:500) diluted in 0.15% saponin/0.2% gelatin-PBS.

After the samples were washed three times for 10 minutes each time in 0.15% saponin-PBS, the color reaction was initiated with 3,3'-diaminobenzidine tablet sets (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) according to the manufacturer's protocol. Stained sections were observed with an Olympus compound microscope (Tokyo, <http://www.olympus-global.com>). The images were captured by means of an Olympus Metamorph digital camera and IPLAB software (Digital Imaging Systems, Buckinghamshire, U.K., <http://www.digitalimaging.com>) and prepared from the digital data files using Adobe Photoshop and Illustrator (Adobe Systems Inc., San Jose, CA, <http://www.adobe.com>).

Alternatively, ultrathin (120–150 nm) cryosections of adult human post-mortem tissue of the ventricular ependyma were double-immunolabeled using antiserum αhE2 and mAb 360 (1:200; Chemicon, Temecula, CA, <http://www.chemicon.com>) directed against prominin-1 and glial fibrillary acidic protein, respectively. The immune complexes were revealed using the Cy3- and Cy2-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Nuclei were stained with 4,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR, <http://probes.invitrogen.com>). The samples were observed using an Olympus BX61 microscope.

### Statistical Analysis

Statistical analyses were performed using the SPSS software package, version 13.0 (SPSS, Chicago, <http://www.spss.com>). The Shapiro-Wilk test was used to determine the distribution of the data. Normally distributed data are expressed as mean ± SD; other data are expressed as median and range and were compared using nonparametric tests. For group differences, the Kruskal-Wallis test was calculated including Bonferroni-corrected Mann-Whitney *U* tests for post hoc analyses. All statistical tests were two-sided, with a significance level of  $\alpha = 0.05$ .



**Figure 1.** Characterization of prominin-1-containing membrane particles from cerebrospinal fluid (CSF) and their potential origin. (A–D): CSF from healthy adult donors was subjected to various treatments followed by immunoblotting for prominin-1 (arrows) using monoclonal antibody 80B258. Caco-2 and Caco-2 membrane extract were analyzed in parallel. (A): Differential centrifugation of CSF (26 ml). (B): Treatment of P4 of CSF (2 ml) in the absence (–) or presence of F. Arrowhead, deglycosylated form of prominin-1. (C): CSF (3 ml) was centrifuged for 30 minutes at 10,000g, and the resulting supernatant containing the P4 particles was subjected to equilibrium sucrose gradient centrifugation. (D): P4 of CSF (1 ml) was incubated with Triton X-100 or Lubrol WX followed by centrifugation for 1 hour at 100,000g to obtain detergent-insoluble (P) and -soluble (S) material. Asterisks, cross-reacting serum proteins. (E): Immunoperoxidase staining of cryosections of human post-mortem brain tissue (temporal horn of lateral ventricle) for prominin-1 using  $\alpha$ HE2 antibody. Arrows indicate the ventricular surface of the ependyma, which shows prominin-1 immunoreactivity. Control, negative control without prominin-1 antibody. Scale bar = 50  $\mu$ m. Abbreviations: F, peptide-N-glycosidase F; P, pellet; P1, pellet after 5 minutes at 300g; P2, pellet after 10 minutes at 1,200g; P3, pellet after 30 minutes at 10,000g; P4, pellet after 1 hour at 200,000g; S, supernatant; S4, 200,000g supernatant.

## RESULTS

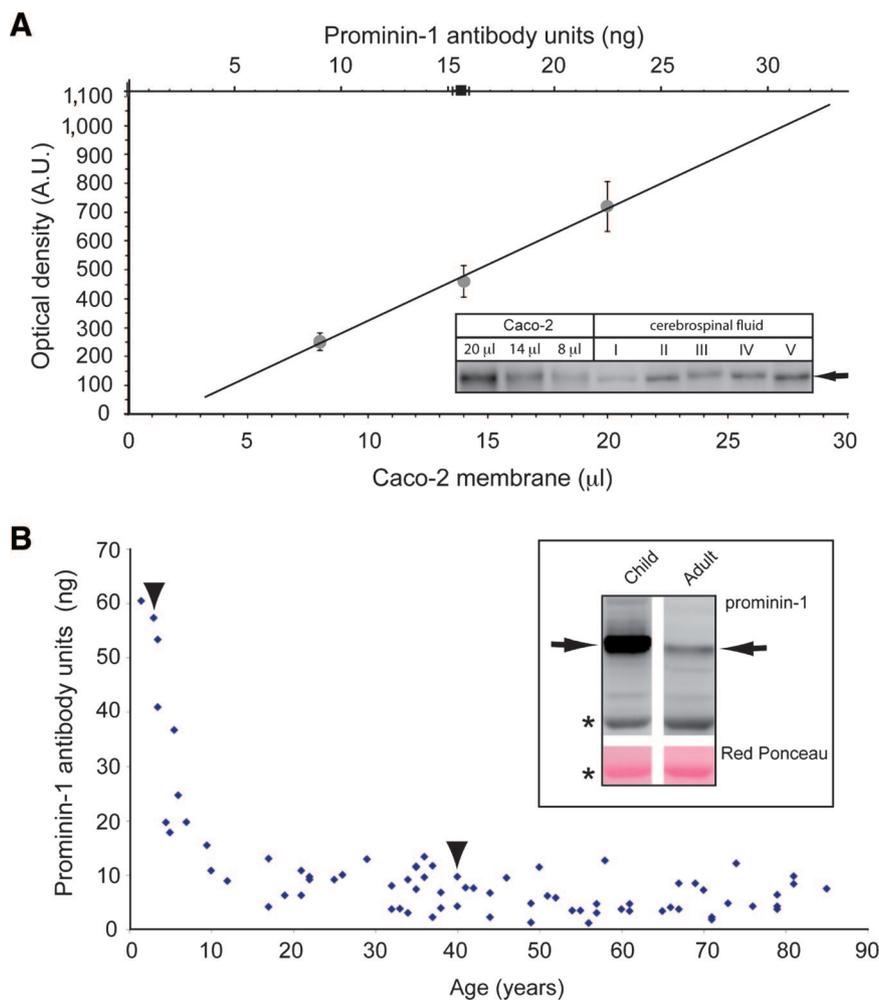
### Occurrence of Membrane Particles in Human CSF That Carry the Stem Cell Marker Prominin-1/CD133

Differential centrifugation of CSF from healthy human subjects followed by immunoblotting of the resulting fractions revealed the presence of prominin-1 in the 200,000g (P4) pellet (Fig. 1A, lane 4, arrow) but not in the other pellets (P1–P3) or in the 200,000g supernatant (S4) (Fig. 1A, lanes 1–3 and 5). The authenticity of the 120-kDa immunoreactive band was verified by comparison with prominin-1 from the human colon carcinoma cell line Caco-2 (Fig. 1A, lane 6) and by *N*-deglycosylation with PNGase F, which yielded a band of 94 kDa (Fig. 1B, lanes 2 and 4, arrowhead), the known apparent molecular mass of deglycosylated prominin-1 [41]. Upon further fractionation by equilibrium sucrose density gradient centrifugation, the prominin-1-containing P4 particles (revealed by immunoblotting) peaked in a fraction with a density of 1.368 g/ml (Fig. 1C, fraction 4, arrow), consistent with previous results [9]. Finally, prominin-1 associated with the P4 particles in the CSF showed the same behavior in nonionic detergents as described previously for cell-associated prominin-1 [14], being soluble in 1% Triton X-100 (Fig. 1D, lane 3, arrow) and insoluble in 1% Lubrol WX (Fig. 1D, lane 4, arrow), a characteristic feature of the prominin-1-containing membrane microdomain [14]. Taken together, these data indicated that CSF from healthy human subjects contains membrane particles that carry the neural stem cell marker prominin-1 and that exhibit characteristics similar to those of the P4 particles previously found in various extracellular fluids [9].

In searching for a possible source of the prominin-1-containing particles, we immunostained the ependyma of the lateral ventricle of post-mortem human brain tissue for prominin-1 (neither of the two individuals analyzed had suffered from any known neurological disease). Consistent with the presence of prominin-1 on the luminal surface of the ependyma in the adult mouse [13] and with the extension of subependymal astrocytic neural stem cells into the lateral ventricle [43, 44], spotty prominin-1 immunoreactivity was detected on the ventricular surface of human ependyma (Fig. 1E, prominin-1, arrows). Similarly, sparse punctate prominin-1 immunoreactivity at the ventricular surface was observed on double immunofluorescence of ultrathin cryosections, which also revealed the presence of glial fibrillary acidic protein (GFAP)-positive cells beneath the ependymal cell layer (supplemental online Fig. 1).

### Age Profile of Membrane Particle-Associated Prominin-1 in the Human CSF

To determine the concentration of prominin-1 in the human CSF, we established a quantitative assay for prominin-1 using standardized immunoblotting with the mAb 80B258, which recognizes an epitope in the first extracellular loop of prominin-1. Immunoblotting of human CSF was performed such that the resulting absorbance values were within the linear range of a standard curve obtained by immunoblotting of increasing amounts of a prominin-1-containing Caco-2 membrane extract (Fig. 2A, lower abscissa, circles). The amount of mAb 80B258 that bound to prominin-1 upon immunoblotting of a defined amount of Caco-2 membrane extract (14  $\mu$ l; 0.1  $\mu$ g of protein per microliter) was quantified (described in Materials and Methods) and found to be  $15.6 \pm 0.4$  ng of antibody (Fig. 2A, upper



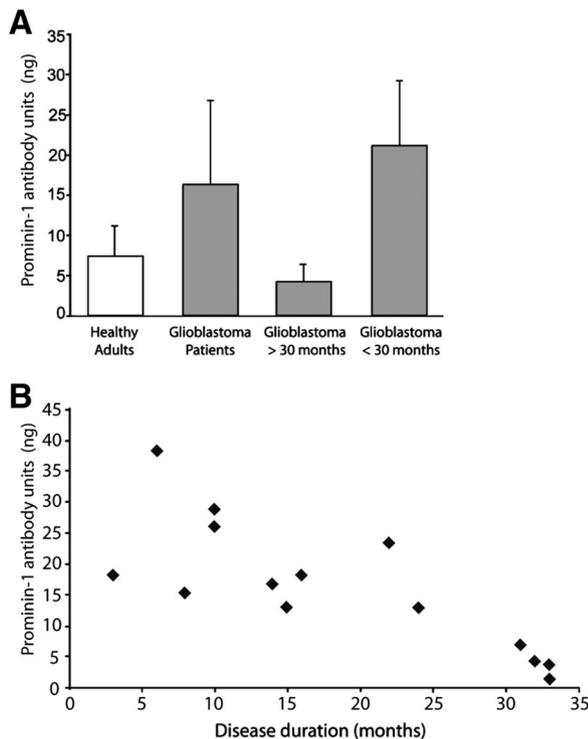
**Figure 2.** Age profile of membrane particle-associated prominin-1 in the human cerebrospinal fluid (CSF). **(A):** Standard curve. Three different amounts (8, 14, and 20  $\mu$ l) of a Caco-2 membrane extract (100 ng of protein per microliter) were subjected to immunoblotting for prominin-1 using monoclonal antibody (mAb) 80B258, immunoreactivity was quantitated by densitometry (standard exposure time, 6 minutes), and the absorbance values obtained were used to construct a standard curve ( $R^2 = 0.9401$ ). Data (circles) are the mean of five representative immunoblots; bars indicate SD. Variation of the exposure time of the five immunoblots from 2 to 12 minutes showed that absorbance values were linear ( $R^2 = 0.9538$ ) from 50 A.U. to at least 1,100 A.U. (solid line) (supplemental online Fig. 2). The scale of the upper abscissa indicates the amounts of bound mAb 80B258 corresponding to the microliters of Caco-2 membrane extract indicated on the lower abscissa, deduced from the finding that upon immunoblotting of 14  $\mu$ l of Caco-2 membrane extract for prominin-1,  $15.6 \pm 0.4$  ng (SD;  $n = 3$ ) of mAb 80B258 was bound to the filter (square symbol and error bars on upper abscissa; details given in Materials and Methods). The inset shows a representative immunoblot with the three Caco-2 standard lanes and five adult individuals. **(B):** Age profile of prominin-1 in CSF of healthy children and adults. Pellets of CSF after 1 hour at 200,000g (1 ml) were subjected to immunoblotting for prominin-1 using mAb 80B258, and the absorbance values obtained were used to calculate the amounts of bound mAb 80B258 according to the relationship shown in (A). The inset shows a representative prominin-1 immunoblot and Red Ponceau S staining of CSF from a child and an adult (individuals indicated by arrowheads in age profile plot). Asterisks indicate membrane-adsorbed albumin. Note the elevated prominin-1 immunoreactivity (arrow) but the similar concentration of albumin in the CSF of the child compared with the adult. Abbreviation: A.U., absorbance units.

abscissa, solid square), which in turn allowed us to express the absorbance values obtained after immunoblotting of human CSF (Fig. 2A, ordinate; representative example given in Fig. 2A, inset) not only as the corresponding microliter values of Caco-2 membrane extract (Fig. 2A, lower abscissa) but also as prominin-1 antibody units (in nanograms) (Fig. 2A, upper abscissa). All immunoblotting data for prominin-1 in human CSF described below (Figs. 2B, 3) are expressed as such prominin-1 antibody units.

Analysis of 61 adults without known neurological disease, who ranged in age between 17 and 85 years (Tables 1 and 2), revealed that the concentration of membrane particle-associated prominin-1 in CSF was essentially constant over adult age and showed remarkably little variation among individuals (Fig. 2B; median, 7.0 ng; mean,  $7.4 \pm 3.8$  [SD] ng of bound prominin-1

antibody; Fig. 2A, inset). On the assumption that under the present conditions of immunoblotting (described in Materials and Methods), one prominin-1 molecule (120 kDa) binds one molecule of IgG (150 kDa), the mean value of 7.4 ng of mAb 80B258 bound to prominin-1 upon immunoblotting of 1 ml of CSF would correspond to 5.9 ng of P4 membrane particle-associated prominin-1 per ml of CSF.

In contrast, analysis of 12 children without known neurological disease, who ranged in age between 1.5 and 12 years (Tables 1 and 2), revealed that the concentration of membrane particle-associated prominin-1 in the CSF of infants was much higher than that of adults (almost 10-fold higher in the first years of life), showing a steep decline postnatally until approximately 10 years of age (Fig. 2B). The higher concentration of membrane particle-associated prominin-1 in the CSF of children



**Figure 3.** Comparison of prominin-1 in the cerebrospinal fluid (CSF) of healthy adults and glioblastoma patients. (A): Pellets of CSF after 1 hour at 200,000g (1 ml) were subjected to immunoblotting for prominin-1 using monoclonal antibody (mAb) 80B258, and the absorbance values obtained were used to calculate the amounts of bound mAb 80B258 according to the relationship shown in Figure 2A. First (left) column: Healthy adults ( $n = 61$ ; age  $\geq 17$ ; data from Fig. 2B); second column: glioblastoma patients ( $n = 14$ ; Tables 1 and 2); third column: glioblastoma patients with a disease duration  $>30$  months ( $n = 4$ ); fourth column: glioblastoma patients with a disease duration  $<30$  months ( $n = 10$ ). Data are the mean  $\pm$  SD. Note the elevated prominin-1 concentration in the CSF of the glioblastoma patients compared with healthy adults (Mann-Whitney  $U$  test:  $p < .001$ ) and the marked difference in CSF prominin-1 concentration between glioblastoma patients with disease duration of  $>30$  months versus  $<30$  months. (B): Concentration of prominin-1-containing particles as a function of disease duration in glioblastoma patients.

reflected a selective increase in this stem cell marker, as the concentration of other proteins (such as plasma-derived albumin; Fig. 2B, inset) in the CSF, the concentration of total protein, and the total cell count (data not shown) were very similar in children and adults, consistent with previous observations [2, 45].

### Membrane Particle-Associated Prominin-1 in Human CSF—A Novel Parameter for Studying CNS Disease Such as Glioblastoma

We next investigated the concentration of membrane particle-associated prominin-1 in the CSF in specific neurological diseases. Given the expression of prominin-1/CD133 in glioblastoma cells [30] and its association with the myelin sheath [15] (Corbeil et al., manuscript under revision), we focused on patients with glioblastoma and remitting-relapsing multiple sclerosis (Tables 1 and 2). We also analyzed patients with polyneuropathy, a disease of the peripheral nervous system, where one might not expect altered levels of prominin-1 in the CSF.

Although the concentration of membrane particle-associated prominin-1 in the CSF of patients with polyneuropathy was essentially the same ( $7.9 \pm 4.0$  ng [ $n = 10$ ; SD] of bound

prominin-1 antibody) as in normal adults, there was a trend toward increased levels in multiple sclerosis patients ( $9.9 \pm 2.6$  ng [ $n = 8$ ; SD] of bound prominin-1 antibody;  $p = .15$ ). Remarkably, we observed significantly increased levels of membrane particle-associated prominin-1 in the CSF of glioblastoma patients ( $16.3 \pm 10.4$  ng [ $n = 14$ ; SD] of bound prominin-1 antibody; Fig. 3A, two left columns; Mann-Whitney  $U$  test:  $p < .001$ ).

Given the relatively large SD for the group of glioblastoma patients, we analyzed the individual values with regard to the duration of the disease and noticed that all values below the mean value for normal adults (i.e.,  $<7.4$  ng of bound prominin-1 antibody) were patients with a disease duration of  $>30$  months, whereas all values above the mean value for normal adults were patients with a disease duration of  $<30$  months (Fig. 3B). The concentration of membrane particle-associated prominin-1 in the CSF was dramatically different between these dichotomized subgroups of glioblastoma patients (Fig. 3A, two right columns;  $>30$  months,  $4.2 \pm 2.2$  ng [ $n = 4$ ; SD];  $<30$  months,  $21.1 \pm 8.1$  ng [ $n = 10$ ; SD]).

The glioblastoma patients were analyzed for other clinical parameters that could potentially affect the level of membrane particle-associated prominin-1 in the CSF. None of the glioblastoma patients suffered from significant hydrocephalus. Four patients showed evidence of meningeal carcinomatosis; however, no correlation between the absence and presence of meningeal carcinomatosis and the level of membrane particle-associated prominin-1 in the CSF was observed (data not shown). Tumor volume showed a broad variance (Tables 1 and 2); a relationship between tumor volume and the level of membrane particle-associated prominin-1 in the CSF was not obvious from the glioblastoma patients analyzed in the present study.

## DISCUSSION

The present demonstration that human CSF contains membrane particles carrying the somatic stem cell marker prominin-1/CD133 opens up new avenues for studying CNS disease. This finding strongly suggests that CSF has a greater potential for the diagnosis and monitoring of neurological diseases than was previously assumed, and the narrow range of membrane particle-associated prominin-1 levels in the CSF of healthy adult individuals makes this a possible application. Our observations not only show that prominin-1-containing membrane particles occur in an internal body fluid but, as far as we are aware, also represent the first demonstration that a somatic stem cell marker present in CNS tissue is released into the CSF.

Prominin-1/CD133 in CSF may therefore provide information about the activity of neural stem cells in the adult brain, not only regarding the physiologically aging brain but also in the context of neurological diseases. Activation of neural stem cells has been implicated in a wide variety of disorders, ranging from neurodegeneration to stroke [46–49], and prominin-1/CD133-expressing cancer stem cells have been shown to play a pivotal role in devastating brain tumors such as glioblastoma [24, 25, 30, 50–53]. Indeed, analysis of glioblastoma patients with a disease duration of  $<30$  months revealed significantly increased levels of membrane particle-associated prominin-1 in CSF, which presumably were due to the presence of prominin-1-positive tumor cells. Alternatively, an upregulation of prominin-1 in normal neural stem cells in the vicinity of the tumor may also occur [54].

Remarkably, the increased levels of membrane particle-associated prominin-1 were no longer observed in patients with a disease duration of  $>30$  months. In this context, it is interesting to note that in a human colon carcinoma cell line studied in vitro, the

release of prominin-1-containing membrane particles was linked to their differentiation [9]. Perhaps the lack of elevated levels of membrane particle-associated prominin-1 in CSF of glioblastoma patients with a disease duration of >30 months reflected a further dedifferentiation of the prominin-1/CD133-positive tumor cells [30], that is, a decrease in the proportion of tumor cells releasing P4 particles relative to those retaining this membrane microdomain [9]. An additional parameter to be considered in this context is oxygen tension, as it has recently been reported that prominin-1/CD133-positive stem and progenitor cells are differentially sensitive to various oxygen concentrations [55, 56].

In healthy humans, neural stem cells may have contributed to the prominin-1-containing membrane particles in CSF. Neural stem cells in the adult have been shown to extend a process into the ventricle that bears a primary cilium [43, 44], a plasma membrane protrusion recently shown to be enriched in prominin-1 and a source of P4 particles in the mouse embryo [11]. The spotty pattern of prominin-1 immunostaining of the human ventricular surface of the lateral ventricle observed in this study is consistent with this notion. Specifically, in addition to ependymal cells [50], neurogenic GFAP-positive astrocytes present in the subventricular zone, the multipotent neural stem cells, may express prominin-1/CD133, as recently suggested for the adult mouse [57], and target it to the primary cilium at the ventricular surface. The observation that there was no significant overlap between the immunostaining for prominin-1 and that for GFAP is not inconsistent with this scenario, because the primary cilium itself would not be expected to contain GFAP, and the astrocytic process extending through the ependymal layer may have been cross-sectioned upon ultrathin cryosectioning, given the cytoarchitecture of the tissue concerned [58]. Further investigation of this issue may require, for example, fluorescence-activated cell sorting using human biopsy material or immunogold EM and three-dimensional reconstruction. Nevertheless, in light of neural stem cells being a possible source of the membrane particle-associated prominin-1 in CSF, it will be interesting to explore the significance of its decline during postnatal development and maturation of the CNS.

CSF constituents (including proteins) have traditionally been considered to be soluble in nature (except for a few cells) [1–5]. The present demonstration that CSF contains specific membrane particles adds another dimension of potential analysis. The prominin-1-containing particles studied here showed a microdomain organization (resistance to the nonionic detergent Lubrol WX) that has previously been found to be characteristic of plasma membrane protrusions, such as microvilli [14]. This raises the possibility that specific membrane dynamics of cells in CNS tissue in health and disease may be revealed by analysis of CSF for these membrane particles. In this regard, myelination and demyelinating disease is particularly interesting, as a specific splice variant of prominin-1 is associated with myelin [15] (Corbeil et al., manuscript under revision). Determination of the CSF levels of membrane particles carrying the myelin-specific splice variant of prominin-1 during the course of the disease may provide *in vivo* information about both demyelination and remyelination processes [59].

Finally, given the implication of cholesterol-based membrane microdomains in signal transduction [60], the association of prominin-1 with a specific membrane microdomain [14, 17] is highly intriguing, in particular with regard to its expression in

stem cells and cancer stem cells. Prominin-1-containing membrane microdomains may contain molecular determinants conveying certain stem cell properties, and their loss (e.g., via the release of membrane particles, as described here) may contribute to cell differentiation [9, 16]. Further investigation is needed to determine whether prominin-1 itself, being a cholesterol-binding protein [14], is involved in the formation of such signal transduction platforms.

Although the present results suggest that analysis of CSF for membrane particle-associated prominin-1 (and other membrane proteins) may potentially be of clinical value, limitations of our study need to be mentioned. Inherent in it being a pilot investigation, the glioblastoma patients analyzed were heterogeneous with regard to clinical and neuroradiological data (Tables 1 and 2). In future studies, it will be important to (a) perform a longitudinal examination of homogeneous glioblastoma patients for a possible relationship between various clinical and neuroradiological parameters and the level of prominin-1-containing particles in CSF; (b) relate these levels to histopathological findings, specifically the state of tumor dedifferentiation (not only for glioblastoma but also astrocytoma of WHO degrees II and III); and (c) relate the level of prominin-1-containing particles in CSF also to the self-renewal versus differentiation potential of the normal neural stem cells from glioblastoma patients. Thus, on a general note, analysis of CSF, obtained from homogeneous patient subgroups with defined diseases and undergoing controlled treatments, for key molecules associated with distinct membrane particles released from specific cellular structures of the CNS is likely to reveal novel insights into neurological disease.

## CONCLUSION

The present study reports for the first time (a) the occurrence of membrane particles—containing the neural stem cell marker prominin-1/CD133—in the human cerebrospinal fluid, (b) its steep decline postnatally, and (c) its elevation in glioblastoma patients. Prominin-1/CD133 in the human CSF might represent a novel marker with potential relevance for studying human central nervous disease.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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