

ARTICLE

## The Stem Cell Marker CD133 (Prominin-1) Is Expressed in Various Human Glandular Epithelia

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**SUMMARY** Human prominin-1 (CD133) is expressed by various stem and progenitor cells originating from diverse sources. In addition to stem cells, its mouse ortholog is expressed in a broad range of adult epithelial cells, where it is selectively concentrated in their apical domain. The lack of detection of prominin-1 in adult human epithelia might be explained, at least in part, by the specificity of the widely used AC133 antibody, which recognizes an epitope that seems dependent on glycosylation. Here we decided to re-examine its expression in adult human tissues, particularly in glandular epithelia, using a novel monoclonal antibody (80B258) generated against the human prominin-1 polypeptide. In examined tissues, we observed 80B258 immunoreactivity at the apical or apicolateral membranes of polarized cells. For instance, we found expression in secretory serous and mucous cells as well as intercalated ducts of the large salivary and lacrimal glands. In sweat glands including the gland of Moll, 80B258 immunoreactivity was found in the secretory (eccrine and apocrine glands) and duct (eccrine glands) portion. In the liver, 80B258 immunoreactivity was identified in the canals of Hering, bile ductules, and small interlobular bile ducts. In the uterus, we detected 80B258 immunoreactivity in endometrial and cervical glands. Together these data show that the overall expression of human prominin-1 is beyond the rare primitive cells, and it seems to be a general marker of apical or apicolateral membrane of glandular epithelia. This manuscript contains online supplemental material at <http://www.jhc.org>. Please visit this article online to view these materials. (J Histochem Cytochem 56:977–993, 2008)

**KEY WORDS**  
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SINCE ITS IDENTIFICATION 10 years ago, the mammalian cholesterol-binding, pentaspan membrane glycoprotein prominin-1 (CD133) has received considerable interest because of its expression by various somatic stem cells originating from distinct organ systems (for reviews, see Fargeas et al. 2006; Bauer et al. 2008). Mouse

prominin-1 was described as a novel marker of neuroepithelial progenitor cells (Weigmann et al. 1997), whereas its human ortholog is a novel antigen—defined by the widely used MAb AC133—with its expression limited to hematopoietic stem and progenitor cells (Yin et al. 1997).

In murine embryos, prominin-1 is observed in developing epithelia of the three germinal layers (Weigmann et al. 1997; Ito et al. 2007), and its expression persists in various adult epithelia including the ependymal cells (Weigmann et al. 1997; Coskun et al. 2008). For instance, it is detected in kidney proximal tubules (Weigmann et al. 1997), ducts of salivary and lacrimal glands (Jászai et al. 2007), epididymis, and developing

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spermatozoa found in seminiferous tubules (Fargeas et al. 2004). Certain non-epithelial cells, e.g., photo-receptor cells and glial cells, express it as well (Maw et al. 2000; unpublished data). In all epithelial cells, prominin-1 is restricted to the apical plasma membrane domain, where it is selectively concentrated in plasma membrane protrusions such as microvilli (Weigmann et al. 1997) and primary cilium (Dubreuil et al. 2007). Thus, although generally considered as a stem cell marker, prominin-1 is widely expressed in differentiated murine tissues.

In the human embryo, prominin-1 (i.e., AC133 epitope) is expressed in various developing epithelia (Corbeil et al. 2000), like in the mouse, but although high levels of prominin-1 mRNA are detected in numerous adult human tissues (Miraglia et al. 1997; Corbeil et al. 2000; Fargeas et al. 2003b; Florek et al. 2005), AC133 expression seems to be downregulated in most adult epithelia (Miraglia et al. 1997), with some notable exceptions such as pancreatic ducts (Lardon et al. 2008). In other organ systems, prominin-1 remains detectable only in very rare cells with stem cell properties (Alessandri et al. 2004; Belicchi et al. 2004; Richardson et al. 2004; Bussolati et al. 2005). The expression by these primitive cells solely could not account for the abundance of prominin-1 mRNA detected in several adult human tissues. This discrepancy might be explained, at least in part, by the fact that the prominin-1 AC133 epitope seems to be dependent on the glycosylated structure (Miraglia et al. 1997), and its glycosylation profile might be distinct in differentiated cells, leading to the lack of its detection (Corbeil et al. 2000; Florek et al. 2005; Hemmoranta et al. 2007). Alternatively, the AC133 epitope might be masked under certain circumstances, particularly when prominin-1 is embedded in a specific membrane microdomain (lipid raft), where protein-lipid and protein-protein interactions are implicated (Röper et al. 2000; Janich and Corbeil 2007). Thus, several parameters might interfere with the IHC detection of the prominin-1 AC133 epitope (Florek et al. 2005; Lardon et al. 2008).

In this study, we reinvestigated the expression profile of prominin-1 in healthy adult human tissues using a novel MAb 80B258 generated against the human prominin-1 polypeptide. Our analysis showed a wide tissue distribution of human prominin-1 as previously reported for its murine counterpart.

## Materials and Methods

### Monoclonal Antibody 80B258 Against Human Prominin-1 (CD133) Polypeptide

The glutathione S-transferase (GST)-hE2 fusion protein containing residues Gly240-Ser388 of the second extracellular domain of human prominin-1 (Miraglia

et al. 1997) was produced and purified as described previously (Florek et al. 2005) and used to immunize Balb/c mice. After initial immunization, animals received two additional injections at 3- to 4-week intervals. The first immunization was performed with Freund's complete adjuvant, whereas booster immunizations were with Freund's incomplete adjuvant. Four days before fusion, mice received a final injection with the same material in PBS. Mouse spleen cells were fused with P3X63Ag8.653 mouse myeloma cells according to standard polyethylene glycol fusion protocol (Harlow and Lane 1988). The cell culture medium was changed 24 hr after fusion from DMEM medium containing 20% FCS (PAA Laboratories; Pasching, Austria) to hypoxanthine, aminopterin, and thymidine selection (HAT) medium (all cell culture reagents were obtained from Gibco Invitrogen; Paisley, UK). After 1 week of HAT selection, medium was changed to hypoxanthine and thymidine (HT) medium. Hybridoma supernatants were initially screened by electrochemiluminescence assay using Multi-Array 96 plates (Meso Scale Discovery; Gaithersburg, MD) coated with GST-hE2 fusion protein (100 ng/well) and then by immunoblotting using an extract of human Caco-2 cells as an antigen source (Corbeil et al. 2000). Positive hybridoma clones were subsequently subcloned by limited dilution. The newly identified MAb 80B258 (isotype: IgG1,  $\kappa$ ) was purified by protein G chromatography. The MAb 80B258 recognizes its antigen in humans but not in mice.

### Cell Culture and Transfection

Chinese hamster ovary (CHO) cells and human colon-derived epithelial Caco-2 cells were cultured as described (Corbeil et al. 2000) and transiently transfected with the eukaryotic expression pCR3.1-Uni plasmid containing the human prominin-1 cDNA under the control of the cytomegalovirus promoter (Miraglia et al. 1997; Corbeil et al. 2000) using the nucleofection method with solution T and program U-23 (Amaxa; Cologne, Germany). [Under these conditions, a good expression of the transgene was observed without the preincubation of cells with sodium butyrate (Corbeil et al. 2000).] After 48 hr, cells were processed for indirect immunofluorescence (see below), or a detergent cell extract was prepared as described previously (Corbeil et al. 1999).

Primary hematopoietic (HSCs) and mesenchymal (MSCs) stem cells were collected from healthy donors after informed consent and approval of the local ethics committee (Medical Clinic and Polyclinic I, University Hospital, Dresden, Germany). Isolation of MSCs was performed as described (Freund et al. 2006a). Mobilized peripheral blood was obtained by subcutaneous injection of 7.5  $\mu$ g/kg granulocyte colony-stimulating factor per day (Granocyte; Chugai Pharma, Frankfurt,

Germany) for 5 days. CD34<sup>+</sup> MACS-immunoisolated HSCs from leukapheresis products (for technical details, see Freund et al. 2006b) were cultured on MSCs growing on fibronectin-coated petri 100-mm dishes ( $12 \times 10^4$  HSCs in 12 ml medium) in serum-free medium (Freund et al. 2006a) supplemented with early-acting cytokines (50 ng/ml stem cell factor, 50 ng/ml fetal liver tyrosine kinase-3 ligand, 15 ng/ml interleukin-3). The coculture HSCs/MSCs were incubated at 37C in a humidified 5% CO<sub>2</sub> atmosphere for 7 days before experimental use. The non-adherent HSCs were collected, and detergent cell extracts (corresponding to one quarter of a dish per loading well) were used for immunoblotting.

#### Endoglycosidase Digestion and Immunoblotting

CHO detergent extracts corresponding to one tenth of a confluent 85-mm dish were incubated overnight at 37C in the absence or presence of 1 unit peptide-N-glycosidase F (PNGase F) according to the manufacturer's instructions (Roche Molecular Biochemicals; Mannheim, Germany). Proteins from either CHO or HSC detergent extracts were analyzed by SDS-PAGE (7.5%) and transferred to polyvinylidene difluoride (PVDF) membranes (pore size, 0.45  $\mu$ m; Millipore Corp., Bedford, MA) using a semi-dry transfer cell system (Cti; Idstein, Germany) as described (Corbeil et al. 2001a). After transfer, membranes were incubated overnight at 4C in blocking buffer (PBS containing 5% low-fat milk powder and 0.3% Tween-20). Human prominin-1 was detected using either mouse MAb 80B258 (1  $\mu$ g/ml) or mouse MAb AC133 (0.25  $\mu$ g/ml, CD133/1; Milteny Biotec, Bergisch Gladbach, Germany) or rabbit  $\alpha$ hE2 antiserum (1:2500; Florek et al. 2005). All primary antibodies were diluted in blocking buffer, and incubation was performed for 1 hr at room temperature. Antigen-antibody complexes were shown using horseradish peroxidase-conjugated secondary antibodies (Dianova; Hamburg, Germany) followed by enhanced chemiluminescence (ECL System; Amersham, Piscataway, NJ).

#### Indirect Immunofluorescence and Confocal Microscopy

Indirect immunofluorescence microscopy analysis of paraformaldehyde (PFA)-fixed transfected CHO cells grown on glass coverslips was performed as described previously (Florek et al. 2005). Briefly, PFA-fixed cells were incubated in SDS buffer (0.005% SDS and 0.2% gelatin in PBS) for 30 min at room temperature and washed with PBS containing 0.2% gelatin for 10 min to remove the residual SDS. Cells were double-labeled for 30 min at room temperature with  $\alpha$ hE2 antiserum (1:500; Florek et al. 2005) and mouse MAb 80B258

(10  $\mu$ g/ml), followed by Cy2-conjugated goat anti-rabbit IgG (H + L) and Cy3-conjugated goat anti-mouse IgG (H + L; Jackson ImmunoResearch Labs, West Grove, PA), all diluted in PBS containing 0.2% gelatin. Coverslips were rinsed and mounted in Moviol 4.88 (Calbiochem; Darmstadt, Germany). Cells were observed with a Zeiss 510 Meta confocal laser-scanning microscope (Jena, Germany). The confocal microscope settings were such that the photomultipliers were within their linear range. The images shown were prepared from confocal data files using LSM 5 LIVE (Göttingen, Germany) and Adobe Photoshop and Illustrator software (San Jose, CA).

#### Flow Cytometry

Three-day-old postconfluent Caco-2 cells were harvested by trypsin treatment for 10 min at 37C. After the inactivation of the trypsin by adding the complete medium, cells were centrifuged for 5 min at  $400 \times g$ . Cells were washed with PBS and either directly labeled with antibodies or fixed with 2% PFA for 15 min followed by incubation in SDS buffer (0.005% SDS and 0.2% gelatin in PBS) for 30 min at room temperature. Fixed cells were washed with PBS containing 0.2% gelatin for 10 min to remove the residual SDS. Cell suspensions (living or fixed cells) were adjusted to  $1 \times 10^6$  cells/ml, and 100- $\mu$ l aliquots were incubated with either the AC133 or 80B258 MAb for 30 min at 4C. For the negative control, the primary antibody was omitted. After washing with PBS, a goat anti-mouse IgG-Alexa Fluor 488 secondary antibody was applied for 30 min at 4C. Unbound antibodies were removed by washing the labeled cells with PBS, and 10,000 events were acquired on a LSRII flow cytometer (BD Biosciences; Franklin Lakes, NJ). Data were analyzed using FlowJo software (TreeStar; Ashland, OR).

#### Tissue Samples

Specimens of adult human tissues were obtained from archives of Department of Pathology, University Hospital in Hradec Králové, and Department of Pathology, University Hospital in Olomouc. For this study, we chose the samples derived either from biopsy or necropsy taken from normal male or female tissues (age range, 36–71 years) that did not show signs of any disease. Uterine samples originated from biopsy of normal endometrium in the proliferative phase (31-, 42-, and 50-year-old women). All samples were derived and processed under general ethical criteria accepted at the University Hospitals in Hradec Králové and Olomouc, and the study was performed in accordance with the Helsinki Declaration of 1975.

Tissues were fixed with 10% formalin (prepared from 40% formaldehyde stock solution; Penta, Prague,

Czech Republic) for at least 1 week at room temperature. Tissue processing was performed in an automated tissue processor (TP1020; Leica, Wetzlar, Germany). Therein, tissues were dehydrated with increasing concentrations of ethanol (70%, 80%, 90%, 2 × 96%, 90 min each), twice in acetone (Penta) for 20 min each, and finally three times with xylene (Penta) for 90 min each, all at room temperature. The dehydrated samples were incubated in paraffin for 3 hr at 56°C and for an additional 6 hr with fresh paraffin. At the end, tissues were embedded in tissue blocks and kept at room temperature. Serial 5- to 6- $\mu$ m-thick sections were cut, mounted on glass slides pretreated with chrome alum-gelatin, and dried overnight at room temperature.

#### IHC

Paraffin-embedded sections were deparaffinized by xylene treatment overnight, hydrated with decreasing concentrations of ethanol (96%, 80%, and 70%) for 10 min each at room temperature, and rinsed twice with distilled water for 10 min each. Two independent techniques were used for the antigen retrieval. Sections were either exposed to microwaves (700 W) in sodium citrate solution (pH = 6.0) for 2 × 5 min or treated with 0.005% SDS in PBS for 30 min. After thorough washing with PBS, samples were incubated with 5% H<sub>2</sub>O<sub>2</sub> (3 × 10 min) to quench the endogenous peroxidase activity. Sections were incubated in blocking buffer containing either 5% normal goat serum (Sigma; Darmstadt, Germany) or 5% normal donkey serum (Jackson ImmunoResearch Labs) in PBS for 20 min at room temperature and exposed to either mouse MAb 80B258 (10  $\mu$ g/ml) or mouse MAb AC133 (2  $\mu$ g/ml; CD133/1; Miltenyi Biotec) diluted in antibody diluent with background reducing components (DAKO Cytomation; Glostrup, Denmark) overnight at 4°C. After washing with PBS, sections were incubated with Biotin-SP-conjugated AffiniPure donkey anti-mouse antibody IgG (H + L, 1:500; Jackson ImmunoResearch Labs) for 45 min at room temperature, rinsed with PBS, and followed by horseradish peroxidase-conjugated streptavidin (1:300; DAKO Cytomation) for 45 min at room temperature. Alternatively, an anti-mouse EnVision peroxidase kit (DAKO Cytomation) was used according to the manufacturer's protocol. Color reactions were performed with the chromogen DAB (2  $\mu$ g/ml; Fluka, Darmstadt, Germany) according to the manufacturer's instructions. After washing with distilled water, DAB precipitate was intensified with 3% CuSO<sub>4</sub> solution for 5 min. Sections were counterstained with Meyer's hematoxylin-eosin or light green (BDH Stains; Poole, UK), dehydrated, and mounted in DPX mounting medium (Fluka). As negative controls, adjacent or similar sections were processed in parallel either without the primary antibody or with a mouse

isotype control (IgG1,  $\kappa$ ; MOPC-21, 10  $\mu$ g/ml; Sigma). Slides were examined with an Olympus BX61 microscope (Hamburg, Germany) and Leica DM5000 B, and the images were prepared using Adobe Photoshop and Illustrator software.

#### Results

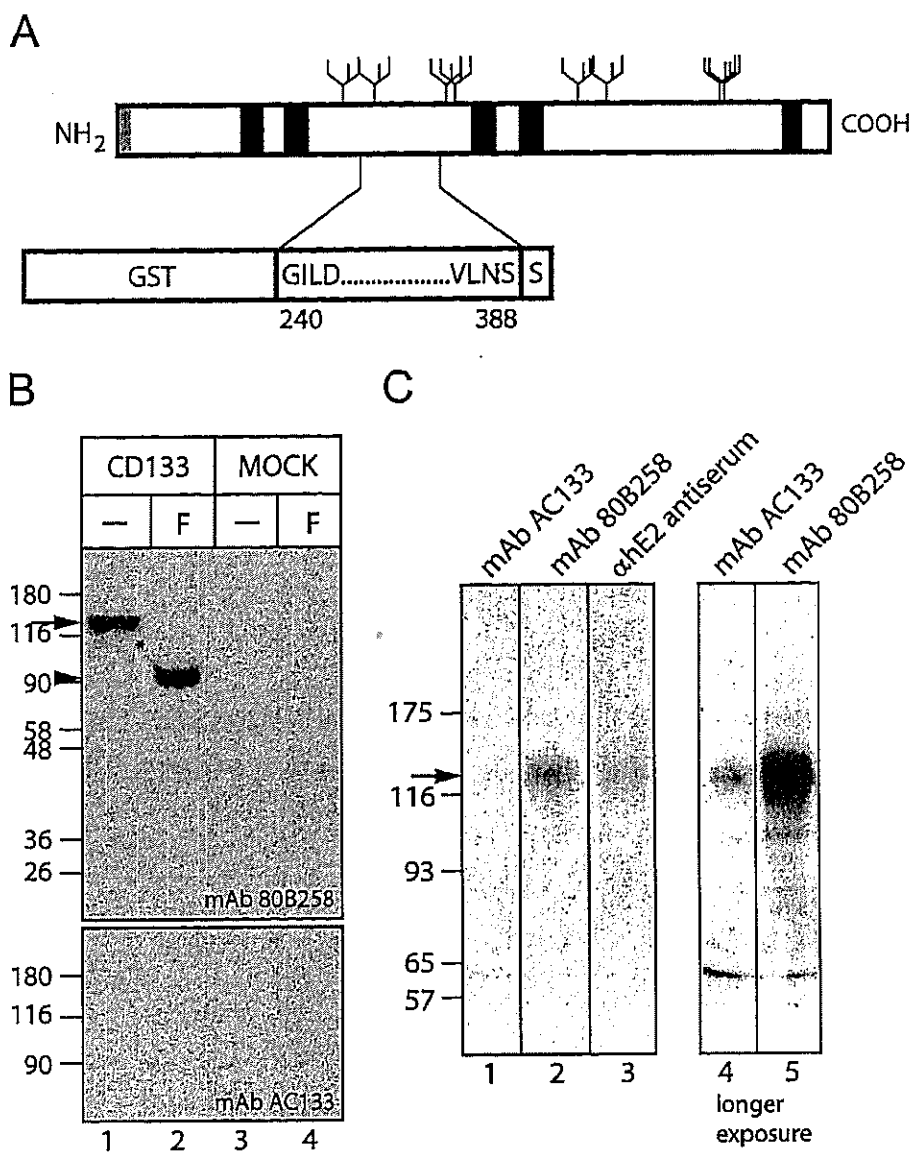
##### A Novel Monoclonal Antibody Directed Against Human Prominin-1 Polypeptide

To be able to reinvestigate the expression of the prominin-1 protein in adult human tissues with tools other than the MAb AC133 (Miltenyi Biotec), which recognizes an epitope thought to be dependent, at least in part, on glycosylation (Miraglia et al. 1997), we generated a novel mouse MAb, designated 80B258, against a human prominin-1 fragment corresponding to amino acid residues Gly<sub>240</sub>-Ser<sub>388</sub> (Figure 1A). In CHO cells transiently transfected with human prominin-1 cDNA, immunoblotting with the MAb 80B258 showed a major band with apparent molecular mass of ~120 kDa (Figure 1B, top panel, Lane 1, arrow) and a very minor ~105-kDa band (Figure 1B, top panel, Lane 1, asterisk), which reflects its overexpression. Indeed, the 105-kDa form, but not 120-kDa form, was sensitive to digestion with endo- $\beta$ -N-acetyl glucosaminidase H (endo H) (data not shown) and therefore represented the high-mannose form localized in endoplasmic reticulum and/or early Golgi compartment. PNGase F treatment converted both forms of human prominin-1 into a product of ~92 kDa (Figure 1B, top panel, Lane 2, arrowhead), indicating that the MAb 80B258 recognizes both the N-glycosylated and deglycosylated forms of the recombinant human prominin-1. The same immunoreactive bands were obtained using the rabbit  $\alpha$ hE2 antiserum (data not shown), which was raised against the same human prominin-1 fragment, as previously reported (Florek et al. 2005). No immunoreactivity was detected when CHO cells were transfected with the expression vector alone (Figure 1B, Lanes 3 and 4). Under the same conditions, the MAb AC133 did not show any immunoreactivity (Figure 1B, bottom panel).

Does the MAb 80B258 recognize prominin-1 under physiological conditions, i.e., without a heterologous expression? To answer this question, we blotted extracts of the human HSCs, which express endogenously prominin-1 (Yin et al. 1997). The 120-kDa immunoreactive band corresponding to prominin-1 was detected with both the MAb 80B258 and the  $\alpha$ hE2 antiserum (Figure 1C, Lanes 2 and 3, respectively, arrow), whereas the AC133 immunoreactivity was barely detectable (Figure 1C, Lane 1), which was nevertheless observable on a longer exposure of the blot (Figure 1C, Lane 4).

Taken together, these data show that the MAb 80B258 recognizes the recombinant and the authentic

**Figure 1** Generation and characterization of the monoclonal antibody 80B258 directed against human prominin-1 polypeptide. (A) Schematic representation of the glutathione S-transferase (GST)- $\alpha$ hE2 fusion protein containing part of the first extracellular loop of prominin-1 (residues Gly240 to Ser388) used as antigen to generate the MAb 80B258. Black boxes indicate transmembrane domains of prominin-1, whereas the signal peptide is in gray. The forks indicate the potential N-glycosylation sites. (B) 80B258 MAb recognizes the recombinant human prominin-1. Lysates of Chinese hamster ovary (CHO) cells transiently transfected with either human prominin-1 (CD133) or, as a control, vector DNA alone (MOCK) were incubated in absence (-) or presence (F) of peptide-N-glycosidase F (PNGase F) and analyzed by immunoblotting with either 80B258 or AC133 MAb. Arrow, endo-H-insensitive 120-kDa form of recombinant human prominin-1; asterisk, endo-H-sensitive 105-kDa form of recombinant human prominin-1; arrowhead, 92-kDa product after N-de glycosylation. (C) 80B258 MAb recognizes the authentic human prominin-1. Lysates of human hematopoietic stem and progenitor cells were analyzed by immunoblotting with either AC133 or 80B258 MAb or  $\alpha$ hE2 antiserum. Arrow, 120-kDa form of human prominin-1. Right panels (Lanes 4 and 5) are a longer exposure of the same blots shown in left panels (Lanes 1 and 2, respectively). The position of prestained apparent molecular mass markers (in kDa) is indicated on the left (B,C).



human prominin-1 by immunoblotting analysis and suggest that the MAb AC133 is not suitable for this technique, which is consistent with the conformational and/or glycosylation dependence of its corresponding epitope (Miraglia et al. 1997). It is not formerly excluded, however, that the human prominin-1 carrying the AC133 epitope represents only a minor subpopulation of total prominin-1 molecules, which is easily detectable on tissue-free cells, i.e., dissociated cells, with sensitive methods such as flow cytometry (Yin et al. 1997).

Unfortunately, the novel MAb 80B258 did not recognize the native form of human prominin-1 on cell surface immunofluorescence of living prominin-1-transfected CHO cells (data not shown). However,

it did show, on treatment of fixed cells with a low concentration of SDS (0.005%), the presence of prominin-1 at the plasma membrane (see online Supplemental Figure SF1A, left panel). The authenticity of this immunostaining was confirmed by its dependence on the presence of human prominin-1 cDNA on transfection of CHO cells (data not shown) and by the similar pattern observed with the  $\alpha$ hE2 antiserum (see online Supplemental Figure SF1A, right panel). The lack of prominin-1 immunolabeling in living cells might be explained by the presence of N-linked glycans located in, or in the vicinity of, the prominin-1 region encompassing Gly<sub>240</sub>-Ser<sub>388</sub> (Figure 1A, forks), which has been used as antigen. Thus, the 80B258 epitope seems to be masked under native labeling conditions. Such restric-

tion might preclude its use for fluorescence-activated cell sorting using living cells. We studied this issue using the human colon-derived epithelial cell line Caco-2, which expresses endogenously prominin-1 (Corbeil et al. 2000; Florek et al. 2005). As suspected, the MAb 80B258, in contrast to MAb AC133 (see online Supplemental Figure SF1B, blue area), did not recognize its corresponding epitope under native conditions by flow cytometry analysis (see online Supplemental Figure SF1B, red area). However, it did using treatment of fixed cells with a low concentration of SDS (see online Supplemental Figure SF1B, green area). Thus, the novel anti-human prominin-1 antibody could not be used for cell isolation.

#### IHC of Human Tissues

Throughout this study, we used two independent techniques to unmask the 80B258 epitope. As pretreatment, sections were either heated in the microwave or incubated with a solution containing a low concentration of SDS. Although the microwave pretreatment is more favorable for antigen retrieval, the tissue architecture is better preserved with SDS preincubation, which under certain circumstances, e.g., examination of delicate plasma membrane structures, is beneficial. For the sake of comprehension, we have outlined in the following sections all relevant cellular structures with either dotted or full lines.

#### Expression of Prominin-1 in the Major Salivary Glands

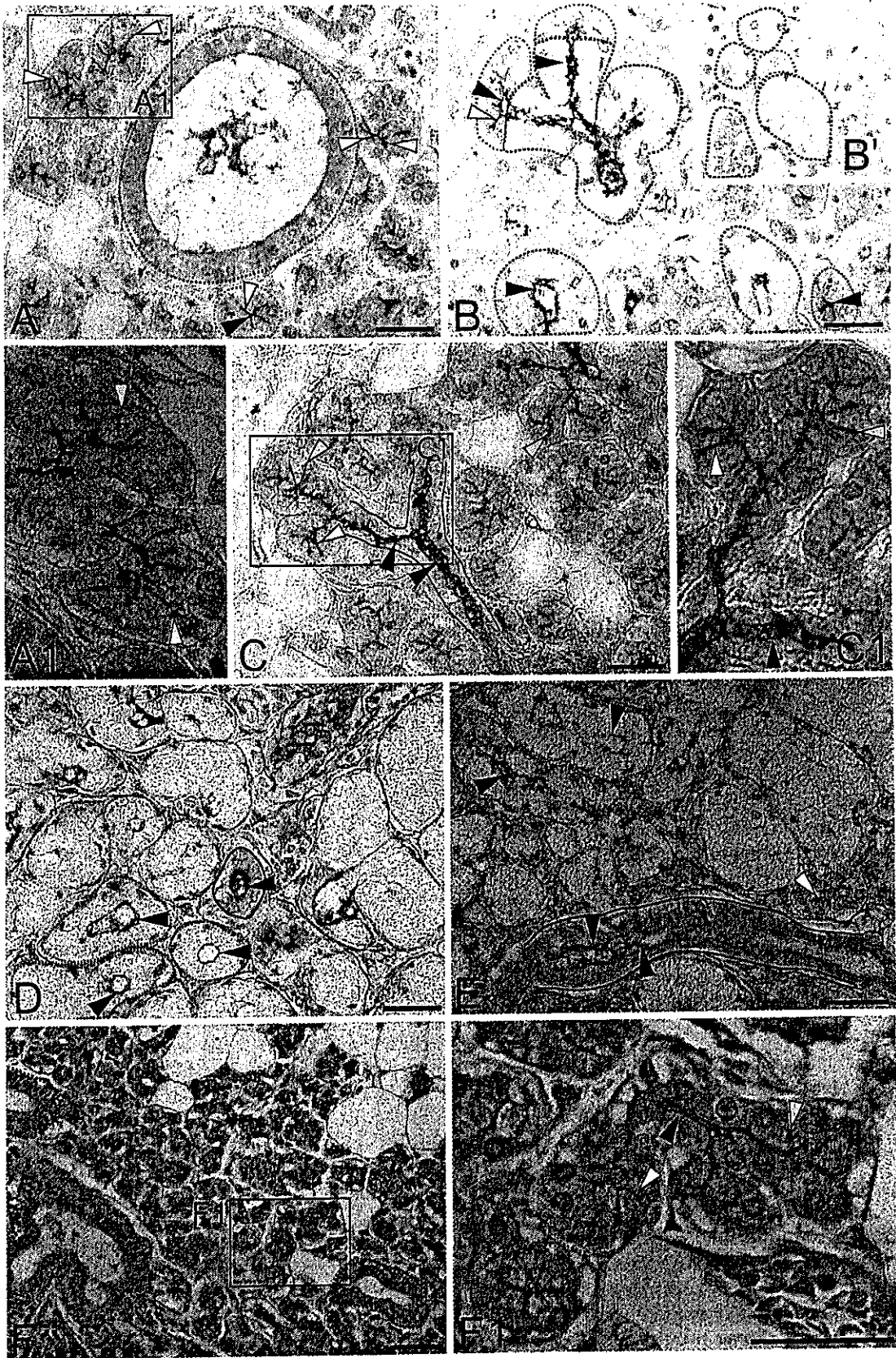
**Submandibular Gland (*Glandula Submandibularis*).** Submandibular glands consist of mostly serous secretory acini, scattered clusters of mucous endpieces with serous demilunes, and a duct system composed of intralobular intercalated and striated ducts, interlobular excretory ducts, and a main excretory duct. IHC analysis of adult human submandibular glands showed 80B258 immunoreactivity at the apical and the upper part of the lateral (apicolateral) membranes of secretory cells (Figures 2A–2C and corresponding insets, black and white arrowheads, respectively) located in serous acini (outlined by a black dotted line). A positive signal was detected as well at the widened endings of intercellular canaliculi (Figures 2A, 2A1, 2C, and

2C1, yellow arrowheads) formed by lateral membranes of serous cells. A similar staining pattern was observed in demilunes of serous cells (Figure 2B, orange dotted line). Cells lining mucous tubuli (blue dotted line) showed an intense apical labeling (Figure 2B, black arrowheads). In the duct portion, intercalated ducts (black line) were strongly positive (Figures 2C and 2C1, blue arrowheads), whereas striated ducts (Figure 2A, red dotted line) and interlobular ducts in connective tissue septa (data not shown) were negative. Likewise, myoepithelial cells surrounding secretory cells and interstitial connective tissue including blood vessels and vegetative nerves were negative (Figures 2A–2C). Finally, it is worth mentioning that rare elongated or rounded cells located in veins were labeled with MAb 80B258 (data not shown).

Technically, in all samples examined including those appearing below, no signal could be observed when the primary antibody was omitted (Figure 2B'; see other panels labeled with a prime) or when a mouse isotype IgG1 control was used (see below; online Supplemental Figures SF2, SF3, and SF4). Furthermore, we noticed that, although the preincubation of samples with SDS solution or microwave pretreatment gave the same results as for the cellular localization of the 80B258 antigen, the latter pretreatment seemed to be more advantageous for antigen retrieval (compare the labeling intensity of serous acini display in panels A1 and C1 of Figure 2). Interestingly, on microwave pretreatment, but not SDS, we could observe rare staining with MAb AC133. For instance, the intercalated ducts of submandibular glands were occasionally labeled AC133 MAb (see online Supplemental Figures SF2C and SF2D, blue arrowheads). Likewise, very rare cells located in veins were also AC133 positive (see online Supplemental Figure SF2C, black arrowheads).

**Sublingual Gland (*Glandula Sublingualis*).** The secretory components of the sublingual gland are composed of mostly mucous endpieces with serous demilunes. The proportion of serous acini is less abundant. Just like for the submandibular gland, 80B258 immunoreactivity was present at the apical and apicolateral (Figure 2E, white arrowhead) membrane of cells lining

Figure 2 Localization of prominin-1 in human salivary glands. Paraffin-embedded sections of submandibular (A–C), sublingual (D,E), and parotid (F) glands were immunolabeled with 80B258 MAb and counterstained with either light green (A–E) or hematoxylin-eosin (F). To unmask the 80B258 epitope, samples were either heated in the microwave (A,B,B') or incubated with SDS solution (C–F) before labeling. (A–F) The 80B258 immunoreactivity was observed in the apical and the top part of lateral membranes of epithelial cells (black and white arrowheads, respectively) located in the serous acini (black dotted line) and serous demilunes (orange dotted line). The restricted lateral membranes of cells forming intercellular canaliculi were positive (yellow arrowheads). The apical localization of 80B258 immunoreactivity was observed in cells lining mucous tubuli (B,D,E, blue dotted line). The luminal surface of the intercalated ducts (black line) in submandibular and parotid glands (C,F1, blue arrowheads) and intralobular duct (black line) in sublingual gland (D,E, blue arrowheads) showed 80B258 immunoreactivity, whereas striated ducts were negative (A,F, red dotted line). The insets in A, C, and F demarcate regions shown at higher magnification in A1, C1, and F1, respectively. (B') Negative control, i.e., without primary antibody. Bar = 50  $\mu$ m.



serous acini and apical membrane of mucous tubuli (Figures 2D and 2E, black arrowheads), and those found either in intralobular ducts (Figures 2D and 2E, blue arrowheads) or small interlobular ducts (data not shown).

**Parotid Gland (*Glandula Parotis*).** The parotid gland is the largest human salivary gland. It contains only serous exocrine acini—the major secretory elements—that are connected to a well-developed duct system composed of intercalated, striated, and interlobular excretory ducts. 80B258 immunoreactivity was detected at the apical and the top part of lateral membranes (Figure 2F, see inset F1, white arrowhead) of secretory cells of serous acini (black dotted line) and the apical membrane of intercalated ducts (Figure 2F1, blue arrowhead). As in submandibular glands, a strong staining was detected in cells located at the widened endings of intercellular canaliculi (Figure 2F1, yellow arrowhead). No signal was detected in striated ducts (Figure 2F, red dotted line).

#### Expression of Prominin-1 in the Sweat Glands of Axilla

**Eccrine Sweat Glands.** The secretory portion of eccrine sweat glands is formed by tubules lined with a pseudostratified epithelium consisting of secretory small clear and large dark cells (Saga 2001). Small intercellular canaliculi are developed between them (Saga 2001). The MAb 80B258 labeled the apical surface of tubules (Figures 3A, 3C, and 3E, black arrowheads) and the lateral membrane of cells forming intercellular canaliculi (Figures 3A and 3C–3E, yellow arrowheads). In the duct portion, 80B258 immunoreactivity was found at the apical membrane of epithelial cells (Figures 3A–3C, blue arrowheads) located in the superficial layer of ducts (red dotted line). Secretion found in their lumen was positive (Figures 3A–3C, white asterisks). Occasionally, we could observe ducts that were negative (data not shown). Myoepithelial cells surrounding secretory cells were negative (data not shown).

**Apocrine and Apoeccrine-like Sweat Glands.** Apocrine sweat glands exhibited 80B258 immunoreactivity at the luminal surface of epithelial cells located in the tubular secretory portion (Figures 3F–3H, black arrowheads). The immunoreactivity was associated with tubular elements that were lined by either columnar (as those outlined by a blue dotted line) or cuboidal epithelium (black dotted line) depending on the state of secretion. A high-magnification view showed that 80B258 immunoreactivity was restricted to the apical domain that appears as a dome-like shape (Figures 3I and 3II, red arrows). Some tubules were nevertheless negative (Figure 3H, white arrow). 80B258 immunore-

activity was also associated with materials secreted in the lumen (Figures 3F and 3G, white asterisks; as negative control, see inset 3F').

Interestingly, 80B258 immunoreactivity showed tubular structures sharing simultaneously the characteristics of eccrine and apocrine sweat glands (Figure 3J). For instance, although these tubules exhibit a large lumen as those found in apocrine glands, we could detect some intercellular canaliculi labeled with the MAb 80B258 (Figure 3J, see insets 3J1 and 3J2, yellow arrowheads) reminiscent to eccrine glands. An intermittent apical staining was also observed (Figures 3J1 and 3J2, black arrowheads) where cells with a "straight" apical surface were positive, whereas others having a dome-shaped apical domain were usually negative (red dotted line). Such situation contrasts to that observed with apocrine glands (Figure 3II). Because of their hybrid morphology, we named them "apoeccrine-like" glands. In this context, it is important to mention that Sato and colleagues have previously reported a third type of sweat glands referred to as "apoeccrine" (Sato et al. 1987, 1989; Sato and Sato 1987). They were described as having some segments similar to eccrine glands (e.g., pseudostratified epithelium, narrow lumen, and presence of small intercellular canaliculi), whereas other segments were similar to apocrine glands (e.g., simple columnar epithelium, large lumen). Whether these structures are the same as those we observed here is still an open question.

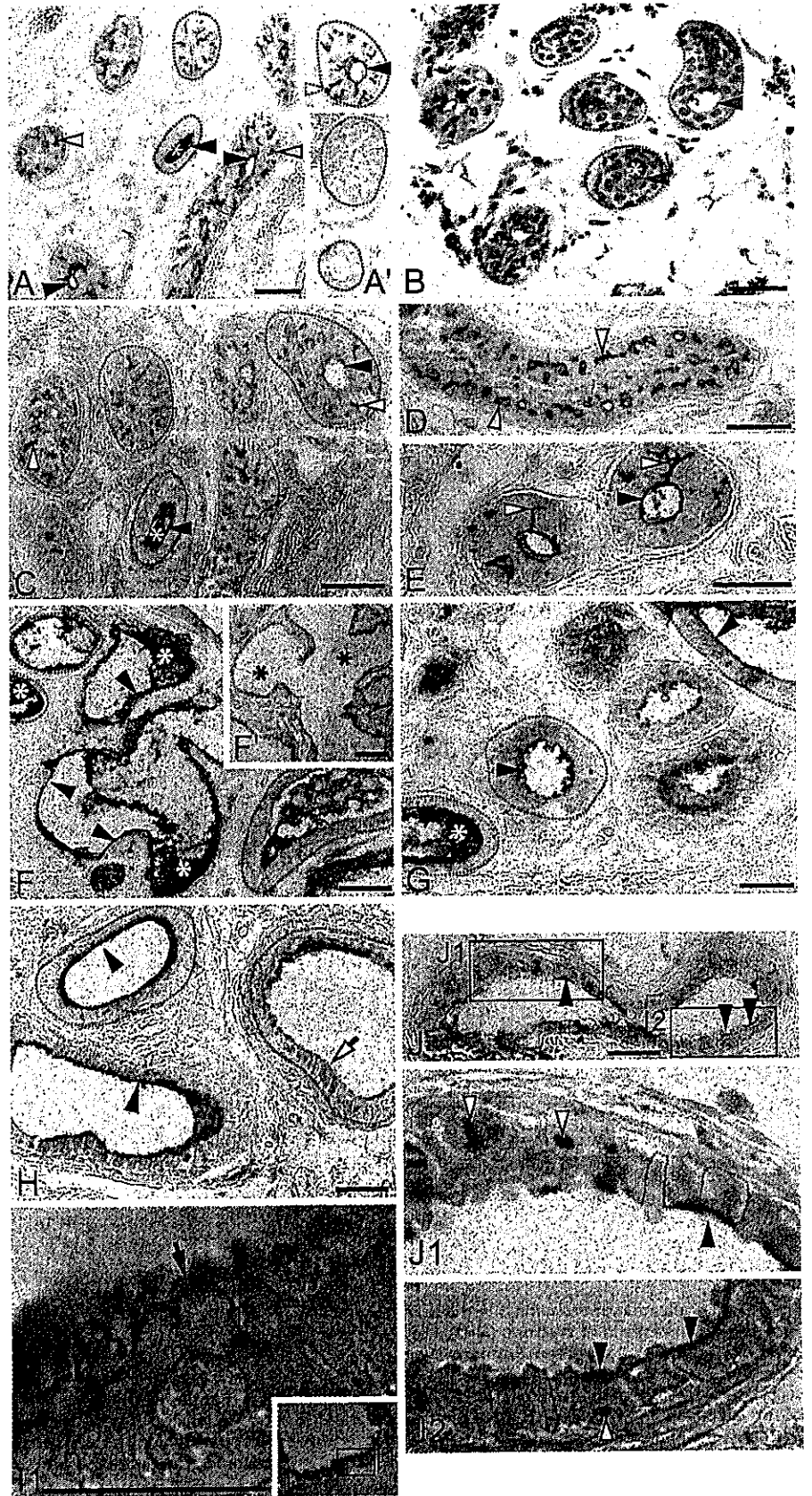
#### Expression of Prominin-1 in the Lacrimal Gland and the Gland of Moll

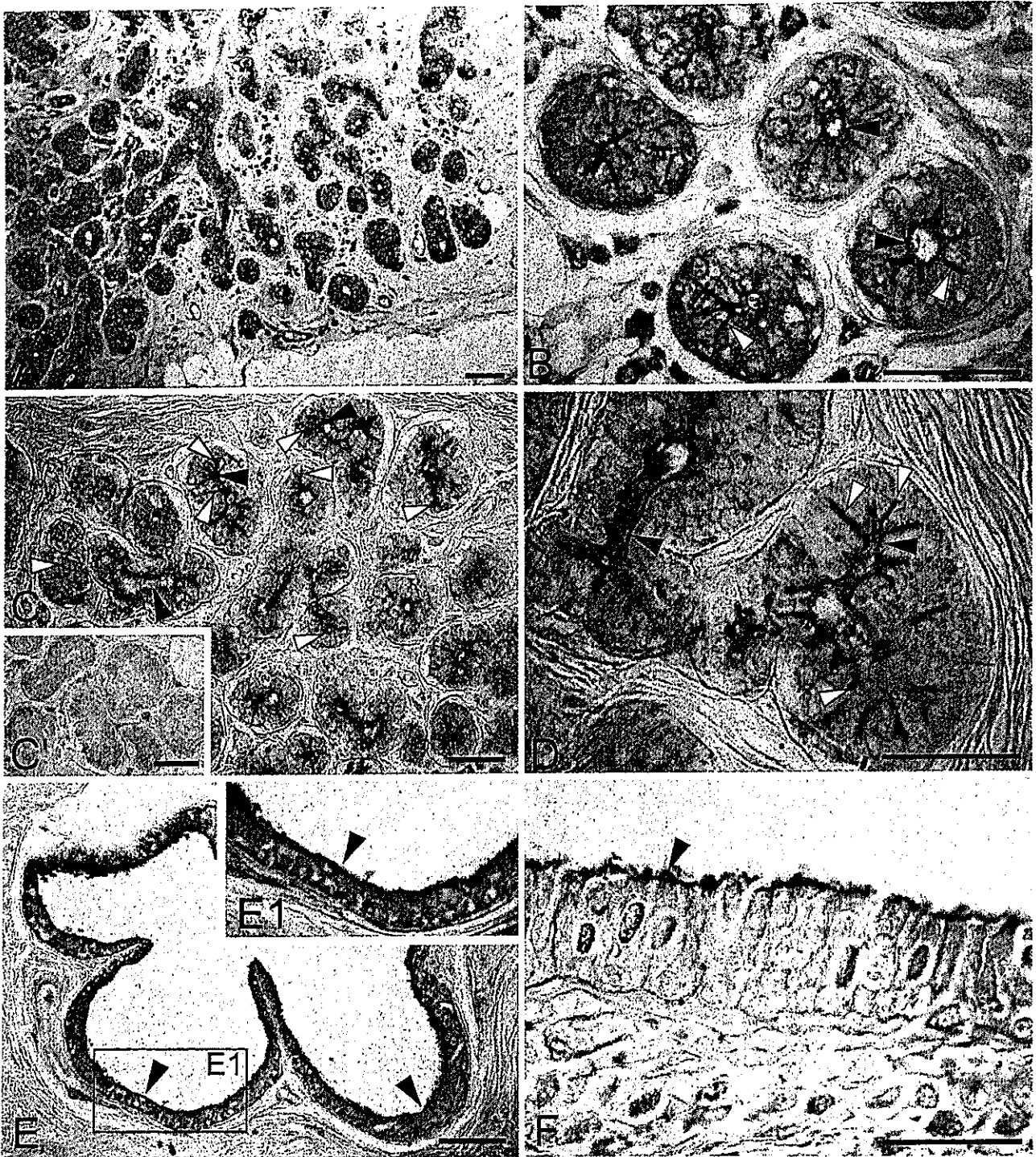
We next studied the expression of prominin-1 in the upper human eyelid, which contains several glands. One of them, the accessory lacrimal gland of Wolfring, is a branched serous tubuloalveolar gland localized in the upper border or above the tarsal plate. It has a similar structure as the large orbital lacrimal gland, but its branched duct system is less complex (Figure 4A). Only 80B258 immunoreactivity was observed at the apical and the top part of the lateral membranes of serous cells (Figures 4B–4D, black and white arrowheads, respectively) including the distended ending membrane of the intercellular canaliculi (Figures 4C and 4D, yellow arrowheads) found in the secretory portion. The apical membrane of epithelial cells associated with the intercalated ducts was positive (Figures 4C and 4D, blue arrowheads).

80B258 immunoreactivity was also observed at the apical membrane of epithelium lining the secretory portion of the apocrine tubular gland of Moll (Figures 4E and 4E1, black arrowheads) and in the secretion found therein (see online Supplemental Figure SF3E, white asterisks). This gland is localized in the edge of the eyelid and opens into the hair follicle of the eyelash, and it is



**Figure 3** Localization of prominin-1 in human sweat glands of axilla. Paraffin-embedded sections containing eccrine (A–E), apocrine (F–I), and apoecrine-like (J) sweat glands were immunolabeled with 80B258 MAb and counterstained with either light green (all panels except B) or hematoxylin-eosin (B). For unmasking the 80B258 epitope, samples were either heated in the microwave (all panels except C) or incubated with SDS solution (C) before labeling. (A–E) The 80B258 immunoreactivity was detected at the luminal surface of cells (black arrowheads) located in the secretory portion (black dotted line) of eccrine sweat glands. The restricted lateral membranes of cells forming intercellular canaliculi (yellow arrowheads), the apical surface of the superficial duct cell layer (blue arrowheads), and secretion (white asterisks) found in the lumen of the duct portion (red dotted line) were positive. (F–I) The 80B258 immunoreactivity was detected at the luminal surface of cells (black arrowheads) and secretion (white asterisks) found in the lumen of the secretory portion of apocrine sweat glands. Note that 80B258 immunoreactivity was associated with tubular structures that are just before or under an extrusion phase of secretion (blue dotted line) and postsecretory or resting state (black dotted line). Some tubules were negative (H, white arrow). The inset in I demarcates a region displayed at higher magnification in I1. In cells in the process of secretion, i.e., those harboring an apical dome-like shape, 80B258 immunoreactivity was confined to the top part of the apical domain (red arrows). (A',F') Negative control, i.e., without primary antibody. Black asterisks indicate the absence of labeling in secreted materials (F'). (J,J1,J2) In apoecrine-like sweat glands, 80B258 immunoreactivity (black arrowheads) was restricted to the cells exhibiting a straight apical plasma membrane (blue dotted line), whereas those with an apical dome-like shape (red dotted line) were negative. The insets in J demarcate two regions shown at higher magnification in J1 and J2. Cells surrounding intercellular canaliculi were positive (yellow arrowheads). Bar = 50  $\mu$ m.





**Figure 4** Localization of prominin-1 in the human lacrimal gland and the gland of Moll of eyelid. Paraffin-embedded sections containing the accessory lacrimal gland of Wolfring (A–D), the gland of Moll (E), and conjunctival epithelium (F) were immunolabeled with 80B258 MAb and counterstained with light green. For unmasking the 80B258 epitope, samples were either heated in the microwave (A,B) or incubated with SDS solution (C–F) before labelling. (A–D) The 80B258 immunoreactivity was observed at the apical and the top part of lateral membranes of serous secretory cells (black and white arrowheads, respectively), and at the widened endings of intercellular canaliculi (yellow arrowheads) of the lacrimal gland. The luminal surface of the intercalated ducts was labeled (blue arrowheads). (C) Negative control, i.e., without primary antibody. (E,F) The 80B258 immunoreactivity was observed at the apical membrane (black arrowheads) of either columnar cells lining secretory tubule of the apocrine gland of Moll (E) or epithelial cells located at the superficial layer of conjunctiva (F). The inset in E demarcates a region displayed at higher magnification in E1. Bar = 50  $\mu$ m.

reminiscent structurally to apocrine glands of the axilla. The lipid secreting holocrine Meibomian sebaceous glands were negative (data not shown).

Finally, some regions of palpebral conjunctival stratified columnar epithelium in the vicinity of the fornix exhibited 80B258 immunoreactivity (Figure 4F, black arrowhead). Neither the MAb AC133 nor isotype IgG1 control gave any immunoreactivity (see online Supplemental Figures SF3F and SF3G, respectively).

Within all sweat gland tissues examined, the AC133 MAb weakly labeled the secreted materials (see online Supplemental Figures SF2I, SF2L, and SF3C, white asterisks) and rarely the apical membrane of apocrine secretory tubules (see online Supplemental Figure SF2L1, black arrowhead), whereas the isotype IgG1 control gave no signal (see online Supplemental Figures SF2J, SF2M, and SF3D).

#### Expression of Prominin-1 in the Uterus, Liver, and Pancreas

**Uterus.** Bioptic samples of normal endometrium in the proliferative phase of the menstrual cycle were examined for prominin-1 expression. In deep portions of the endometrium (pars spongiosa), uterine glands exhibited strong 80B258 immunoreactivity at the apical surface of the epithelium (Figure 5A, black arrowheads). Interestingly, higher-magnification views including a tangential section showed that the immunoreactivity was associated with motile cilia (Figures 5B, 5B1, 5B2, and 5C, blue arrowheads) and other small plasma membrane-associated structures, e.g., microvilli (Figure 5B2, red arrowhead). The top part of the endometrium (pars compacta) containing straight narrow glands and some segments of epithelium lining the uterine cavity were either negative or weakly positive (data not shown).

In the cervix, i.e., the lower and narrow portion of the uterus, 80B258 immunoreactivity was observed at the dome-shaped apical domain (Figures 5D, 5D1, and 5D2, arrowheads) of epithelial cells of the mucous cervical glands (dotted line). Neither the MAb AC133 nor isotype IgG1 control gave any immunoreactivity (data not shown).

**Liver (Hepar).** In the liver, 80B258 immunoreactivity was detected at the apical membrane of cells forming canals of Hering, which are located at the periphery of classic hepatic lobule (Figures 6A and 6B, white arrowheads). In the portal space/tract (red dotted line), intraportal bile ductules lined by a simple cuboidal epithelium (Figures 6A–6E, black dotted line) and small interlobular bile ducts lined by a cuboidal or low columnar epithelium (Figure 6F) were positive at their luminal surface (Figures 6A–6F, blue arrowheads). Large bile ducts lined with simple columnar epithelium (Fig-

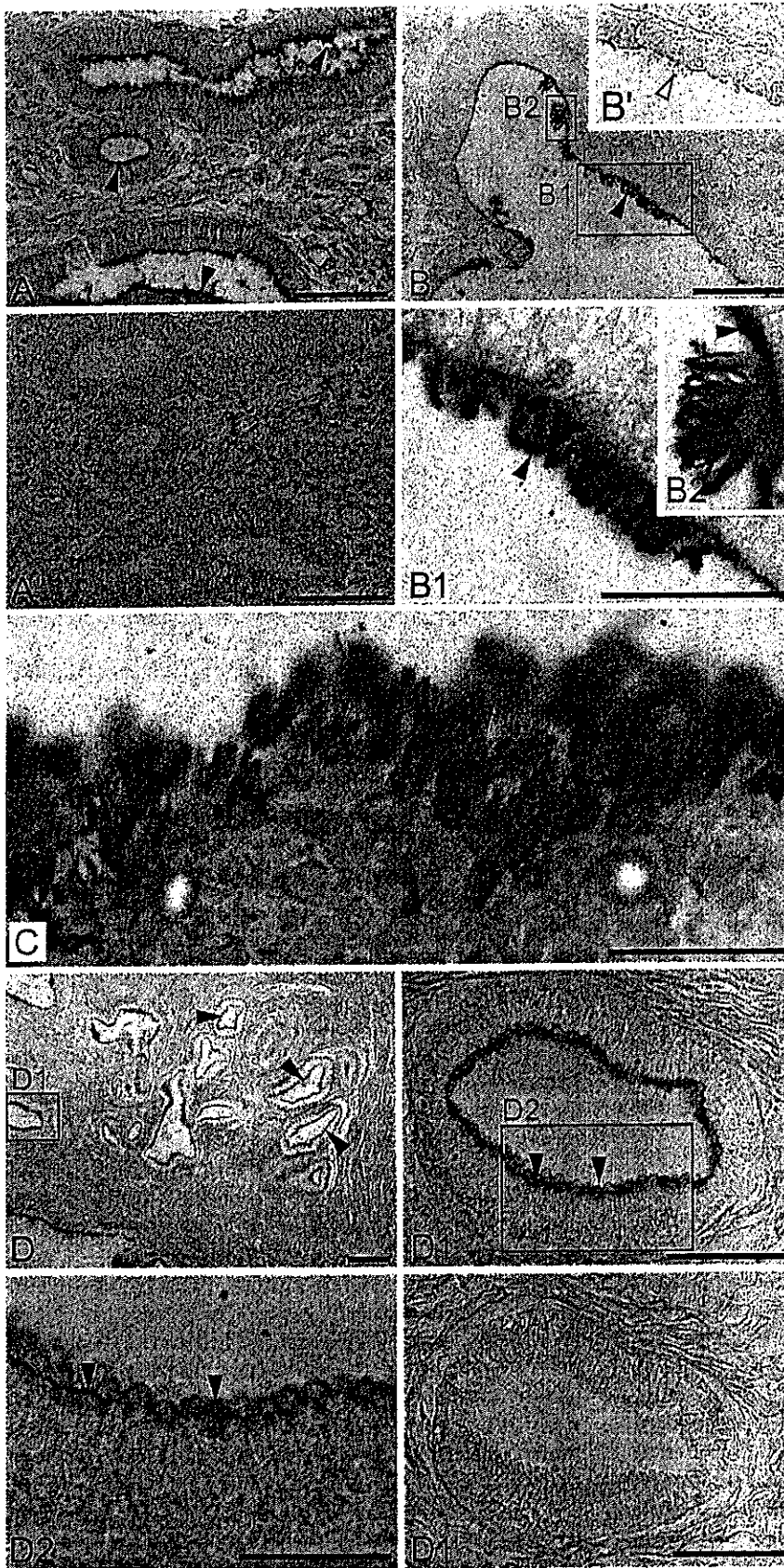
ures 6G and 6H), connective tissue, blood vessels located in the portal spaces, the apical bile canalicular membrane of hepatocytes, and gall bladder were negative (data not shown). Neither the MAb AC133 nor isotype IgG1 control gave any immunoreactivity (see online Supplemental Figures SF4A–SF4C).

**Pancreas.** Recently, we as well as others have shown that prominin-1 is expressed in the apical membrane of cells present in intercalated ducts of the pancreas (Immervoll et al. 2008; Lardon et al. 2008). We could reproduce these data with MAb 80B258 (Figures 7A and 7C, blue arrowheads). Interestingly, we also detected 80B258 immunoreactivity in the apical domain of centroacinar cells (Figures 7B and 7D, black arrowheads), i.e., pale-stained cells lining the inner part of acini (dotted line) of pancreas secretory portion. Interlobular ducts in the connective tissue septa and islets of Langerhans were negative in agreement with previous reports (data not shown). On microwave pretreatment, but not SDS, MAb AC133 gave a weak and occasional staining at the apical membrane of cells present in intercalated ducts (see online Supplemental Figures SF4E–SF4G, blue arrowheads) as previously reported using paraffin sections (Lardon et al. 2008). Rare cells located in the interstitium were found to be positive for AC133 (see online Supplemental Figure SF4H, black arrowhead). The nature of these cells is unknown. No labeling was observed with the isotype IgG1 control (see online Supplemental Figures SF4I and SF4J).

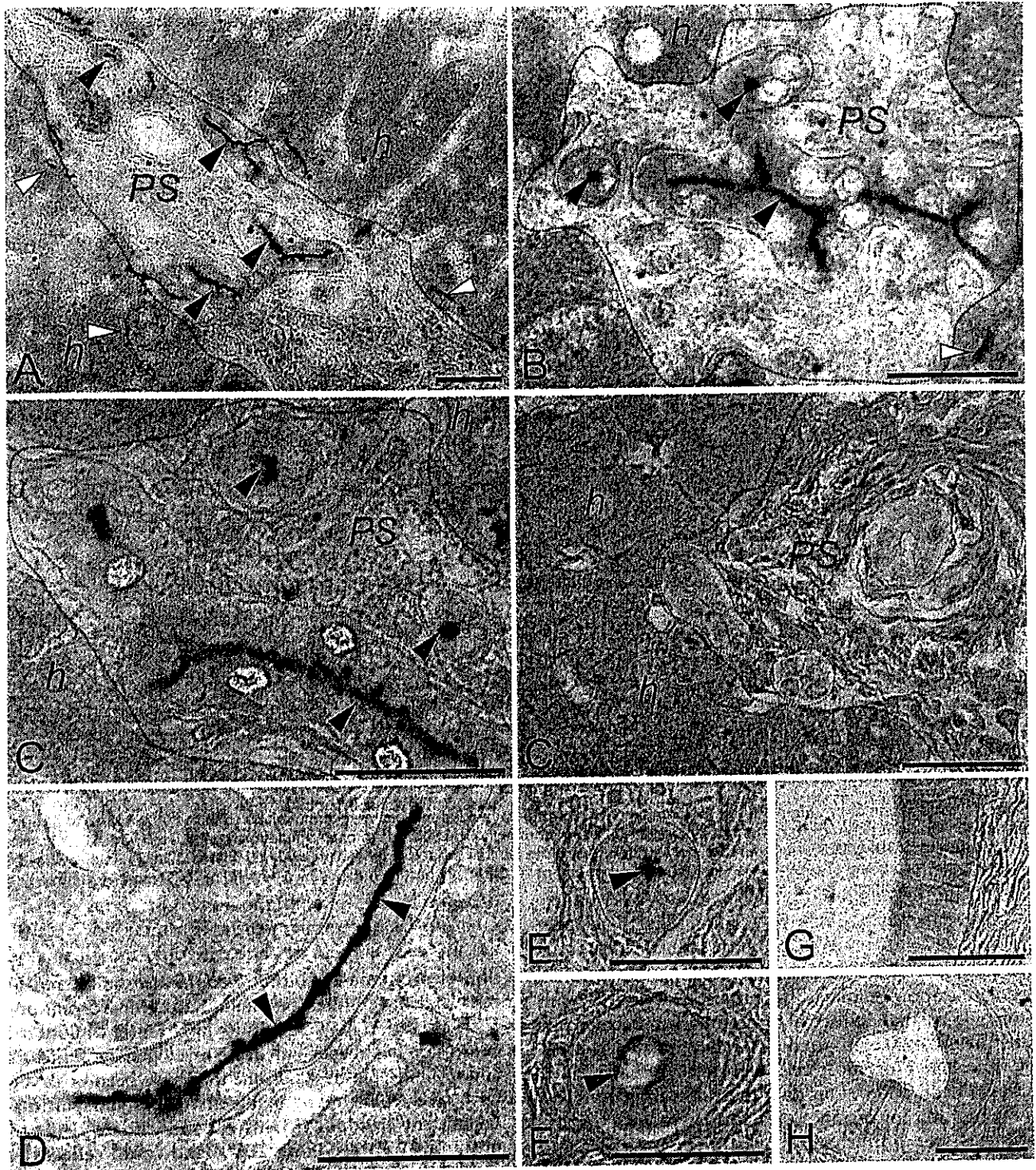
#### Discussion

This study showed that prominin-1 is widely expressed in healthy adult human tissues, which is in agreement with the distribution of its transcript (Miraglia et al. 1997; Florek et al. 2005; see online Supplemental Table ST1), thus mimicking the situation previously observed in the mouse (Weigmann et al. 1997). The lack of its detection observed in previous studies—based on MAb AC133 (Miraglia et al. 1997; for review see Fargeas et al. 2006; see online Supplemental Figures SF2–SF4)—might be explained by the dependence of the corresponding AC133 epitope on conformational and/or glycosylation modifications.

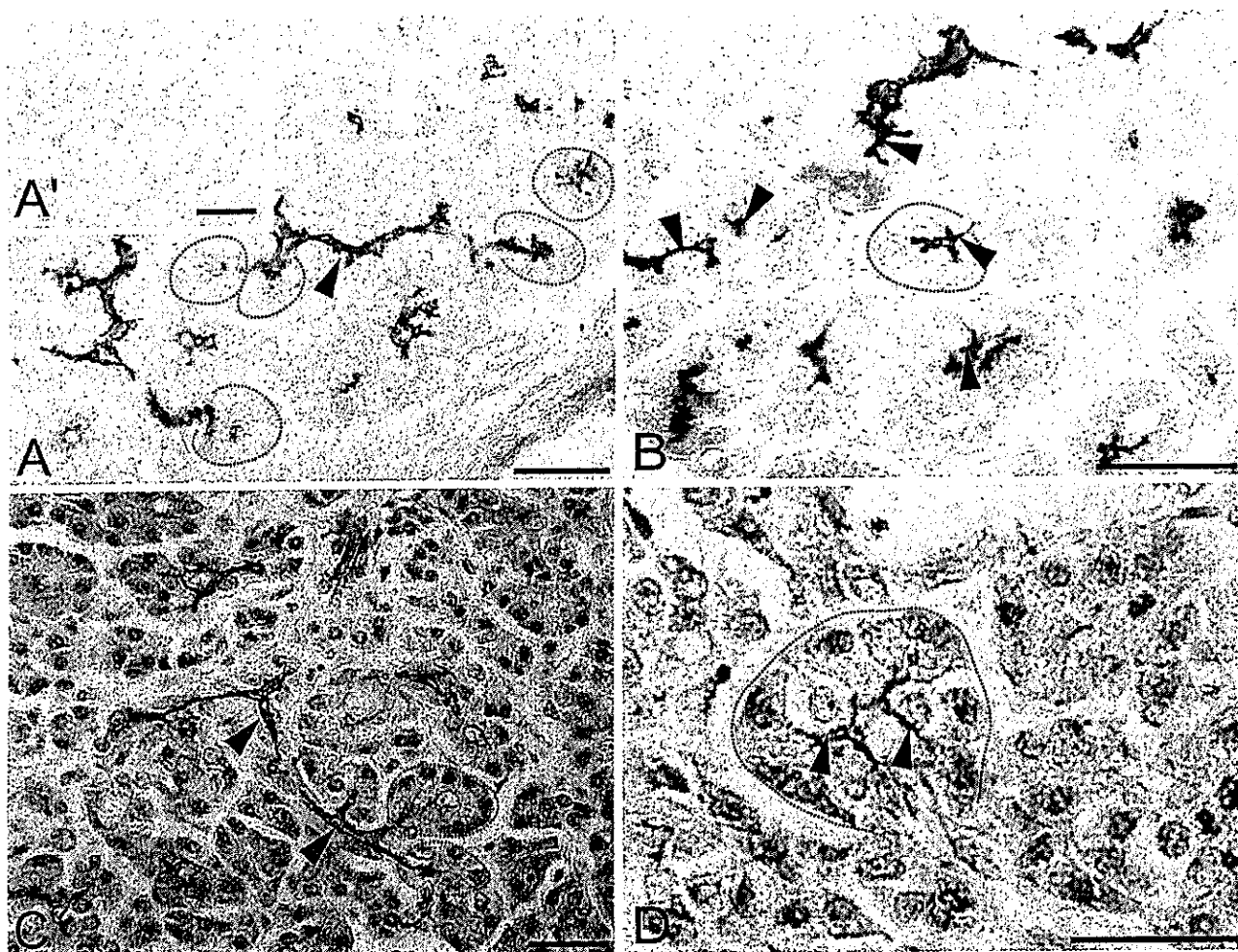
Technically, the AC133 epitope might be sensitive to the paraffin embedding procedure. Indeed, we have previously shown that MAb AC133 failed to give reproducible immunostaining on paraffin sections of healthy human pancreatic samples, although it worked well on cryosections (Lardon et al. 2008). Likewise, Immervoll et al. (2008) have suggested very recently that the prominin-1 AC133 epitope might also be expressed in various adult epithelia, but its detection on paraffin sections needed very sensitive methods including harsh conditions for antigen retrieval. Physiologically,



**Figure 5** Localization of prominin-1 in human normal endometrial and cervical glands. Paraffin-embedded sections of materials derived from bioptic samples were immunolabeled with 80B258 MAb and counterstained with light green. For unmasking the 80B258 epitope, samples were incubated with SDS solution before labeling. (A–C) The 80B258 immunoreactivity was observed at the luminal surface (black arrowheads) of epithelial cells lining endometrial glands that are located in pars spongiosa of the endometrium (A). Insets in B demarcate two regions shown at higher magnification in B1 and B2. Numerous motile cilia (blue arrowheads) and other smaller membrane structures (red arrowhead) were labeled. A tangential section shows the strong labeling of motile cilia (C, blue arrowheads). (D) 80B258 immunoreactivity was observed at the luminal surface of epithelial cells (black arrowheads) lining cervical glands outlined by a black dotted line. The insets in D and D1 demarcate regions shown at higher magnification in D1 and D2, respectively. (A', B', D1', D2') Negative control, i.e., without primary antibody. White arrowhead indicates immuno-negative cilia (B). Bar = 50  $\mu$ m.



**Figure 6** Localization of prominin-1 in the human liver. Paraffin-embedded sections were immunolabeled with 80B258 MAb and counterstained with light green. For unmasking the 80B258 epitope, samples were either heated in the microwave (A,B) or incubated with SDS solution (C–H) before labeling. (A–F) 80B258 immunoreactivity was detected at the apical membrane of cells present in the canals of Hering (A,B, white arrowheads), intraportal ductules (A–E, blue arrowheads), and small interlobular bile ducts (F, blue arrowhead). (G,H) No 80B258 immunoreactivity was detected in larger bile ducts. (C) Negative control, i.e., without primary antibody. h, hepatocytes; PS, portal space, outlined by a red dotted line. Bar = 50  $\mu$ m.



**Figure 7** Localization of prominin-1 in the human pancreas. Paraffin-embedded sections were immunolabeled with 80B258 MAb and counterstained with light green (A,B) or hematoxylin-eosin (C,D). For unmasking the 80B258 epitope, samples were incubated with SDS solution before labeling. (A–D) 80B258 immunoreactivity was detected at the luminal surface of cells located in intercalated ducts (blue arrowheads) and the apical membrane of centroacinar cells (black arrowheads) of the secretory portion outlined by a dotted line. (A) Negative control, i.e., without primary antibody. Bar = 50  $\mu$ m.

we cannot exclude that the AC133 epitope is down-regulated upon cellular differentiation, with the result that only a small subset of prominin-1 molecules carried the AC133 epitope, particularly those expressed by stem and progenitor cells (see below; Bhatia 2001; Fargeas et al. 2006). Our data with salivary glands and the pancreas, for instance, are consistent with this hypothesis (see online Supplemental Figures SF2 and SF4). Given the postulated dependence of the AC133 epitope on a glycosylated structure (Miraglia et al. 1997), the general glycosylation profile of cells might change on differentiation, as suggested in epithelial and non-epithelial cell models (Ogier-Denis et al. 1988; Hemmoranta et al. 2007), leading to the lack of the AC133 epitope but not the prominin-1 molecule as such (Corbeil et al. 2000; Florek et al. 2005). The glycosyla-

tion of prominin-1 molecules could also be altered in cells that have undergone malignant transformation. Such alteration may explain its apparently discordant expression in patients with acute myelogenous leukemia and myelodysplastic syndrome that have been observed with two MAbs recognizing distinct glycosylation-dependent epitopes (MAbs AC133 and AC141) of prominin-1 (Green et al. 2000) and could reflect the different stages of differentiation of these malignant cells. A similar conclusion was drawn on solid cancer (Florek et al. 2005). Alternatively, a switch during the cellular development of a particular prominin-1 splice variant carrying the AC133 epitope to another one lacking it might occur as well. Up to 12 splice variants of prominin-1 have been described so far (Fargeas et al. 2003a,2007). Finally, it is important to point out that

the special molecular environment of prominin-1 within the plasma membrane might also influence the detection of certain epitope(s). For instance, its incorporation into a densely packed cholesterol-based lipid microdomain might mask some of them, particularly those in the vicinity of the membrane (Röper et al. 2000; Janich and Corbeil 2007). Thus, numerous parameters might interfere with the IHC detection of the prominin-1 AC133 epitope, and importantly, the lack of its detection needs to be evaluated with some caution. On a more general note, the term "AC133" shall be used to describe human prominin-1 bearing the AC133 epitope as previously proposed (Fargeas et al. 2003a), and thus the AC133 antigen is not necessary synonymous with prominin-1.

Our study showed that prominin-1 is expressed in various glandular epithelia (see online Supplemental Table ST1), where it is confined to the apical or apicolateral membranes of polarized cells that were fully differentiated and adapted to their function, i.e., secretion (in the case of secretory cells) or modification and conducting the secretion (in the case of duct cells). The free surface of these cells is covered with numerous microvilli, for which prominin-1 has its own tendency to be concentrated, and it might be involved in their organization (for review, see Corbeil et al. 2001b). The presence of prominin-1 in motile cilia found in the endometrium (Figure 5) is another example of its selective localization in plasma membrane protrusions. The latter observation together with the fact that prominin-1 binds to the plasma membrane cholesterol within a specific lipid microdomain (Röper et al. 2000) suggest that the lipid composition and/or organization of the membrane of the motile cilium is distinct to that found in the planar portion, as recently shown for the primary cilium in MDCK cells (Janich and Corbeil 2007).

The overall expression of prominin-1 based on MAb 80B258 is generally consistent with previous RNA data obtained from Northern and dot blot analyses (for details, see online Supplemental Table ST1). However, some discrepancy might be observed given that the expression of prominin-1 is often confined to a particular structure, e.g., cilium, which is not abundant regarding the whole tissue that is normally used to prepare the mRNA samples. The uterus is a good example.

Our data concerning salivary and lacrimal glands (Figures 2 and 4, respectively) document for the first time the source of small membrane vesicles containing prominin-1 found in the human saliva and tears (Marzesco et al. 2005; Jászai et al. 2007). Moreover, the expression of prominin-1 by sweat glands and 80B258 immunoreactivity associated with the secretion therein (Figure 3) indicate that prominin-1 might be found also in sweat. Although the physiological function of these extracellular membrane vesicles is

currently unknown, they might offer an interesting tool for diagnostic purposes of certain solid cancers, e.g., salivary gland cancer, as recently proposed for central nervous system diseases (Huttner et al. 2008). Likewise, analyzing the prominin-1 content, in conjunction with other constituents of the saliva, might be useful for monitoring functional recovery of salivary glands on tissue engineering/replacement. The physiological function of prominin-1 is still not clarified as well. However, its concentration in plasma membrane protrusions (microvillus and motile cilium) present within the apical membrane of glandular (this study; Jászai et al. 2007) and other epithelial cells (Weigmann et al. 1997; Marzesco et al. 2005; Dubreuil et al. 2007) and the occurrence in extracellular membrane vesicles (Marzesco et al. 2005; Florek et al. 2007) potentially derived from these plasmalemma protrusions (Dubreuil et al. 2007; for review, see Fargeas et al. 2006) suggest that this cholesterol-binding glycoprotein might play a role in the secretion process and/or its regulation.

The general expression of prominin-1 in adult human tissues, like in the mouse, is clearly beyond the stem and progenitor cells. However, within the prominin-1-positive cell population might reside a minute population of cells harboring stem cell properties, which are playing a role in vivo in the replacement of damaged and/or aging cells. Such rare primitive cells could be immuno-isolated on tissue dissociation with MAb AC133 as reported previously (Uchida et al. 2000; Tamaki et al. 2002; Yu et al. 2002; Schwartz et al. 2003; Alessandri et al. 2004; Belicchi et al. 2004; Richardson et al. 2004; Bussolati et al. 2005). Alternatively, prominin-1-positive cells (particularly those associated with glandular epithelium) might have a high propensity to dedifferentiate on presentation of appropriate cues. In both scenarios, which are not mutually exclusive, prominin-1 emerges as an important marker with significant clinical value for somatic stem cell isolation (Bornhäuser et al. 2005; Torrente et al. 2007).

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