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Original Article

Somatic Stem Cell Marker Prominin-1/CD133 Is Expressed in Embryonic Stem Cell–Derived Progenitors

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ABSTRACT

Prominin-1/CD133 is a plasma membrane marker found in several types of somatic stem cells, including hematopoietic and neural stem cells. To study its role during development and with differentiation, we analyzed its temporal and spatial expression (mRNA and protein) in preimplantation embryos, undifferentiated mouse embryonic stem (ES) cells, and differentiated ES cell progeny. In early embryos, prominin-1 was expressed in trophoblast but not in cells of the inner cell mass; however, prominin-1 transcripts were detected in undifferentiated ES cells. Both ES-derived cells committed to differentiation and early progenitor cells coexpressed prominin-1 with early lineage markers, including the cytoskeletal markers (nestin, cytokeratin 18, desmin), fibulin-1, and valosin-containing protein. After spontaneous differentiation at terminal stages, prominin-1 expression was downregulated and no coexpression with markers characteristic for neuroectodermal, mesodermal, and endodermal cells was found. Upon induction of neuronal differentiation, some prominin-1–positive cells, which coexpressed nestin and showed the typical morphology of neural progenitor cells, persisted until terminal stages of differentiation. However, no coexpression of prominin-1 with markers of differentiated neural cells was detected. In conclusion, we present the somatic stem cell marker prominin-1 as a new parameter to define ES-derived committed and early progenitor cells. STEM CELLS *2005;23:791–804*

INTRODUCTION

Prominin-1 (CD133 or, for the human orthologue, AC133 antigen) [1] is a 115-/120-kDa cholesterol-binding glycoprotein [2, 3] that belongs to a growing family of pentaspan membrane proteins expressed throughout the animal kingdom [4] (for review, see [5]). Prominin-1 is expressed on several primitive cells such as hematopoietic stem and progenitor cells derived from bone marrow, fetal liver, and peripheral blood [6, 7], neural and endothelial stem cells [2, 7–9], and developing epithelium [2, 7, 10] (for review, see [11]). The expression of prominin-1 is not limited to stem and progenitor cells but also occurs in adult epithelial cells, e.g., kidney proximal tubules [2], and in nonepithelial cells, notably rod photoreceptor cells [12].

Irrespective of cell type, prominin-1 is specifically associated with plasma membrane protrusions [2, 7, 12–14], and it binds to plasma membrane cholesterol [3]. In epithelial cells, prominin-1 is selectively concentrated in microvilli present at the apical plasma membrane. The apical localization of prominin-1 is inde-

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pendent of the presence of functional tight junctions [2, 13], which are known to prevent the lateral movement of transmembrane proteins and lipids of the extracellular membrane leaflet between the apical and basolateral plasma membrane [15, 16]. It was suggested that the specific retention of prominin-1 in microvilli may play an important role in the establishment and maintenance of apical-basal polarity of epithelial cells [13, 17]. Although the physiological function of prominin-1 has not yet been established, recent studies using biochemical, morphological, and genetic approaches suggest a role for this glycoprotein in the morphogenesis or physiology of plasma membrane protrusions [3, 5].

Until now, prominin-1 was only described to be associated with somatic stem or progenitor cells. The in vitro models of undifferentiated embryonic stem (ES) and embryonic carcinoma (EC) cells allowed us to investigate prominin-1 expression in pluripotent ES cells and during in vitro differentiation. Mouse blastocysts were used to comparatively analyze the expression and localization of prominin-1 in early embryo-derived cells in vivo. The specific expression pattern suggested prominin-1 as a new marker to define ES-derived committed and early progenitor cells in vitro.

Materials and Methods

Culture of Undifferentiated ES and EC Cells and Embryoid Body Differentiation

R1 ES cells [18] were cultivated on the feeder layer (FL) of inactivated mouse embryonic fibroblasts in culture medium I (Dulbecco's modified Eagle's medium [DMEM] supplemented with 15% fetal calf serum [FCS] [Invitrogen GmbH, Karlsruhe, Germany, http://www.invitrogen.com]) and human leukemia inhibitory factor (LIF) [19] containing additives I (2 mM L-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin [all from Invitrogen], and 5×10^{-5} M β-mercaptoethanol [Serva, Heidelberg, Germany, http://www.serva.de]). After dissociation with 0.2% trypsin/0.02% EDTA, cells were plated onto 0.1% gelatin-coated 60-mm tissue culture plates (Nunc, Wiesbaden, Germany, http://www.nuncbrand.com) and cultivated as undifferentiated cells in subconfluent monolayer for 6 and 48 hours, respectively. To generate colonies, cells (n = 2,000/60-mm plates) were kept in culture medium I for 7 days and differentiated in the absence of LIF for 2–28 days. Reverse transcription–polymerase chain reaction (RT-PCR) and immunofluorescence analyses were performed at defined time points. P19 EC cells [20] were cultivated under the same conditions in culture medium I containing additives I but in the absence of LIF for up to 28 days.

For embryoid body (EB) formation, R1 cells (n = 600) were cultivated first in hanging drops for 2 days and then in bacteriological Petri dishes for 3 days in culture medium II (Iscove's modified Dulbecco's medium [Invitrogen] supplemented with 20% FCS, 450 µM α-monothioglycerol [MTG] [Sigma, St. Louis, http://www.sigmaaldrich.com]) containing additives I. At day 5, EBs were plated onto 0.1% gelatin-coated 60-mm tissue culture plates [19] and differentiated in culture medium II containing additives I for up to 23 days. Samples for RT-PCR and immunofluorescence analyses were collected in stages at 5 + 2, 5 + 9, 5 + 16, and 5 + 23 days.

Differentiation of ES Cells into Neuronal Cells

Neuronal differentiation of R1 cells was performed according to Okabe et al. [21] with modifications [22]. Briefly, R1 cells (n = 200) were cultured as EBs in hanging drops and in bacteriological Petri dishes containing culture medium II supplemented with additives I for 2 days each. At day 4, EBs (n = 20 to 30) were transferred to gelatin-coated 60-mm tissue culture plates and differentiated in culture medium II (without MTG) containing additives I. One day later, the medium was exchanged with differentiation medium I (DMEM/F12 [Invitrogen] supplemented with "ITSFn" additives, including 5 µg/ml insulin, 30 nM sodium selenite, 50 µg/ml transferrin [all from Sigma], and 5 µg/ml fibronectin [Invitrogen]). The medium was replaced every second day. At days 4 and 8, EBs were dissociated by treatment with 0.1% trypsin/0.08% EDTA in phosphate-buffered saline (PBS) for 1 minute, collected by centrifugation, replated onto poly-L-ornithine/laminin–coated tissue culture plates, and cultured in differentiation medium II (DMEM/F12 containing 20 nM progesterone, 100 µM putrescin, 1 µg/ml laminin, 25 µg/ml insulin, 50 µg/ml transferrin, 30 nM sodium selenite [all from Sigma], basic fibroblast growth factor [bFGF] [10 ng/ml], and epidermal growth factor [EGF] [20 ng/ml; Strathmann Biotech, Hannover, Germany, http://www.biotec-ag.de]) for 6 days until days 4 and 13. The medium was replaced every second day, but bFGF and EGF were added daily. At days 4 and 14, neuronal cells were induced by differentiation in Neurobasal medium supplemented with 2% B27 (all from Invitrogen), 10% FCS, interleukin-1β (IL-1β) (200 pg/ml; PeproTech, London, http://www.peprotech.com; added daily), and db-cAMP (700 µmol; Sigma, added every 4 days beginning at days 4 + 14 until days 4 + 32) [22].

Isolation and Cultivation of Blastocysts

Female NMRI mice (8–10 weeks old) in the proestrus stage were used for mating overnight and examined for the presence of vaginal plugs at day 0.5 postconceptionem (p.c.). Embryos (3.5 days p.c.) were recovered from uteri by flushing with PBS supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ (Ca/Mg-PBS), and 20% FCS. Blastocysts were kept in suspension for 4 days or plated onto 0.1% gelatin-coated four-well dishes (Nunc) in embryo culture medium (I:II = 1:1) containing additives I.

Semiquantitative and Quantitative RT-PCR

Transcript abundance of prominin-1, nanog, and nestin was analyzed by semiquantitative RT-PCR. Total RNA was isolated from cells or tissues by the single-step extraction method [23], reverse transcribed, and amplified (Perkin Elmer, Überlingen, Germany, http://www.perkinelmer.com). PCR products were resolved by electrophoresis in 2% agarose gel and stained with ethidium bromide as described previously [19]. Blastocysts were collected in lysis buffer (Dynal Biotech, Oslo, Norway, http://www.dynal.no), and mRNA was isolated using Dynalbeads mRNA DIRECT micro kit (Dynal Biotech). mRNA isolation and RT amplification were performed according to the manufacturer's instructions. The following oligonucleotide primers were used (antisense and sense primer, annealing temperature, number of cycles, length of the amplified fragment): nanog (5'-CTGGGAACGCCTCATCAA-3', 5'-CATCTTCTGCTTCCTGGCAA-3', 63°C, 40 cycles, 239 bp), prominin-1 (5'-ACCAACACCAAGAACAAGGC-3', 5'-GGAGCTGACTTGAATTGAGG-3', 60°C, 40 cycles, 349 bp), and nestin (5'-CTACCAGGAGCGCGTGGC-3', 5'-TCCACAGCCAGCTGGAACTT-3', 60°C, 35 cycles, 220 bp). The housekeeping gene β-tubulin (5'-TCACTGTGCCTGAACTTACC-3', 5'-GGAACATAGCCGTAAACTGC-3', 60°C, 28 cycles, 317 bp) was used as internal standard for both RT-PCR and quantitative PCR (Q-PCR) reactions. The mRNA level of a given gene was normalized to the corresponding β-tubulin level, and the percentage of mRNA level relative to the highest transcript level was presented [19].

Q-PCR reactions were performed as previously described [24] on RNA extracted from ES and EC cells and fetal and adult tissues using primers for fibulin 1 (Fbln1, 5'-TGCCCACCTTTCGAGAGTTC-3'; 5'-CGAAGGTTCCCTTCTGTGATG-3') and, as a control, M5 β-tubulin (5'-GAAGAGGAGGCCTAACGGCAGAGAGCCCT-3'; 5'-GAGTGCCTGCCATGTGCCAGGCACCATTT-3').

Identification of Differentiation Markers by Serial Analysis of Gene Expression

Four serial analysis of gene expression (SAGE) catalogs (available at http://www.ncbi.nlm.nih.gov/sage/) were compared to identify differentiation-responsive markers from undifferentiated ES (R1) [25] and EC (P19) cells and from EC cells after differentiation to a committed state (P19: 3 + 0.5 days) or to a differentiated state (3 + 3.0 days) [24]. We had previously identified 1,498 tags that demonstrated significant changes ($p < .05$) in abundance with differentiation of P19 cells [24], but when the ES and EC catalogs were combined, we were able to query approximately 60,000 unique tags (i.e., short nucleotide sequences [14 bp] that are used to identify transcripts). Catalog comparisons were performed using SAGE 2000 software after uploading DNA sequences from GenBank (nonredundant [NR] and expressed sequence tags [EST]; Release 131.0, available at http://www.ncbi.nlm.nih.gov/sage [National Center of Biotechnology Information]). Databases were queried for *Gene-to-Tag* matches as previously described [24]. The link to the NR rodent database produced direct matches to named genes. In the cDNA mode, the SAGE 2000 analysis software identifies and extracts only those tags that match cDNAs at the 3' end of a sequence. These data were then transferred into Excel (Microsoft, Redmond, WA, http://www.microsoft.com) and searched for key words (cytoskeleton, intermediate filament, filamin, and tubulin but not actin). Those tags/transcripts that contained these identifiers were consolidated, and only those transcripts that increased in abundance with differentiation were analyzed further. Tags that only matched ESTs with similarity to cytoskeletal-associated proteins or that were poorly abundant (<2 tags in the committed or differentiated EC libraries) were excluded from these analyses. Statistical analyses between libraries were as previously described [24].

Immunofluorescence and Confocal Imaging Analyses

Immunofluorescence analysis of undifferentiated cells, differentiated derivatives, and cultivated blastocysts was done as described [13]. Briefly, cells grown on coverslips were washed with Ca/Mg-PBS, first at room temperature (r.t.) and then on ice, and labeled for 30 minutes at 4°C by addition of the monoclonal rat 13A4 (anti–prominin-1 antibody; 10 μg/ml) or rabbit αI3 antiserum [2] diluted in immunofluorescence buffer (Ca/Mg-PBS containing 0.2% gelatin). Unbound antibodies were removed by five washes with ice-cold immunofluorescence buffer. The coverslips were either fixed with methanol:acetone (7:3) at –20°C for 10 minutes or with 3% paraformaldehyde at r.t. for 30 minutes. Goat serum (10% in PBS) or bovine serum albumin (BSA) (1% in PBS) was used to inhibit unspecific labeling. The fixative was removed by three washes with Ca/Mg-PBS, and the residual formaldehyde was quenched for 30 minutes with 0.1 M glycine in PBS. The following primary antibodies (definition, dilution) were used: Nestin (rat 401, 1:3), stage-specific embryonic antigen-1 (SSEA-1) (MC-480, 1:10; both from Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww), βIII-tubulin (clone TU-20, 1:150), oligodendrocyte-specific protein (1:150; both from Chemicon, Hofheim, Germany, http://www.chemicon.com), cytokeratin 18 (clone KS-B17.2, 1:100), E-cadherin (anti-uvomorulin, clone Decma-1, 1:100; both from Sigma), desmin (1:50; DakoCytomation, Carpinteria, CA, http://www.dakocytomation.us), glial fibrillary acidic protein (GFAP) (clone G-A-5, 1:150), vimentin (clone Vim 3B4, 1:20; both from Roche Molecular Biochemicals, Mannheim, Germany), fibulin-1 (1:50), valosin-containing protein (VCP) (1:50), frizzled-related protein 2 (FRP-2) (1:50; both from Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), platelet-endothelial cell adhesion molecule-1 (PECAM-1) (1:20; Abcam, Cambridge, U.K.,

http://www.abcam.com), and vascular endothelial (VE)-cadherin (1:20; R&D Systems, Wiesbaden, Germany, http://www.rndsystems.com). Cells were incubated with the antibodies at 37°C in a humid chamber for 60–90 minutes. After three washes with Ca/Mg-PBS, cells were incubated with fluorochrome-conjugated secondary antibodies (Cy3 anti-mouse immunoglobulin M [IgM], rat immunoglobulin G [IgG], or goat IgG [1:600] and/or fluorescein isothiocyanate [FITC] anti-mouse IgG or IgM, rabbit IgG, and rat IgG [1:100]; Jackson ImmunoResearch, Dianova, Hamburg, Germany, http://www.jacksonimmuno.com). As a control for the cell-surface labeling of prominin-1, R1 cells grown on coverslips (48 hours) and cultivated blastocysts (3.5 + 4 days) were fixed with 3% paraformaldehyde (PFA) at r.t. for 30 minutes, permeabilized with saponin (0.2%), and incubated with rat monoclonal antibody (mAb) 13A4 (anti–prominin-1 antibody; 10 μg/ml) according to Corbeil et al. [7]. To control for unspecific binding of the primary antibody, R1 (48-hour) cells were labeled by the secondary antibodies Cy3 goat anti-rat (used for 13A4 antibody) and FITC mouse anti-rabbit (used for rabbit polyclonal antibody), respectively. Hoechst 33342 (5 μg/ml) was used to label the nuclei. Specimens were analyzed for percentage of labeled cells per Hoechst 33342–positive cells with the fluorescence microscope (Nikon, GmbH, Düsseldorf, Germany, http://www.nikon-instruments.jp/eng) or the confocal laser-scanning microscope (CLSM 410, Carl Zeiss, Jena, Germany, http://www.zeiss.com) by using the following excitation lines/barrier filters: 364 nm/450 to 490 band pass (BP) (Hoechst 33342), 488 nm/510 to 525 BP (FITC), and 543 nm/570 long pass (LP) (Cy3).

Immunohistochemistry of EBs

EBs collected by sedimentation at day 5 were washed twice with PBS and fixed in Bouin's solution (75 ml picric acid, 25 ml 4% formaldehyde, 5 ml acetic acid) for 2 hours at r.t. After two washes with 70% ethanol, samples were processed through graded ethanol (96%, 80%, 70%, and 50%, 5 minutes each at r.t.) and EBs were embedded in paraffin, sectioned at 5-μm slices, and mounted on silanized slides using conventional techniques. Slides were deparaffinized at 60°C for 2 hours, rehydrated at r.t. in xylene (5 minutes) and isopropanol (5 minutes), processed through graded ethanol (see above), and washed in distilled water (3 minutes) and three times in PBS (5 minutes each). The further processing of immunohistochemical analysis was performed as described above beginning with serum or BSA treatment to inhibit unspecific labeling.

Bromodeoxyuridine Labeling

The proliferation of prominin-1–labeled cells after 48 hours (R1, P19) and 7 days (R1) of cultivation and of R1 EB outgrowths (5 + 9 days) was estimated by bromodeoxyuridine (BrdU) labeling. BrdU (10 μmol/l) was added to the cells for 1 hour. Fixation and staining were done according to manufacturer's instructions (Roche). Cells were immunolabeled for prominin-1 followed by Hoechst 33342 incubation. Areas (n = 10) of each coverslip were randomly selected, and the percentage of BrdU-labeled prominin-1–positive cells was estimated.

Membrane Preparation and Western Blotting Analysis

Undifferentiated R1 cells, R1 cells cultivated in the absence of LIF and after EB formation (5 + 2, 5 + 16, and 5 + 23 days), and P19 cells were analyzed. Cells were washed three times with PBS, scraped from the dish in ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), and centrifuged at 1,000g for 5 minutes. The cell pellets were then frozen at –80°C. Membrane preparations were performed at 4°C. Briefly, cell pellets (75–200 μl) were resuspended in 1 ml homogenization buffer (300 mM sucrose, 1 mM EDTA, 10 mM HEPES-KOH [pH 7.5], 1 mM PMSF, 2 μg/ml leupeptin, 10 μg/ml aprotinin), and the cell suspension was passed eight times back and forth through a 0.70 × 30-mm needle attached to a 1-ml syringe followed by eight passages through a ball-bearing homogenizer (12-μm clearance, European Molecular Biology Laboratory, Heidelberg, Germany, http://www.embl.org). The homogenate was centrifuged at 1,000g for 10 minutes, and the resulting postnuclear supernatant was centrifuged for 1 hour at 40,000g in an Optima MAX ultracentrifuge (Beckman, Fullerton, CA, http://www.beckman.com) using a TLA 120.2 rotor. The membrane pellet was resuspended in 1% SDS, 20 mM sodium phosphate, pH 7.5, and boiled for 2 minutes. Protein concentration was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL, http://www.piercenet.com).

Proteins (100 μg) were analyzed by SDS-PAGE and transferred to poly-vinylidene difluoride membranes (Millipore Corp., Bedford, MA, http://www.millipore.com; pore size, 0.45 μm) using a semidry transfer cell system (Cti, GmbH, Idstein, Germany, http://www.elektrophorese-pro.de). After transfer, membranes were incubated at 4°C in blocking buffer (PBS containing 5% low-fat milk powder and 0.3% Tween 20) overnight. Prominin-1 was then detected using rat mAb 13A4 (1 μg/ml) followed by the incubation with horseradish peroxidase–conjugated secondary antibody, all diluted in blocking buffer. Antigen-antibody complexes were visualized using enhanced chemiluminescence (ECL system, GE Healthcare, Chalfont St. Giles, U.K., http://www1.amershambiosciences.com). For quantification, nonsaturated exposures were scanned with a Powerlook 110 scanner (Umax) and quantified using MacBas software (Raytest Isotopenmessgeräte GmbH, Pforzheim, Germany, http://www.raytest.com).

Fluorescence-Activated Cell Sorter Analysis

Undifferentiated ES cells (R1, 6 hours, 48 hours, 7 days) cultivated in the presence of LIF or 14 days after LIF removal (R1, 7 + 14 days) and P19 cells (48 hours) were dissociated with accutase (PAA Laboratories GmbH, Linz, Austria, http://www.paa.at)

for 5–10 minutes at r.t., washed in Ca/Mg-PBS (two times), and incubated with the primary antibodies against anti–prominin-1 (see above). After rinsing in Ca/Mg-PBS (three times), cells were treated with the fluorescence-labeled secondary antibody Alexa Fluor 488 anti-rat IgG (1:100, Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Fluorescence was measured with the FACSAria (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) flow cytometer equipped with FACSDiva Software. Quantitative analysis was done with FCS Express (De Novo Software, Thornhill, Ontario, Canada, http://www.denovosoftware.com).

Results

Transcript Abundance of Prominin-1, Nanog, and Nestin

Prominin-1 transcripts were assayed as a function of the differentiation status in undifferentiated ES and EC cells, in cells committed to differentiation (48 hours), and after differentiation. Given the strong expression of prominin-1 in kidney [2], this tissue was used as positive control (i.e., 100%). Nanog was used as a marker for undifferentiated ES cells, whereas nestin was used for committed and neural progenitor cells.

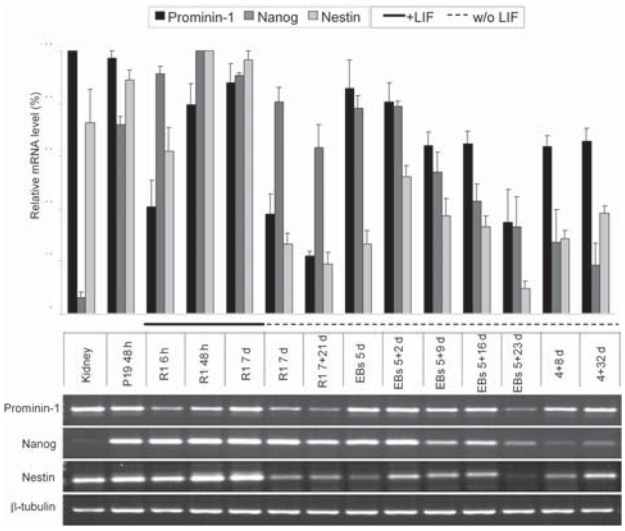

Figure 1. Prominin-1, nanog, and nestin transcript levels analyzed by semiquantitative reverse transcription–polymerase chain reaction. Nanog, prominin-1, and nestin transcripts were determined in adult kidney tissue. P19 (48 h) cells, undifferentiated R1 cells (6 h, 48 h), R1 cells cultivated as colonies for 7 days (7 d) in the presence of LIF, R1 cells further cultivated as monolayer in the absence of LIF for 7 and 21 days (w/o LIF; 7 d, 21 d), EBs cultivated in suspension for 5 days (5 d), EB outgrowths (5 + 2 d, 5 + 9 d, 5 + 16 d, 5 + 23 d), and ES-derived cells after neuronal differentiation at stages 4 + 8 d and 4 + 32 d were analyzed. The housekeeping gene β-tubulin was used as internal standard. Each value ($n = 3$ experiments) represents mean ± SEM. Abbreviations: EB, embryoid body; ES, embryonic stem; LIF, leukemia inhibitory factor.

In R1 ES cells cultured for 6 hours after passage (without FL), a low level of prominin-1 transcript was observed (~40% of the positive control), but the amount doubled when the cells were kept in an undifferentiated state for up to 48 hours (Fig. 1). The amount of prominin-1 transcript was also elevated in R1 colonies cultured for 7 days and in early EBs (5 days; 5 + 2 days), but it slightly decreased in EB outgrowths differentiated for 9 and 16 days and after neuronal differentiation (stages 4 + 8 days and 4 + 32 days). Interestingly, the cultivation of R1 cells for 7 and 21 days in the absence of LIF and spontaneous differentiation of EB outgrowths for 23 days reduced the prominin-1 transcript level twofold to threefold (Fig. 1). In P19 EC cells, the maximum level of prominin-1 transcript was found in cells cultured for 48 hours (Fig. 1). A downregulation of prominin-1 transcript was also observed in P19 cells cultured for a longer period, that is, 21 days (data not shown).

As expected, nanog transcripts were highly abundant in undifferentiated R1 cells cultured for 6 and 48 hours (the latter was set to 100%) and nearly absent in kidney (Fig. 1). Similar levels were measured in ES cells cultured in the presence of LIF for 7 days, in 5-day EBs, and in early EB outgrowths. Upon LIF removal or after spontaneous and neuronal differentiation, nanog was downregulated (Fig. 1). In parallel, we analyzed the level of nestin transcript, a neural stem cell–associated intermediate filament protein [26] but also a potential marker of multilineage progenitors [27]. Nestin transcripts were found abundantly in undifferentiated R1 cells at 48 hours (set to 100%) and in R1 cells grown for 7 days in the presence of LIF. Undifferentiated R1 cells cultured for only 6 hours showed lower level of nestin transcript (~60%). A significant decrease of nestin transcript was observed in R1 cells differentiated for 7 days in the absence of LIF and at terminal differentiation stages (10%–20%; 21 days, 5 + 23 days). The nestin transcript level was at an intermediate level after neuronal differentiation at progenitor and terminal stages (30%–40%; 4 + 8 and 4 + 32 days; Fig. 1).

These data revealed that the transcript level of prominin-1 generally parallels that of nestin in ES cells, i.e., upregulation during the commitment to differentiation (R1 48 hours and 7 days with LIF) and downregulation after differentiation (R1 7 days and 21 days without LIF). Together with the observation that the level of nanog transcript is largely constant in undifferentiated R1 cells, irrespective of the commitment status (6 hours, 48 hours, and 7 days), these observations suggested that prominin-1 and nestin may play a role during commitment and early differentiation of ES cells in vitro; but to further characterize the cells, additional markers are necessary.

Identification of Molecular Markers That Are Differentiation Responsive

To identify potential markers of early differentiation, we queried four SAGE library catalogs for potential early genes. Our rationale was that cytoskeletal-associated proteins are such markers,

because they generally affect the distribution of membranous organelles and determine cell shape and cell polarity, which are hallmarks of differentiation. By identifying tags/transcripts that were absent or poorly expressed in undifferentiated ES and EC cells and that significantly ($p < .05$) increased in abundance with differentiation, we postulated that these proteins would be useful markers for early differentiation (ECs 3 + 0.5 and 3 + 3.0 represent cells that differentiated for 0.5 and 3.0 days, respectively, after plating of 3-day EBs).

A total of 169 cytoskeleton-associated tags could be specifically identified from the SAGE libraries. Several gene products (e.g., Tubulin 1a or 16 [Tuba1/Tuba6], Internexin, a neuronal intermediate filament protein, alpha [Ina]) showed significant decreases in tag abundance with differentiation (data not shown), but only five tags showed increased abundance with differentiation (Table 1). Three of these tags were associated with cytokeratin 18 (Krt1-18) and 19 (Krt1-19) or fibulin 1 (Fbln1) transcripts; however, each of these tags also matched one other sequence within the public domain (Table 1). It was therefore unclear if these tags actually corresponded to cytoskeletal-associated proteins. However, cytokeratins 8 and 18 are some of the earliest cytoskeletal components known to be coordinately expressed at four- to eight-cell-stage embryos [28], suggesting that these products represent authentic differentiation markers. Unlike Krt1-18, cytokeratin 8 (Krt2-8) did not show a significant differentiation response among the SAGE libraries: only four tags were present in one P19 SAGE catalog (3 + 0.5 days), and this tag sequence (AGCATTCATA) was absent in the three other catalogs. By Q-PCR, we also found that fibulin 1 is in fact differentiation-responsive (data not shown). Fibulin-1 is highly expressed in P19 cells at day 3 + 0.5, before decreasing by day 3 + 3 (Table 2), and it is prominent in fetal and adult hearts and lungs.

One other potential differentiation-responsive tag corresponded to VCP, which has been shown to be important for membrane fusion, nuclear trafficking, and cell proliferation at the level of both cell division and apoptosis [29]. Because this specific VCP tag increases with in vitro differentiation and is abundant in mouse forelimb SAGE libraries, we have included this product as a differentiation-responsive transcript.

Colocalization of Prominin-1 with Proteins Characteristic for Early Differentiating ES and EC Cells

Next we investigated the expression of prominin-1 protein and its distribution in undifferentiated and early differentiating R1- and P19-derived cells by indirect immunofluorescence using rat mAb

Table 1. Comparative SAGE analyses and targeted in silico analysis of ES (R1) and EC (P19) cells and differentiating EC-derived cells

Tag sequence	Gene	R1 (tpm)	P19 (tpm)	3 + 0.5 (tpm)	3 + 3.0 (tpm)
CAAACTGTGC	Krt1-18 keratin complex 1, acidic, gene 18	22	15	549	94
	5730406I15Rik RIKEN cDNA 5730406I15 gene	22	15	549	94
CTGTGGAAGA	Krtap16-9 keratin-associated protein 16-9	80	77	98	132
	4632415H16Rik RIKEN cDNA 4632415H16 gene	80	77	98	132
GACATCAAGT	Krt1-19 keratin complex 1, acidic, gene 19	7	0	59	132
	Mus musculus 12-day embryo eyeball cDNA, RIKEN full-length enriched library, clone: D230036K21, product: unknown EST, full insert sequence	7		59	132
TGCCAGGCAC	Tubb5 tubulin, beta 5	174	154	235	264
CAGCTGGCCA	Fbln1, Fibulin-1	58	46	313	113
	6030460N08Rik, RIKEN cDNA	58	46	313	113
GCCTGTTCCA	Vcp, Valosin-containing protein	145	308	314	415[a]

[a]Significance relative to ES R1 cells.
Abbreviations: EC, embryonic carcinoma; ES, embryonic stem; tpm, tags per million.

Table 2. BrdU labeling index of prominin-1–positive P19 (48-hour), R1 (48-hour), and differentiated R1 EB-derived (5 + 9-day) cells

Cell line and cultivation stage	Percentage of BrdU-positive cells of total Hoechst 33342–labeled cells	Percentage of BrdU-positive cells in the prominin-1–labeled cell population
P19 48 h	59.5% ± 5.8%	58.1% ± 5.95%
R1 48 h	50.8% ± 6.7%	31.3% ± 5.1%[a]
R1 EBs 5+9 d	34.3% ± 4.8%[a,b]	16.4% ± 3.4%[a,c]

For each value, 10 areas of each coverslip ($n = 4$ to 6 per experiment) of two independent experiments were analyzed for the percentage value of BrdU-positive (+) cells of Hoechst 33342–labeled cells (middle column) and the percentage of BrdU-positive cells in the prominin-1–labeled population (right column). Values represent mean ± SD. Statistical significance was tested by the paired Student's *t*-test. Significance values:
[a]$p \le .001$ versus 48-hour P19.
[b]$p \le .001$ versus 48-hour R1 cells.
[c]$p \le .01$ versus 48-hour R1 cells.
Abbreviations: BrdU, bromodeoxyuridine; EB, embryoid body.

13A4 [2], which recognizes an epitope localized in the second extracellular loop of prominin-1 [12]. Antibodies against proteins characteristic of undifferentiated cells, e.g., SSEA-1 [30], and of early differentiated cells, e.g., CK18, nestin, desmin, FRP-2, fibulin-1, and VCP, were also used.

In undifferentiated R1 cells cultivated in the presence of LIF for 6 hours (or in the presence of mouse embryonic feeder layer cells, data not shown), fewer than 10% of prominin-1–positive cells were observed (Figs. 2A, 3A, 3B), although 28% of the cells were nestin-positive (Fig. 2A). However, in R1 cells that are committed to differentiation (48 hours), prominin-1 immunoreactivity was easily detectable. Interestingly, prominin-1–positive cells mainly localized at the periphery of R1 cell colonies (Figs. 2B–2H). In contrast, undifferentiated cells expressing SSEA-1

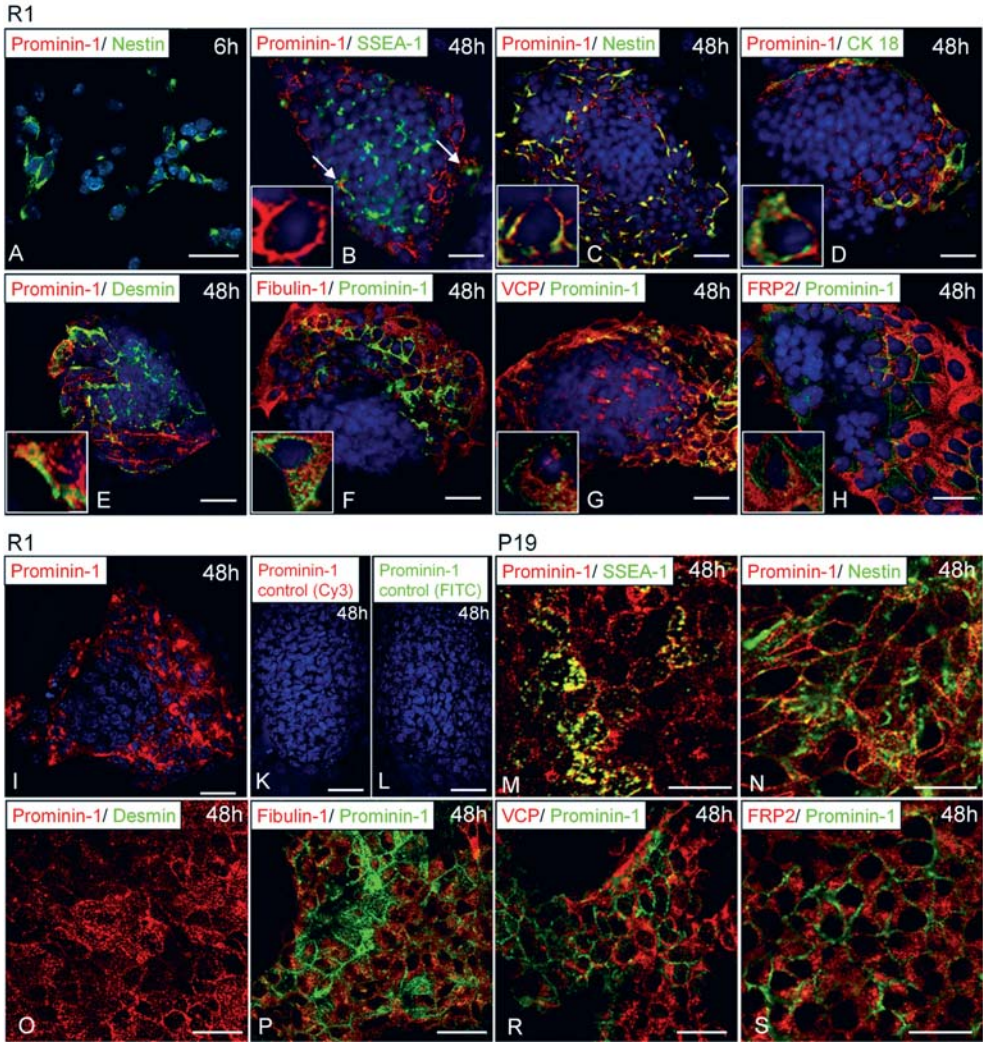

Figure 2. Double immunofluorescence and confocal imaging analysis of prominin-1 (cell-surface labeling) and various intracellular marker proteins in R1 and P19 cells cultured for 6 hours (R1) and 48 hours (R1, P19). **(A):** Six-hour R1 cells showed no prominin-1–positive cells, whereas nestin-positive cells (green) were detected in approximately 28% of cells. **(B):** R1 (48-hour) cells showed almost no coexpression of prominin-1 (red) and SSEA-1 (green). SSEA-1–positive cells were localized in the center of ES cell aggregates, whereas prominin-1–positive cells were restricted to peripheral regions of the ES cell colonies. Prominin-1 was localized to the cell membrane (inset). **(C–H):** R1 (48-hour) cells showed partial coexpression of **(C)** prominin-1 (red) and nestin (green), **(D)** CK18 (green), and **(E)** desmin (green) and areas colabeled by **(F)** prominin-1 (green) and fibulin-1 (red), **(G)** VCP (red), and **(H)** FRP-2 (red), respectively, at the periphery of R1 cell aggregates. Hoechst 33342 (blue) was used to visualize cell nuclei. Insets show intracellular localization of proteins at higher magnification. As a control for the cell-surface labeling of **(B–H)** prominin-1, R1 (48-hour) cells after fixation with paraformaldehyde were saponin-permeabilized and incubated with prominin-1 antibody. Prominin-1 labeling was again detected at **(I)** the peripheral region of cell aggregates (red). **(K–L):** To control unspecific binding, R1 (48-hour) cells were labeled only with the secondary antibodies (Cy3 goat anti-rat antibody [for rat monoclonal 13A4 antibody] [K], fluorescein isothiocyanate mouse anti-rabbit [for rabbit polyclonal antibody] [L]). **(M–S):** P19 cells cultured for 48 hours showed prominin-1 (red) expression in almost all cells and a partial coexpression with **(M)** SSEA-1 (green) and **(N)** nestin (green) but no colabeling with **(O)** desmin (green). P19 cells were colabeled by **(P)** prominin-1 (green) and fibulin-1 (red), **(R)** VCP (red), and **(S)** FRP-2 (red), respectively. Bars = 30 μm. Abbreviations: ES, embryonic stem; FRP, frizzled-related protein; SSEA, stage-specific embryonic antigen; VCP, valosin containing protein.

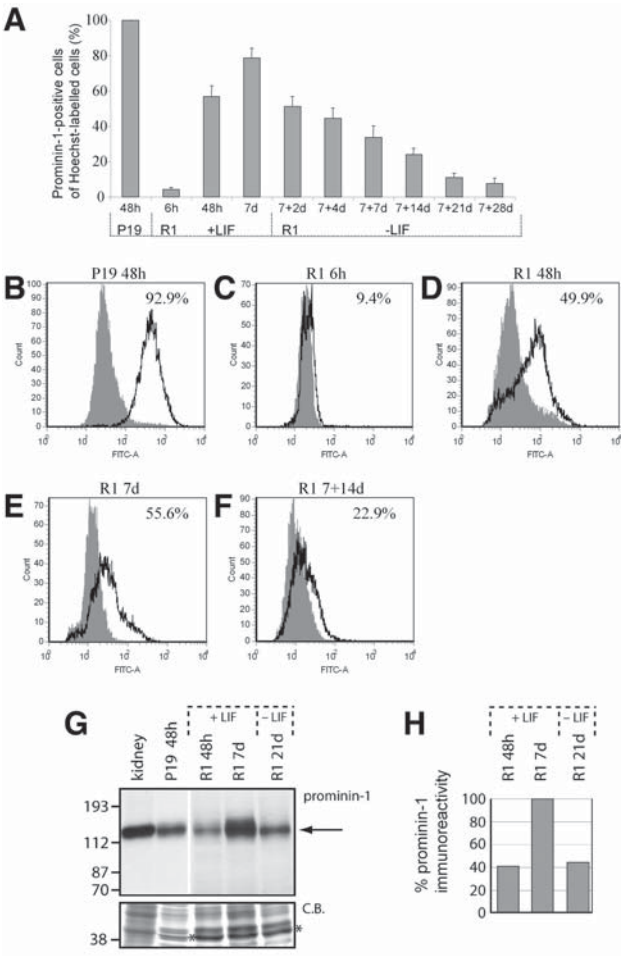

Figure 3. Semiquantitative immunofluorescence, FACS, and Western blot analysis of prominin-1 in P19 (48-hour) and R1 cells. **(A):** Semiquantitative immunofluorescence analysis for prominin-1–positive Hoechst 33342–labeled P19 cells (48 hours); R1 cells cultured in the presence of LIF (6-hour, 48-hour, and 7-day colonies); and R1 cells differentiated for 2–28 days in the absence of LIF. For each value, 10 areas (~1,000 cells) of each coverslip ($n = 3$) of three independent experiments were analyzed. Values represent mean ± SD. **(B–F):** FACS analysis of prominin-1 immunoreactivity in **(B)** P19 cells (48 hours), **(C)** R1 6-hour cells, **(D)** R1 48-hour cells, **(E)** R1 7-day colonies, and **(F)** R1 cells after differentiation for 14 days (7 + 14 days) in the absence of LIF. The histograms represent the fluorescence of the prominin-1–immunolabeled cells (black) compared with corresponding control cells (gray; labeled with only secondary antibody) after gating the cells in a forward/side-scatter dot plot. **(G):** Western blotting analysis of prominin-1 (top panel, arrow) in undifferentiated R1 cells (48-hour and 7-day colonies) and R1 cells after further differentiation for 21 days (7 + 21 days). Adult kidney membrane and P19 (48-hour) cells were used as positive control. In each sample, the total amount of protein was visualized by Coomassie-blue staining (C.B.) (bottom panel). **(H):** Quantification of prominin-1 immunoreactivity during the differentiation of R1 cells. Prominin-1 immunoreactivity detected in **(G)** was quantified by densitometric scanning, normalized to the amount of protein detected by C.B. (reference band indicated by asterisks) **(G)**, and plotted as percentage of prominin-1 immunoreactivity detected in undifferentiated R1 cells (7-day colonies). Each value represents the mean of two independent experiments. Abbreviations: FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; LIF, leukemia inhibitory factor.

were located in central areas of the aggregates (Fig. 2B). No coexpression of prominin-1 and SSEA-1 was observed (Fig. 2B, inset), except in singular cells at the clone periphery (Fig. 2B, see arrow), demonstrating that prominin-1 is not present in undifferentiated SSEA-1–positive ES cells and localizes to a distinct cell population at the periphery of ES cell colonies.

In contrast to SSEA-1, nestin was highly coexpressed with prominin-1 at peripheral regions of ES cell clusters cultured for 48 hours (Fig. 2C). A partial coexpression of prominin-1 was also observed with fibulin-1 (Fig. 2F), VCP (Fig. 2G), and FRP-2 (Fig. 2H) and the intermediate filament proteins CK18 (Fig. 2D) and desmin (Fig. 2E). Like prominin-1, CK18, which is expressed during embryonic development in early endodermal and epithelial cells [31], was expressed by cells located mostly at the periphery of R1 cell aggregates (Fig. 2D). Control experiments with saponin permeabilization revealed that prominin-1 labeling was indeed located at the periphery of the ES cell aggregates (Fig. 2I), consistent with the results of prominin-1 cell-surface labeling without membrane permeabilization (Figs. 2B–2H). Control experiments showed no unspecific binding of the secondary antibodies in R1 (48-hour) cells (Figs. 2K, 2L). This coexpression pattern of prominin-1 with markers of early differentiation states suggests that prominin-1 may be involved in commitment and early differentiation of ES cells.

With regard to undifferentiated P19 EC cells, which do not grow as compacted cell colonies as R1 cells but rather grow in a homogeneous monolayer, prominin-1 immunoreactivity was detected in nearly all cells cultured for 48 hours (Figs. 2M–2S). The typical punctuate membrane staining observed suggests its association with microvilli, as previously reported [13].

Some prominin-1–positive P19 cells expressed SSEA-1 (Fig. 2M), which contrasts with the situation observed with R1 cells (Fig. 2B). Several prominin-1–positive P19 cells also expressed nestin (Fig. 2N). No desmin was observed in undifferentiated P19 cells (Fig. 2O). Cytoplasmic proteins typical for ES cells committed to differentiation, such as fibulin-1, VCP, and FRP-2, were also detected in most prominin-1–positive P19 cells (Figs. 2P–2S, respectively).

Proliferation Analysis of Prominin-1–Positive Cells

To analyze the proliferation of prominin-1–positive cells, R1 and P19 cells were tested for BrdU incorporation. P19 cells cultured for 48 hours showed the highest BrdU labeling index (59.5%), followed by R1 cells (50.8%), whereas EB outgrowths after spontaneous differentiation showed a significantly lower value (34.4%) (Table 2). The maximal amount of BrdU-labeled/prominin-1–positive cells was found in P19 cells (58.1%), whereas significantly lower numbers of BrdU-labeled/prominin-1–positive cells were detected in undifferentiated R1 cells (31.3%) and in EB outgrowths after differentiation (16.4%) (Table 2).

These observations suggest that in undifferentiated P19 EC cells with a short cell cycle phase of 9–11 hours [32], prominin-

1 is expressed in approximately 60% of the cells, whereas in R1 ES cells with a longer cell cycle phase (~12–14 hours), half of the cycling cell population expressed prominin-1. In differentiating ES cells (5 + 9 days), a lower percentage of prominin-1–positive cells is BrdU-labeled (Fig. 4A), suggesting that the proliferation efficiency of prominin-positive cells decreases when ES cells start to differentiate.

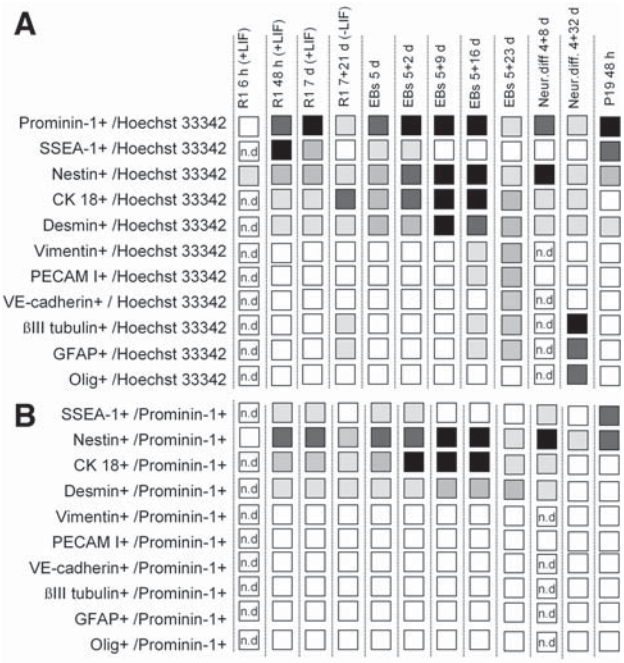

Figure 4. Semiquantitative imaging analysis of immunoreactivity in R1 cells at different stages of differentiation and in P19 cells. **(A):** Undifferentiated R1 cells (6 hours, 48 hours) and R1 cells cultivated as colonies for 7 days; differentiated R1 cells further cultured in the absence of LIF for 21 days; 5-day-old EBs; EB outgrowths at stages 5 + 2 d, 5 + 9 d, 5 + 16 d, and 5 + 23 d; R1 cells after neuronal differentiation at stages 4 + 8 d and 4 + 32 d; and 48-hour P19 cells were analyzed for immunofluorescence signals, and the percentages of immunopositive cells relative to the total number of Hoechst 33342–labeled cells were determined. For each value, 10 areas of each coverslip (n = 2 to 3) of three independent experiments were analyzed. Approximate percentage of immunolabeling was expressed as follows: **(B):** Coexpression profiles of cells positive for SSEA-1, nestin, cytokeratin 18, desmin, vimentin, E-cadherin, platelet-endothelial cell adhesion molecule-1, vascular endothelial–cadherin, βIII-tubulin, glial fibrillary acidic protein, and Oligodendrocyte (in %) in the prominin-1–labeled population (prominin-1 labeling = 100%). Specific proteins were investigated in the same cell populations and differentiation stages as described in **(A)**. The approximate percentage of immunolabeling was estimated according to **(A)**. Abbreviations: EB, embryoid body; GFAP, glial fibrillary acidic protein; LIF, leukemia inhibitory factor; n.d, not determined; PECAM-1, platelet-endothelial cell adhesion molecule-1; SSEA, stage-specific embryonic antigen; VE, vascular endothelial.

Prominin-1 Protein Abundance During In Vitro Differentiation

Our RT-PCR studies showed that (6-hour) R1 cells contained prominin-1 transcripts at 40% of the levels found in kidney tissue (Fig. 1); however, mRNA and protein levels were highly abundant in 48-hour R1 cells. Prominin-1 is therefore upregulated when undifferentiated ES cells become committed to differentiation. To confirm this finding, we performed semiquantitative immunofluorescence, Western blotting, and fluorescence-activated cell sorter (FACS) analysis using anti–prominin-1 mAb 13A4.

Because all P19 cells were labeled by prominin-1, these cells were used as positive control (n = 100%) in the semiquantitative immunolabeling assay (Fig. 3A). Prominin-1 staining was detected in approximately 60% of 48-hour R1 cells and 80% of 7-day colonies but was clearly reduced after differentiation in the absence of LIF (Fig. 3A).

FACS analysis confirmed the immunofluorescence data (Figs. 3B–3F). P19 cells cultured for 48 hours displayed prominin-1 immunoreactivity in approximately 93% of cells (Fig. 3B). R1 cells (6 hours) represented less than 10% of the undifferentiated cells (Fig. 3C), whereas an increase of prominin-1–positive cells was measured in R1 cells cultured for 48 hours (~50%; Fig. 3D) and after clonal growth in the presence of LIF in 7-day colonies (~55%; Fig. 3E). Therefore, FACS analysis clearly supported the immunofluorescence results, showing that undifferentiated ES cells grow as a heterogeneous population of cells. Spontaneous differentiation for 7 + 14 days resulted in a reduction of prominin-1 immunoreactivity by FACS analysis (~23%; Fig. 3F).

Western blotting analysis confirmed the immunofluorescence and FACS data (Figs. 3G, 3H). Significant protein levels were determined in P19 (48-hour) and R1 (48-hour, 7-day) cells, and a downregulation of prominin-1 was obvious in spontaneously differentiated R1 cells (Fig. 3G) in agreement with immunofluorescence and PCR data. Prominin-1 immunoreactivity was quantified during the differentiation of R1 cells. The prominin-1 immunoreactivity of R1 cells (cultivated as colonies for 7 days) was set to 100%, whereas R1 cells cultivated for 48 hours and differentiated for 21 days showed approximately 40% prominin-1 immunoreactivity (Fig. 3H).

Finally, to discriminate between various prominin-1 splice variants [33], we have performed Western blotting analysis using the αI3 antiserum [13] instead of mAb 13A4. The αI3 antiserum is directed against the cytoplasmic C-terminal domain of prominin-1.s1 variant [1, 2] and does not detect any other known prominin-1 splice variant [33]. The αI3 antiserum, like the mAb 13A4 (Fig. 3G), recognized the prominin-1 band in P19 and R1 cells, indicating that this band was the s1 variant (data not shown).

Prominin-1 Is Expressed in ES-Derived Progenitors but Not in Differentiated Cells

Next, we analyzed the colocalization of prominin-1 with lineage-specific proteins in R1 cells during differentiation at early and terminal stages. Prominin-1–positive cells were detected at peripheral regions

of early EB outgrowths without any colabeling with SSEA-1 2 days after plating of 5-day EBs (Fig. 5A). Central areas of EB outgrowths were prominin-1–negative (Fig. 5B, arrow); however, approximately 50%–75% of prominin-1–positive cells at the periphery of EB outgrowths coexpressed nestin (Figs. 4B, 5B). CK18 showed a high level of coexpression with prominin-1 (Fig. 5C), whereas desmin showed only a partial colocalization (Figs. 4B, 5D).

Because of the colocalization of prominin-1 with proteins characteristic for neural (nestin), epithelial (CK18), and (partially) mesodermal (desmin) cell types in early differentiating ES cells, we analyzed the lineage-specific expression of prominin-1 after continued differentiation of R1 cells. Lineage-specific markers of glial (GFAP), neuronal (βIII-tubulin), epithelial (E-cadherin), endothelial (VE-cadherin, PECAM-I), and meso-

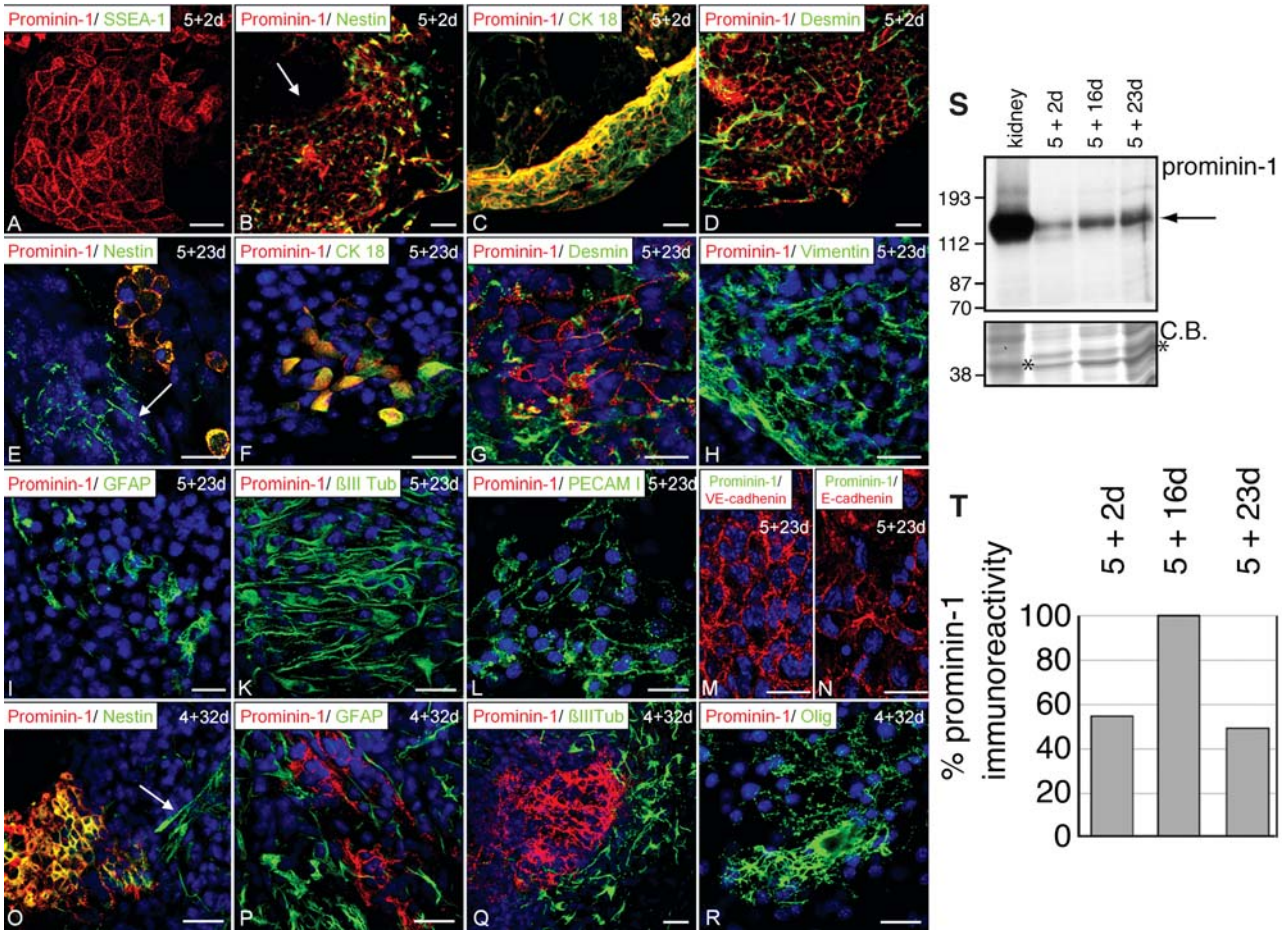

Figure 5. Double immunofluorescence and confocal imaging analysis of prominin-1 (cell-surface labeling) and various intracellular marker proteins in R1-derived EBs (5-day) differentiated for 2 (5 + 2 d, **A–D**) and 23 (5 + 23 d, **E–N**) days after EB plating, 32 days after neuronal differentiation (4 + 32 d, **O–R**), and Western blot analysis **(S, T)**. **(A–D):** At 5 + 2 d, no SSEA-1 labeling (green) in prominin-1–positive cells (red) **(A)** was detected. Prominin-1–positive cells were not found in central areas of EB outgrowths (**B**, arrow) but localized at peripheral regions of EB outgrowths **(B–D)** and showed coexpression with nestin (green, **B**) and CK18 (green, **C**) and a partial coexpression with desmin (green, **D**). **(E–N):** In EB-derived cells differentiated for 23 days (5 + 23 d), coexpression of prominin-1 (red, **E–L**; green, **M, N**) with nestin (green, **E**), CK18 (green, **F**), and desmin (green, **G**) but no coexpression with vimentin (green, **H**), GFAP (green, **I**), βIII tubulin (green, **K**), platelet-endothelial cell adhesion molecule-1 (green, **L**), vascular endothelial–cadherin (green, **M**), and E-cadherin (green, **N**) was detected. **(O-R):** R1 cells after neuronal differentiation at terminal stage (4 + 32 d) showed coexpression of prominin-1 only in nestin-positive epithelial-like cells (green, **O**) but no coexpression in nestin-positive neuron-like cells (green, arrow, **O**) and in GFAP-labeled (green, **P**), βIII tubulin–labeled (green, **Q**), and oligodendrocyte-labeled protein (Olig) (green, **R**) cells. Hoechst 33342 (blue) was used to visualize cell nuclei. Bars = 30 µm. **(S):** Western blotting analysis of prominin-1 (top panel, arrow) in R1-derived EBs at stages 5 + 2 d, 5 + 16 d, and 5 + 23 d. Adult kidney membrane was used as positive control. In each sample, the total amount of protein was visualized by Coomassie-blue staining (C.B.) (bottom panel). **(T):** Quantification of prominin-1 immunoreactivity in R1-derived EBs at various stages. Prominin-1 immunoreactivity detected in **(S)** was quantified by densitometric scanning, normalized to the amount of protein detected by Coomassie-blue staining (reference band indicated by asterisks in [**S**]), and plotted as percentage of prominin-1 immunoreactivity detected in EBs at stage 5 + 16 d. Each value represents the mean of two independent experiments. Abbreviations: EB, embryoid body; GFAP, glial fibrillary acidic protein; PECAM-1, platelet-endothelial cell adhesion molecule-1; SSEA, stage-specific embryonic antigen.

dermal (vimentin) cells were analyzed at a terminal stage (5 + 23 days; Figs. 5E–5N).

At the terminal differentiation stage, 5%–25% of R1-derived cells were labeled by prominin-1 (Fig. 4A), and a partial coexpression of prominin-1 in nestin-positive (Fig. 5E), CK18-positive (Fig. 5F), and desmin-positive (Fig. 5G) cells was found. However, significant differences in the morphology of cells labeled by prominin-1 and nestin (Fig. 5E), respectively, were observed. Whereas most cells that coexpressed prominin-1 and nestin showed an epithelial morphology, most cells labeled only by nestin (prominin-1–negative) revealed the typical morphology of neuron-like cells. Several cells at this terminal differentiation stage were also labeled by vimentin (Fig. 5H), GFAP (Fig. 5I), βIII-tubulin (Fig. 5K), PECAM-I (Fig. 5L), VE-cadherin (Fig. 5M), and E-cadherin (Fig. 5N); however, no coexpression of prominin-1 was observed with any of these proteins (Fig. 4B). Western blotting confirmed the upregulation of prominin-1 in EB outgrowths at stage 5 + 16 days but showed a downregulation at stage 5 + 23 days (Fig. 5S). The quantification of prominin-1 immunoreactivity (maximum value at stage 5 + 16 days was set to 100%) revealed approximately 50% prominin-1 immunoreactivity at stages 5 + 2 days and 5 + 23 days (Fig. 5T).

Because of the relatively high level of coexpression of prominin-1 with nestin (known as neural stem cell marker) in R1-derived cells (Figs. 2C, 4B, 5B), we analyzed prominin-1 expression specifically during neuronal differentiation [22]. R1-derived nestin-positive progenitor cells at early stage of neural differentiation (4 + 8 days) showed high coexpression with prominin-1 (Fig. 4B), whereas after induction of neuronal differentiation, glial (GFAP), neuronal (βIII-tubulin), and oligodendrocytic (oligodendrocyte-specific protein) cells were all prominin-1–negative (Figs. 5O–5R). Prominin-1 and nestin were coexpressed in some epithelial-like clusters (Fig. 5O), but prominin-1 was never found in nestin-positive cells of neuronal morphology (Fig. 5O, see arrow).

We also tested the colocalization of prominin-1 with endodermal (hepatic and pancreatic) lineage markers, including α-fetoprotein, albumin and cytokeratin 19, Islet-1, and insulin, respectively, but did not see prominin-1 labeling in any of these cells (data not shown).

In conclusion, we found that prominin-1 expression is maintained when ES cells undergo commitment to differentiation into neural, epithelial, and (partially) mesodermal cells, suggesting a role of prominin-1 for the specification of these precursor cells. At terminal stages of differentiation, however, prominin-1 is never coexpressed with proteins that are typically found in specialized cell populations with the exception of prominin-1 expression in nestin-positive cell types, which define potential neural precursor cells. This might suggest a specific role of prominin-1 expression at least in neural precursor cells.

Prominin-1 Is Expressed in Trophectoderm Cells of Mouse Blastocysts

Given that prominin-1 is expressed in early progenitor cells, we have investigated the presence of this marker in early embryos in vivo. RT-PCR analysis showed the absence of prominin-1 transcript in 3.5-day p.c. blastocysts (Fig. 6A). Under the same condition, nestin was barely detectable, whereas nanog attained maximal levels (Fig. 6A). Remarkably, after plating of blastocysts

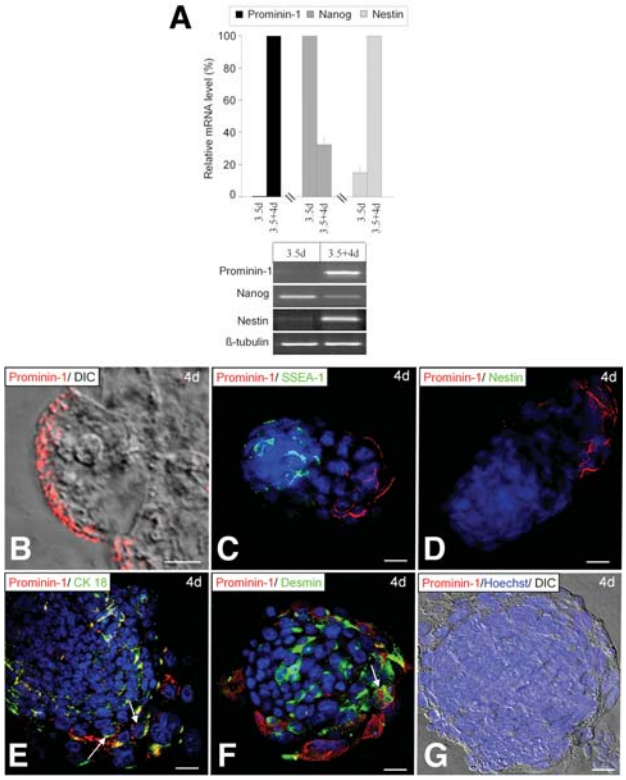

Figure 6. Prominin-1 expression in mouse blastocysts. **(A):** The abundance of prominin-1, nanog, and nestin transcripts in blastocysts isolated at day 3.5 postconceptionem (3.5 d) and in blastocysts cultivated for an additional 4 days (3.5 + 4 d) was assayed by reverse transcription–polymerase chain reaction. The mRNA level of prominin-1 and nestin determined in cultivated blastocysts (3.5 + 4 d) and of nanog in isolated blastocysts (3.5 d) was set to 100%, and the respective other value was expressed relative to this. The housekeeping gene β-tubulin was used as internal standard. Each value (n = 3 experiments with 15 to 20 blastocysts) represents mean ± SEM. **(B–G):** Double immunofluorescence analysis of prominin-1 (cell-surface labeling) and various proteins in blastocysts cultivated for 4 days (3.5 + 4 d) (n = 15). Prominin-1 was localized at the apical domain of trophoblast cells (**B**, red overlay with DIC). Cells were double-labeled for **(C)** prominin-1/stage-specific embryonic antigen-1 (SSEA-1), **(D)** prominin-1/nestin, **(E)** prominin-1/CK18, and **(F)** prominin-1/desmin and analyzed by confocal microscopy. Partial coexpression of prominin-1 with **(E)** CK18 and **(F)** desmin was found in the trophoblast cells (see arrows). Corresponding phase micrograph of **(G)** a blastocyst cultivated for 4 days is shown, in which Hoechst 33342 (blue) was used to visualize cell nuclei. Bar = 30 μm **(B, D–G)** and 10 μm **(B)**. Abbreviation: DIC, differential interference contrast.

followed by 4 days of culture, the level of prominin-1 as well as nestin transcript showed maximal levels (Fig. 6A), whereas the amount of nanog transcript was decreased (Fig. 6A).

To verify prominin-1 abundance in cells of the inner cell mass (ICM), the founder cells of ES cells, we investigated prominin-1 expression in plated blastocysts by indirect immunofluorescence (Fig. 6). Using either cell-surface immunolabeling (Figs. 6B–6F) or immunolabeling after PFA fixation and saponin permeabilization (data not shown), prominin-1 (red) was detected in trophoblast cells but not in ICM cells (Figs. 6B–6F). Prominin-1 was localized at the apical domain of trophoblast cells (Fig. 6B), and no coexpression with SSEA-1 (green, localized in ICM cells) was found (Fig. 6C). Comparable results are obtained upon saponin permeabilization (not shown). Nestin was not detected in ICM and trophoblast cells (Fig. 6D), but a partial colocalization of prominin-1 (red) with CK18 (green; Fig. 6E) and with desmin (green; Fig. 6F) was found in trophoblast cells (see arrows).

Discussion

The present data show a unique expression pattern of prominin-1 in ES-derived committed and early progenitor cells. Whereas a high transcript level of the pluripotency-sustaining factor nanog in undifferentiated ES cells [34, 35] was associated with a low expression level of prominin-1, maintenance of ES cells in the presence of LIF (without feeder cells) for 48 hours resulted in the upregulation of prominin-1 at both the mRNA and protein levels. This undifferentiated but committed status of ES cells was also characterized by high nestin expression. After differentiation in the absence of LIF, prominin-1, nanog, and nestin were all downregulated, although to different degrees. Relatively high prominin-1, nanog, and (lower) nestin levels were maintained in EBs, but nanog and nestin were downregulated, whereas prominin-1 was abundant at an intermediate stage characterized by multilineage progenitor cells, including neural, epithelial, and (partially) mesodermal cell types [27]. This specific pattern of prominin-1 expression in early progenitor cells leads us to propose prominin-1 as a novel marker of ES-derived progenitor cells. Our hypothesis is supported by the coexpression of prominin-1 with proteins identified by SAGE as early differentiation markers of ES cells, including cytokeratins, fibulin-1, and VCP.

Cytokeratins are known to play a role in early development. CK18 is expressed in mouse embryos from the eight-cell stage, with a maximum of expression at the blastocyst stage, and in epithelial cells after birth and in adults; in addition, CK18-deficient mice die at embryonic day 9.5 [36]. The partial coexpression of prominin-1 with CK18 in early differentiating ES cells and committed progenitors may suggest the involvement of both proteins in the determination of epithelial cell types.

Fibulin-1, an extracellular matrix protein involved in epithelial-mesenchymal transition [37], is specifically expressed during early embryogenesis in the neural crest, endocardial cushion tissue, and myotome [38-40]. The partial coexpression of fibulin-1 and prominin-1 in committed ES cells would suggest an early differentiation status of prominin-1–positive cells, whereas the high coexpression of fibulin-1 with prominin-1 in EC cells would point to fibulin-1 as a tumor-related marker. Indeed, fibulin-1 has been detected at high levels in human ovarian epithelial tumors [41]. Recently, Singh et al. [42] isolated a $CD133^+$ cancer stem cell subpopulation (which also expressed nestin) from human brain tumors and injected only approximately 100 $CD133^+$ cells that resulted in tumor formation. This may explain the expression of prominin-1 in nearly all P19 cells.

VCP transcripts were also highly abundant in ES-derived cells. The coexpression of prominin-1 with VCP in ES and EC cells might also be connected with the high proliferation capacity of these cells, because VCP is involved in cell-cycle regulation and a variety of membrane functions [43].

The continued differentiation of ES cells into the neuronal lineage resulted in a restricted expression of prominin-1 to nestin-positive cells. The coexpression of nestin and prominin-1 in potential neural progenitor cells may be comparable to the earlier observations of prominin-1 expression in mouse neuroepithelial stem cells [2] and the detection of AC133 antigen (the human prominin-1 orthologue) in human neuroepithelial stem cells. However, prominin-1 expression is not restricted to a neural stem/progenitor cell population, because embryonic epithelia of lung and gut and adult kidney and epididymis also showed high levels of prominin-1 expression [2, 7, 33]. Similarly, nestin expression is not restricted to neural stem or progenitor cells [26, 44] but was also expressed in ES-derived multilineage progenitor cells that have the potential to develop into neuroectodermal, endodermal, and mesodermal cell types [27].

Our hypothesis that prominin-1 plays a role in the definition of committed and early progenitors is also supported by our finding of partial coexpression of prominin-1 with FRP-2 in ES-derived cells. FRPs play a role in the Wnt signaling pathway as receptors for Wnt by regulating the intracellular levels of β-catenin [45]. Soluble FRP-2 (sFRP-2), a Wnt antagonist, is expressed in neuroepithelial cells of the developing nervous system in vivo [46] and plays a role as modulator of neural differentiation of ES cells in vitro [47]. Constitutive expression of the *Sfrp-2* gene promoted neural gene expression and the formation of morphologically differentiated neurons [47]. sFRP-2 was found to be widely expressed in the developing embryo, but in adults, sFRP-2 is expressed in the heart, brain, lung, and kidney [48]. All these tissues showed relatively high nestin transcript levels (unpublished data).

The high abundance of prominin-1 in early embryonic cell types led us to further analyze its expression in mouse blastocysts in vivo. Although prominin-1 was absent in the undifferentiated ICM cells, it was found in trophoblast cells. Therefore, one could speculate that prominin-1 plays a role in processes related

to implantation. Such a role would be suggested by the detection of AC133-2 (a subtype of the human AC133 antigen) in human trophoblast cells and trophoblast-derived cell lines in vivo [49]. Other early differentiation markers, CK8 and CK18, were also restricted to trophoblast cells and never found in the ICM of blastocysts [28]. Although we found a coexpression of prominin-1 with CK18 in trophoblast cells of blastocysts, until now, we have no indication for a specific role of prominin-1 in early implantation.

However, the different patterns of expression of prominin-1 in ES and ICM cells could be also related to the different growth conditions of the cells in vivo and in vitro. Unlike trophoblast cells in vivo, the ICM-derived ES cells grow in vitro as monolayer of an epithelial-like cell population. The transition of ICM cells to a different growth status of ES cells in vitro might be connected with a modification of cellular properties, which are associated with prominin-1 expression.

Our findings that ES cells were labeled by prominin-1 as soon as they are committed to differentiation and the coexpression of prominin-1 with early lineage markers suggest a role for prominin-1 in commitment and early differentiation. Hence, we propose prominin-1 as a new marker to characterize ES-derived committed and early progenitor cells.

Acknowledgments

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