AC133-2: A Novel Isoform of Human AC133 Stem Cell Antigen

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Running Title
A Novel Isoform of Human AC133 Antigen
Summary

Human AC133 antigen, also called CD133, was recently identified as a hematopoietic stem cell marker. However, the molecular structure and function of this protein has remained unclear. Here, we cloned and identified a novel isoform of AC133, which we named AC133-2. In comparison to the reported AC133 cDNA, which is referred to herein as AC133-1, a small exon of 27 nucleotides is deleted in AC133-2 by alternative mRNA splicing. Similar to the previously characterized AC133 antigen, recombinant AC133-2 expressed in 293 cells was glycosylated and transported to plasma membrane. AC133-2 mRNA was found predominant in a variety of human fetal tissue, adult tissues and several carcinomas. In contrast, AC133-1 mRNA was more prominent in fetal brain and adult skeletal muscle, but was not detected in fetal liver and kidney, adult pancreas, kidney and placenta, suggesting different roles for the two isoforms in fetal development and mature organ homeostasis. We demonstrate here that AC133-2 is the isoform expressed on hematopoietic stem cells derived from fetal liver, bone marrow, and peripheral blood. The results indicate that AC133-2, not AC133-1, has been the cell surface antigen recognized by anti-AC133 monoclonal antibodies used for isolation of hematopoietic stem cells. To further investigate its expression in other stem cell populations, we found AC133-2 co-expressed with β1 integrin in the basal layer of human neonatal epidermis. AC133-2+/β1 integrin+ cells proliferated and differentiated in culture, which coincided with a loss of AC133-2 and gain in a terminal differentiation marker involucrin. Taken together, these results suggest AC133-2 is expressed in multiple stem cell niches and may provide a means to isolate specific stem cell sub-populations from human tissues.
Introduction

Human AC133 antigen is a glycoprotein with a molecular weight of ~120 kDa. Based on its predicted amino acid sequence, AC133 contains an extracellular N-terminus, two large extracellular loops, five transmembrane domains, two small cysteine-rich cytoplasmic loops, and a cytoplasmic C-terminus (1). AC133 antigen was first detected on CD34^{bright} hematopoietic stem cells using a monoclonal antibody (mAb) named clone AC133 that was raised against human CD34^{+} cells (2). AC133 antigen has since been widely used to facilitate the analysis and isolation of hematopoietic primitive cells (3, 4, 5). Subsequently, Peichev et al. showed that endothelial progenitor cells co-express AC133 antigen and the endothelial cell specific receptor KDR in subpopulations of CD34^{+} cells derived from fetal liver, bone marrow, cord blood and peripheral blood (6, 7). Recently, human central nervous system-stem cells were also reported to express AC133 antigen (8). A characteristic feature of this protein is its rapid down-regulation during cell differentiation (7, 9), which makes it a unique cell surface marker for identification and isolation of stem cells and progenitor cells.

A structural and sequence related protein, prominin, was identified as the mouse orthologue of human AC133 antigen (9). The two polypeptides share ~65% amino acid sequence identity. It has been shown that prominin is phylogenetically conserved from mammals to zebrafish, and in fruit flies and nematodes (10, 11). Prominin is selectively expressed in microvilli of various embryonic and adult epithelial cells, and in plasma membrane protrusions of nonepithelial cells including murine CD34^{+} bone marrow progenitor cells (9, 12). Studies have shown that prominin specifically interacts with membrane cholesterol (11), suggesting prominin has a role in membrane organization and membrane-to-membrane interactions. Recently, Corbeil et al. reported that human AC133 antigen was also expressed in the plasma membrane of human epithelial cell line Caco-2 (9). Human retinal degeneration has been associated with a frameshift mutation in AC133 gene, which results in a truncated protein that fails to reach to the cell surface.
Whether human AC133 has similar functions as prominin in membrane organization remains unclear.

Although human AC133 antigen has been used as a cell surface marker to identify and isolate certain stem cell and progenitor cell populations (2, 6, 8), the molecular mechanism of how this protein functions remains unclear. In this study we described the identification and characterization of a novel splice isoform of human AC133 antigen. We examined its tissue distribution at the mRNA level, and protein expression in vitro and in vivo. Our results suggest a role of this isoform in fetal development and adult tissue and organ homeostasis, and its application as a useful cell surface marker for human stem cells.

Experimental Procedures

Cell Lines and Reagents— Retinoblastoma cell line WERI-RB-1 and irradiated 3T3 mouse embryo cells were purchased from American Tissue Culture Collection. 293 cells were kindly provided by Dr. Yashushi Numaguchi, Children’s Hospital, Boston. All primers were synthesized by Sigma Gynosis. Unless otherwise mentioned, all other reagents were purchased from Sigma.

Exon/Intron Structure Analysis— Sequence homology searches in the GenBank™ database were performed using the most recent version of Blast software at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Exon 3/ intron junctions were amplified by polymerase chain reaction (PCR) using forward primer: 5’-ATGCTGCGTTGGTCTGGAGTTCACT-3’ and reverse primer: 5’-CAGCAGCCCGAGGACAGCATAGAA-3’, and Takara LA-PCR kit (PanVera Corp). The genomic DNA fragment was sequenced in the Molecular Core Facility, Children’s Hospital, Boston.

Cloning of AC133 Isoforms— The coding region of AC133-2 was amplified from cDNA of WERI-RB-1, lung carcinoma LX-1, fetal liver or adult kidney tissues by PCR using forward
primer: 5’-GCACCTCGAGTGAGGATCTTGCTAGCTATG-3’ and reverse primer: 5’-GAGTGTTAGTGGATGAGGATCTTGCTAGCTATG-3’. The PCR condition used was: 94°C for 1 min, 57°C for 1 min, 72°C for 3 min. Authenticity was confirmed by DNA sequencing (Molecular Core Facility, Dana Farber Cancer Institute). The PCR products were subcloned into pcDNA3.1 (-) (Invitrogen) at restriction sites Xho I and Kpn I.

**mRNA Expression**— To separate the two AC133 isoforms, forward primer 5’-CAGAAGGCA TATGAATCC-3’ and reverse primer 5’-CACCACATTTTTACAGC-3’ were used in PCR to amplify short DNA fragments. Hot start PCR was then performed for 35 cycles using Taq and pfu DNA polymerase at 1:5 enzymatic activity ratio. Each PCR cycle included: 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec, followed by extension at 72°C for 5 min after cycling. PCR products were analyzed in ethidium bromide-stained 3% MetaPhor-agarose gels (BioWhittaker Molecular Applications). cDNA panels of multiple human tissues and tumors were from CLONTECH.

**Flow Cytometry**— 293 cells transfected with AC133-2 cDNA construct were trypsinized, and resuspended in PBS containing 2 mM EDTA and 0.5% BSA. Single cell suspensions were labeled with PE-conjugated mAb AC133/2 (Miltenyi Biotech), or PE-conjugated mouse IgG1 isotype control (R&D Systems) according to the manufacturer’s instruction. Cells were then analyzed or sorted on a FACS Vantage SE flow cytometer (Becton-Dickinson). 10,000 to 50,000 events were acquired in list mode with CellQuest software (Becton-Dickinson). The listmode files were analyzed on a PC computer with WinMDI 2.8 software (The Scrippps Research Institute).

**Immunofluorescence and Confocal Microscopy**— Near confluent 293 cells grown on glass coverslips were transfected with 2 µg of the AC133-2 cDNA and 10 µl of Lipofectamine 2000
(Life Technology Inc.). After 48 h, cells were carefully rinsed with PBS and fixed in 3% paraformaldehyde in PBS for 30 min at room temperature. WERI-RB-1 cells grown in suspension were directly plated on glass coverslips in 50 µl PBS/0.5% BSA for 20 min before fixation. Incubation and washes were done directly on the coverslips. Indirect immunofluorescence staining was performed as previously described (9). Cells were observed with a BioRad uRadience2000/Nikon Eclipse 800 (Bio-Rad Laboratories) confocal laser scanning scope using BioRad LaserSharp2000 imaging software. The images shown were prepared from confocal image files using Adobe Photoshop 5.5 software.

Cell Surface Biotinylation and Immunoprecipitation—Biotinylation was performed as reported (9), except that Sulfo-NHS-LC-LC-biotin (Pierce Chemical) was used. Protein concentrations of the cell lysates were quantified by Bio-Rad Protein Assay. 400 µg protein was incubated with mAb AC133/2 at 10 µg/ml for overnight. Immune complexes were collected with protein A/G-agarose (Santa Cruz Biotechnology). Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis on a 4-12% gradient gel (Invitrogen), and transferred to a PVDF membrane (Millipore). The blot was blocked with 5% low fat milk/0.3% Tween-20. Biotinylated proteins were identified by horseradish-conjugated NeutrAvidin (Pierce Chemical) and detected by enhanced chemiluminescence western blotting detection reagent (KPL).

LX-1 Lung Carcinoma Implantation and Tumor Cell Isolation—Frozen LX-1 tumor fragment was acquired from the National Cancer Institute. 2 x 2 x 2 mm³ size tumor cubes were implanted in the axillary region of 6–7 week-old nude (nu/nu) mice (13). After 3 to 4 weeks, about 2 x 2 x 2 cm³ size tumors were dissected. Tumor tissue was minced in PBS and gently homogenized in a 50-ml tube (Fisher Scientific) with a fitted pestle. Tumor cells were then
sequentially filtered through 100 μm and 40 μm mesh. Single tumor cells were subjected to flow cytometry or cell surface biotinylated followed by immunoblotting to detect AC133-2 protein.

**AC133-2 Expression in Human Skin Epidermis**— Human neonatal foreskins were obtained from routine circumcisions performed at Brigham and Women’s Hospital, Boston under an IRB approved protocol. Epidermal basal keratinocytes were isolated as reported (14). Isolated cells were labeled with PE- or FITC-conjugated isotype controls, PE-conjugated AC133/2 mAb, or FITC-conjugated CD29, a mAb against β1 integrin subunits (Beckman Coulter). Cells were analyzed by flow cytometry or indirect immunofluorescence staining. AC133-2+/β1+ cells purified by fluorescence-activated cell sorting (FACS) were cultured on a feeder layer of 3T3 mouse embryo cells using the method of Rheinwald (15). To monitor the loss of AC133-2 expression, very confluent keratinocytes were further grown in DME/F12 containing 2% FBS for a week before FACS analysis. For indirect immunofluorescence staining, cultured keratinocytes were plated on glass cover slips pre-seeded with irradiated 3T3 cells, and grown in the same conditions as described above. Confluent cells were fixed and stained with anti-keratin (clone LP34) or anti-involucrin (NeoMarkers) as described (16).

**Results**

**Cloning and Identification of AC133-2**— To make a Northern blot cDNA probe for human AC133 antigen, we amplified a N-terminal fragment of 458 bp (GenBank Accession No. AF027208) by RT-PCR from the retinoblastoma cell line WERI-RB-1, from which the first AC133 cDNA was cloned (1). DNA sequencing data revealed that one out of four clones had a deletion of a 27bp segment corresponding to positions 314 to 340. This did not result in a frame shift in the coding sequence, but created a 9 amino acid deletion (Fig. 1A, 1B). Using RT-PCR with primers flanking the start and stop codons of AC133, a novel full-length cDNA clone was
isolated and sequenced. This novel sequence, which will be referred to as human AC133-2, differs from the previously published AC133, which will be referred to as human AC133-1, by the 27 bp deletion (Fig. 1A). A literature search revealed that a homologous mouse sequence to human AC133-1 had previously been reported by Maraglia et al. (17). It was named as mouse AC133, which will be referred to as mouse prominin-1 in this paper (Fig. 1B). Interestingly, this sequence is 27 bp longer than the previously characterized mouse prominin, which will be referred to here as prominin-2. The relationship between the gene structure of prominin-1 and –2 has not been reported. The corresponding nine amino acids in human AC133-1 are highly homologous to that in mouse prominin-1 (Fig. 1B). Human AC133-2 and mouse prominin-2 encode proteins that lack a 9 amino acid segment at same location in the N-terminal extracellular region just proximal to the first transmembrane domain. The deletion produces no significant change in the predicted membrane spanning domains of AC133-2, compared to AC133-1, by hydrophobicity analysis (data not shown). The discovery of human AC133-2 and the existence of its mouse counterpart prominin-2 indicate that AC133-2 may be a result of alternative mRNA splicing.

*Human AC133-2 is a Novel Splice Variant*— Exons of AC133 gene were identified by aligning human genomic sequences with AC133-1 or –2 cDNA sequences using the database at the National Center for Biotechnology Information. We found two human chromosome 4 clones C0024K08 and RP11-45J21 (GenBank™ accession number AC005598 and AC067999) that each contains most of the exons of the AC133 gene. Exons were also confirmed by blasting AC133-2 cDNA against the recently completed human genome. The AC133 gene consists 27 exons spanning a region of about 200 kb. Exon 1 contains the 5’UTR and the start methionine, while the stop codon is in exon 26. Exons 1 to 26 range in size from 27 to 217 bp, whereas exon 27 is considerably larger, containing at least 1143 bp. All exon/intron junctions conform to the GT-donor/AG-acceptor rule (20) (Table 1, see supplemental data). Our analysis revealed that AC133-
2 resulted from an deletion of exon 3 of 27 nucleotides, flanked by splice acceptor and donor consensus sequences (Fig. 1C). Using a forward primer in the intron prior to exon 3 and a reverse primer in exon 4, exon 3 and its surrounding intron sequences were amplified by PCR from human genomic DNA, and confirmed by DNA sequencing. These results indicate that AC133-2 is a novel alternatively-spliced isoform of AC133-1.

AC133-2 is a Membrane-associated Glycoprotein—To determine if AC133-2 cDNA could be transported to the cell surface, as shown for the endogenous AC133 (1), we analyzed the protein product and its cellular localization in 293 cells transfected with AC133-2 cDNA. Cell Surface AC133-2 was detected by flow cytometry (Fig. 2A) using a commercially available PE-conjugated mAb designated AC133/2 (1, 2). More than 95% transfected cells expressed AC133-2. In contrast, mock transfected 293 cells did not show any immunoreactivity to this antibody (data not shown). Localization of AC133-2 to plasma membrane of transfected 293 cells was observed by indirect immunofluorescence staining with the same antibody followed by confocal laser scanning microscopy (Fig. 2B, a, b). The cellular localization of endogenous AC133 antigen in retinoblastoma cell line WERI-Rb-1 was similar (Fig. 2B, c, d). Cell surface distribution of AC133-2 was further confirmed by biotinylation of the intact cells, followed by immunoprecipitation with mAb AC133/2. Recombinant AC133-2 was detected by NeutrAvidin blotting as a single band with an apparent molecular mass of ~115 kDa (Fig. 2C). Deglycosylation with PNGase F yielded a ~94 kDa band, which is the predicted size from AC133-2 amino acid sequence. The 9 amino acid deletion in AC133-2 should not affect the degree of N-glycosylation compared to AC133-1, because none of the consensus sequences for glycosylation sites are within the deleted region.

Tissue mRNA Expression of AC133 Isoforms—Similar to AC133-1, the transcript of AC133-2 is approximately 4 kb as determined by Northern blotting (data not shown). However, the size
difference between AC133-1 and –2 mRNA is too small to be resolved by this technique. Specific primers flanking the truncated sequences were used to amplify AC133-1 fragment of 180 bp and AC133-2 of 153 bp in the same PCR reaction. The two fragments were then separated electrophoretically on a 3% MetaPhor agarose gel. The identity of the cDNA fragments was confirmed by DNA sequencing. To determine the constitutive mRNA expression profiles of AC133 isoforms, PCR was performed on commercially available cDNA panels. Since the CLONTECH multiple tissue cDNA panels have been normalized against several housekeeping genes, mRNA expression level between samples can be directly compared.

AC133-2 mRNA was ubiquitously expressed in a variety of human fetal and adult tissues (Fig. 3A). It was predominant in fetal liver, skeletal muscle, kidney, and heart, as well as adult pancreas, kidney, liver, lung and placenta. AC133-1 mRNA was not detectable in fetal liver and kidney, adult kidney, pancreas and placenta. However, AC133-1 was abundantly expressed in fetal brain, but poorly expressed in adult brain. In skeletal muscle and heart, the relative abundance of the two isoforms was reversed between fetal and adult tissues. Interestingly, AC133-2, but not AC133-1, was high in fetal liver, low in bone marrow, and barely detectable in peripheral blood (Fig. 3B). These relative levels of AC133-2 mRNA in hematopoietic tissue are consistent with reported AC133 protein levels (3). Low levels of AC133-1 transcripts were observed only after AC133+ cells were purified from fetal liver, bone marrow, cord blood and G-CSF immobilized peripheral blood using the mAb AC133/2 conjugated magnetic beads. The ratios of AC133-1 and AC133-2 transcripts were identical among these samples (data not shown). Also, AC133-2 was easily detected in several tumors, such as lung carcinoma LX-1, pancreatic adenocarcinoma GI-103, colon adenocarcinoma CX-1 and breast carcinoma GI-101 (Fig. 3C). In contrast, AC133-1 was strongly expressed in retinoblastoma cells WERI-RB-1, the tumor from which this isoform was originally cloned.

In vivo AC133-2 Protein Expression — Due to the lack of isoform-specific antibody, we have not investigated the protein tissue distribution profile of AC133 isoforms. We know AC133-2
protein is expressed on hematopoietic stem cells, in which AC133-2 is the predominant transcript (Fig. 3B), because AC133+ cells have been isolated from hematopoietic tissues (2, 3, 4, 5, 6). To show that AC133-2 protein is expressed in vivo in other tissues, we analyzed cell surface expression of this protein in lung carcinoma LX-1. LX-1 tumors expressed only the AC133-2 mRNA (Fig. 3C). Mononuclear cells isolated from peripheral blood and WERI-RB-1 retinoblastoma cells were included as a negative and a positive control, respectively. Flow cytometric analysis showed that about 80% of LX-1 tumor cells were recognized by AC133 mAb (Fig. 4B). In addition, the endogenous AC133-2 protein was detected by immunoprecipitation from biotinylated LX-1 tumor cells (Fig. 4D). AC133-2 appeared as a single band with a similar molecular mass to the AC133 antigen expressed in WREI-RB-1 cells. The relative protein levels of AC133-2 in LX-1 and WERI-RB-1 determined by immunoprecipitation and flow cytometry were similar.

Expression of AC133-2 in a Human Stem Cell Niche— The wide tissue distribution of AC133-2 (Fig. 3A, B) suggests that the utility of this antigen for stem cell analysis and purification is not restricted to hematopoietic tissue. To determine if AC133-2 was expressed in other stem cell niches, we chose to isolate basal keratinocytes from human neonatal foreskin epidermis due to its availability. Epidermal stem cells reside in the basal layer of epidermis, in which cells mainly express α2β1, α3β1, and α5β1 integrins (5). Only AC133-2 was detected in epidermal cells (Fig. 5A, lane 1). Flow cytometric analysis showed that over 90% of the isolated epidermal cells were positive for β1 integrin subunits, indicating they were basal cells (Fig. 5B). In the total isolated cell population, the percentage of AC133-2+ cells was the same as the AC133-2+/β1 integrin+ cells, suggesting AC33-2+ cells were a subpopulation of the epidermal basal cells (data not shown). About 10% of the basal cells were positive for PE-conjugated mAb AC133/2 and FITC-conjugated mAb against β1 integrin subunits. Previous studies have shown that about the same
numbers of the basal keratinocytes have stem cell properties (21, 22). Expression of AC133-2 was then further confirmed by indirect immunofluorescence staining of FACS-sorted AC133+ cells with a different mAb (Fig. 5C). Over 95% cells were AC133-2 positive, and were also β1 integrin-positive (data not shown).

**Down-regulation of AC133-2 in Culture**—To test whether isolated AC133+ cells could proliferate and differentiate in culture, isolated epidermal basal cells were either labeled with PE-conjugated AC133 mAb or doubled with PE-conjugated AC133 mAb and FITC-conjugated anti-β1 integrin mAb, and then AC133+ cells or AC133+/β1 integrin+ cells were isolated by two rounds of fluorescence-activated cell sorting (Fig. 6B, left panel). The positive cells formed visible colonies as early as 6 to 10 days after culture initiation on irradiated 3T3 embryonic cells. Colonies formed faster among AC133+ cells than AC133+/β1 integrin+ cells, perhaps because antibody binding to β1 integrins decreased the efficiency of cell attachment through integrin receptors. Cells continued to grow and formed tightly adherent epithelioid colonies and underwent stratification, which was difficult to see under phase contrast (Fig. 6Aa) but was manifested by indirect immunofluorescence staining with a mAb to anti-keratin 5, 6 and 18 (Fig. 6Ac). Clearly, all cultured cells were keratinocytes. The flat stratified cells in layers also expressed involucrin (Fig. 6Ad), a terminal differentiation marker (22). Isotype-matched control monoclonal Ab did not show any immune reactivity (Fig. 6Ab). Flow cytometry showed that AC133-2 expression was completely lost in cultured basal cells after confluent keratinocytes were grown in basal medium containing low serum for 6 days (Fig. 6B, right panel). This was confirmed by indirect immunofluorescence staining with anti-AC133 mAb (data not shown).
Discussion

In this study we have identified and characterized a second isoform of AC133. As a result of mRNA alternative splicing, the novel isoform, AC133-2, differs from the previously identified isoform, AC133-1, by absence of exon 3. As a consequence, AC133-2 encodes 856 amino acid residues with a deletion of 9 amino acids in the N-terminal extracellular domain.

How might loss of the 9 amino acids affect the structure and function of AC133-2? The nine amino acid deletion does not interfere with the signal peptide, asparagine-linked glycosylation sites, or any of the five transmembrane domains. Similar to the endogenous human AC133-1 antigen (1) and mouse prominin-2 (12), human AC133-2 can localize to the plasma membrane, indicating a possible role in cell-cell interactions or ligand receptor interactions. Whether the two isoforms are functionally redundant or serve distinct functions remains unclear, as little is known about their biological functions. Nevertheless, since both AC133 isoforms contain 5 tyrosine residues in the cytoplasmic C-terminal domain, the biological functions may depend on an interaction with an unknown ligand(s) as well as intracellular proteins, and thereby relay AC133 isoform-specific signaling. We speculate that the deletion in the AC133-2 isoform may affect ligand binding affinity and specificity, and therefore delineate its biological function from AC133-1.

The mRNA distribution profiles of AC133-1 and –2 suggest distinct roles for the two isoforms in development and mature organ homeostasis. Whereas AC133-2 was expressed ubiquitously, AC133-1 was not detectable in fetal liver and kidney, adult pancreas, kidney and placenta. AC133-1 was strongly expressed in fetal brain but not in adult brain tissue, implying a possible role for AC133-1 in fetal brain development. AC133-2 was more abundant than AC133-1 in fetal skeletal muscle and heart but much less than AC133-1 in adult counterparts, suggesting AC133-2
may be involved in fetal development in these tissues. In addition, we found that AC133-2 was strongly expressed in four poorly differentiated human tumors, including lung carcinoma LX-1, pancreatic adenocarcinoma GI-103, colon adenocarcinoma CX-1 and breast carcinoma GI-101, but not in prostatic adenocarcinoma PC3, lung carcinoma GI-117, ovarian carcinoma GI-102, and colon adenocarcinoma GI-112. Variable expression of AC133 isoforms in tumors may be associated with the degree of differentiation of the tumor cells.

In vivo, AC133 antigen was first detected in hematopoietic CD34\textsuperscript{bright} cells using a mAb raised against CD34\textsuperscript{+} cells from human blood (1). AC133-1 cDNA was originally cloned from a retinoblasoma cell line WERI-RB-1, and has been the only reported transcript encoding AC133 antigen (2). Here, for the first time, we demonstrate that AC133-2 transcript is predominant in hematopoietic tissue, while AC133-1 is the predominant transcript in WERI-RB-1 cells (Fig. 3). Based on abundant evidence in the literature (2, 3, 4, 5, 6), it appears that the AC133-2 isoform has been the antigen recognized in many Ab-based selection protocols to isolate hematopoietic stem or progenitor cells.

In addition, we show that AC133-2 is expressed in human stem cell niches other than hematopoietic tissue. About 10\% basal keratinocytes in foreskin epidermis co-express AC133-2 and \( \beta_1 \) integrin subunits, consistent with previously reported numbers of stem cells in human skin epidermis (14). We showed that isolated AC133-2\textsuperscript{+}/\beta_1 integrin\textsuperscript{+} cells from the epidermal basal layer can self-renew, differentiate in culture, and that AC133-2 is lost as the cells differentiate into non-stratified and stratified involucrin-expressing keratinocytes. These results demonstrate that AC133-2 serves as a marker of undifferentiated cells. Previously, isolation of skin epidermal stem cells relied solely on \( \beta_1 \) integrin as a marker, which cannot efficiently differentiate stem cells and other basal cells due to subtle difference in their integrin levels (20). Therefore, AC133-
2 together with β1 integrin will facilitate direct isolation of purer populations of epidermal stem cells.

The expression pattern of AC133-2 suggests that stem cells/progenitor cells are present in more human fetal and adult tissues than previously thought. Accumulating evidence supports the concept that the utility of AC133 antigen as a stem cell marker is not restricted to hematopoietic tissue (8, 21). Recently, Uchida et al. reported the isolation of human central nervous system-stem cells (hCNS-SC) by using a monoclonal antibody to AC133 antigen and other specific markers for hCNS-SC (8). Our study supports that AC133 antigen is an excellent marker for stem and progenitor cells when used in combination with other tissue and cell specific markers. The AC133-2 isoform may be of significance in the analysis and isolation of stem and progenitor cells from specific tissues and thereby facilitate functional characterization and application to tissue engineering and regenerative medicine.

In conclusion, we have isolated and characterized a novel isoform of the AC133 antigen. Our findings clarify the sequence identities of the two isoforms on the surface of stem cells and progenitor cells. The discovery of AC133-2 isoform increases complexity of this novel family of 5-transmembrane cell surface glycoproteins. Further investigation is needed to unmask the biological significance of these two AC133 isoforms.
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Footnotes

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Figure legends

**Fig. 1. Sequences of AC133-1 and AC133-2 isoforms.** A, 27 bp is deleted in the human AC133-2 DNA sequence compared to AC133-1. B, The resultant 9-amino acid deletion is indicated by dotted lines (top). Analogous region in mouse prominin-1 and –2 is shown (bottom). C, The intron/exon boundaries conform to the splice donor/acceptor consensus (bold).

**Fig. 2. Expression of recombinant AC133-2 protein in 293 cells.** A, 293 cells were transfected with AC133-2 cDNA construct or empty vector (mock) for 48 h. Cells were analyzed by flow cytometry. Peak (a) in the histogram represents cells stained with PE-conjugated mIgG1 isotype control. Peak (b) are cells stained with PE-conjugated AC133/2 mAb. B, Membrane localization of recombinant AC133-2. AC133-2 cDNA transfected 293 cells (a, b) or WERI-RB-1 cells (c, d) were labeled with PE-conjugated mIgG1 (a, c), or AC133/2 mAb (b, d), followed by FITC-conjugated anti-mouse IgG (green). Single optical xy plane sections at center of cells are shown. C, AC133-2 cDNA or mock transfected 293 cells were biotinylated with sulfo-NHS-LC-LC-biotin prior to immunoprecipitation from cell lysates using AC133/2 mAb. Immune complexes were treated with or without 1.5 unit of PNGase F at 37°C for overnight followed by NeutrAvidin blotting.

**Fig. 3. Tissue distribution of AC133-1 and AC133-2 mRNA.** AC133-1 and –2 were amplified by PCR, and then separated on 3% Metapho-agarose gel stained with ethidium bromide. Sample mRNA level was quantitated by glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression in the lower panels. The variation in G3PDH level is because of the multiple housekeeping gene normalization method used by Clontech. The DNA markers in kb are shown on the left. A, AC133-1 and –2 in human fetal and adult tissues. B, AC133-1 and AC133-2 in human fetal liver, bone marrow and peripheral blood. C, AC133-1 and AC133-2 in human tumors,
including breast carcinoma GI-101, prostatic adenocarcinoma PC3, lung carcinoma GI-117 and LX-1, ovarian carcinoma GI-102, pancreatic adenocarcinoma GI-103, colon adenocarcinoma GI-112 and CX-1, and retinoblastoma cells WERI-RB-1. Results are consistent in at least three independent PCR reactions.

**Fig. 4. In vivo AC133-2 protein expression.** Peripheral blood mononuclear cells (A), WERI-RB-1 retinoblastoma cells (B), or LX-1 lung carcinoma cells (C) were labeled with PE-conjugated mIgG1 (a) or PE-conjugated AC133/2 mAb (b), and analyzed by flow cytometry. D, cells were biotinylated with sulfo-NHS-LC-LC-biotin prior to immunoprecipitation from cell lysates using AC133/2 mAb. Immune complexes were separated on SDS-PAGE, transferred to PVDF membrane and probed by NeutrAvidin. AC133-2 protein band is indicated by a arrowhead.

**Fig. 5. AC133-2 is expressed in subpopulation of epidermal basal cells.** Epidermal basal cells were isolated from neonatal foreskins digested with thermolysin and trypsin. A, AC133 isoforms were amplified by RT-PCR from RNA of the isolated epidermal cells (lane 1), or control WERI-RB-1 cells (lane 2). B, epidermal cells were double labeled with isotype controls (left panel), or PE-AC133/2 mAb to AC133-2 and FITC-CD29 to β1 integrin subunits (right panel). Cells were analyzed by flow cytometry. The upper right quadrant shows cells stained for both AC133-2 and β1 integrin subunits. C, AC133+ cells labeled with only PE-AC133/2 mAb were sorted by two rounds of FACS, plated on glass coverslips and stained with another mAb AC133/1 to AC133-2, followed by Texas-Red anti-mouse IgG (red). Green fluorescence of β1 integrin subunits colocalize with red fluorescence of AC133-2 (not shown). Single optical xy plane sections at center of the cells are shown. The results are representative of three independent experiments.
Fig. 6. Down-regulation of AC133-2 in culture. A, AC133⁺/β1 integrin⁺ cells isolated by fluorescence activated cell sorting (FACS) were grown on irradiated 3T3 embryonic feeder cells. Cultured cells were photographed under phase contrast (a), or stained with matched isotype control mIgG1 (b); or with anti-keratin mAb clone LP34 (c), or with anti-involucrin mAb (d) followed by FITC-conjugated mIgG. B, Purity of AC133⁺/β1 integrin⁺ cells isolated by two rounds of FACS was determined by flow cytometry (left panel). The isolated cells were grown to confluence in complete medium and continued to grow in basal medium for a week. Basal cells were then labeled with PE-conjugated AC133 mAb and FITC-conjugated anti-β1 integrin mAb CD29, and subjected to flow cytometry. The results are representative of three independent experiments.
Acknowledgement:

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Figures

Fig. 1.

A

| Sequence | Description |
|----------|-------------|
| 314-340  | Hu AC133-1  |
|          | Hu AC133-2  |

B

| Species           | Protein Sequence |
|-------------------|------------------|
| Human AC133-1     | KDYDKEVLKIVYAEI |
| Human AC133-2     | KDYDKEPIVYAEI   |
| Mouse prominin-1  | SVDSEKIVLAKYAEI |
| Mouse prominin-2  | SVDSEKIVLAKYAEI |

C

Exon 3

Fig. 2

A

PDF chart with events vs. PE-AC133.

B

Images showing 293/AC133-2 and WERI-RB-1.

C

Table showing Mock and AC133-2 with different treatments.
Fig. 5

A

C

B

Fig. 6

A

B

AC133-1
AC133-2

PE-mlG2a

FITC-mIgG1

10^0 10^1 10^2 10^3 10^4

10^1 10^2 10^3 10^4

PE-AC133

FITC-CD29

AC133-PE

FITC-CD29

FITC-CD29

FITC-CD29
Supplemental Data:

Table 1. Partial Exon/Intron Organization of the Human AC133 Gene

| Exon No | Nucleotide No | Exon Size (bp) | 5’ Splice Donor | 3’ Splice Acceptor |
|---------|---------------|----------------|-----------------|-------------------|
| 1       | 1–257         | TCC CAG AAG   | gtaagtgct       |                   |
| 2       | 258–313       | TAT GAC AAG   | gtaatLtlt       | tttttccag ATA CTT TGA |
| 3       | 314–340       | GGT CTA AAG   | gtaagaCtt       | ctttcacag CCA GAA ACT |
| 4       | 341–546       | AAT AAT AAG   | gcaagttca       | gaatggca ATT GTC TAC |
| 5       | 547–667       | ACT CCA GAG   | gtaaaaCcc       | ttccgtcag CAT TGG CAT |
| 6       | 668–731       | ATC TGA ACA   | gtgagttaa       | ttttttcag CAA ATC AAA |
| 7       | 732–821       | TGG CAA CAG   | gtgaCgatt       | tggcatgtag GTA TCA ATT |
| 8       | 822–1039      | CTG AGG CAG   | gtagcgagg       | ttccccatag CGA TCA AGG |
| 9       | 1040–1114     | GTC CCA CAG   | gtgagtttt       | aattttcag CTT CCA CCC |
| 10      | 1115–1178     | TGG TAG CAG   | gtagaCttt       | attttttcag GCC TAT CAA |
| 11      | 1179–1338     | TTC ATA CTG   | gtaggtgCt       | tggggttcag GTA TCA AAA |
| 12      | 1339–1491     | CCT CAT GGT   | gtaggtgCt       | cctccctcag GTG GCT TGG |
| 13      | 1492–1615     | TAA TTC CCG   | gtaaatatt       | ttttttcag TGG AGT TGG |
| 14      | 1616–1719     | AGT TTA CAG   | gtagCttaa       | gtttttcag GTT TTG GAT |
| 15      | 1720–1804     | ATT AAT GAG   | gtaataCgga       | ttccccacag TGA CTG CAA |
| 16      | 1805–1948     | TTG GCT CAG   | gtagCgcgc       | ttccgcag CAT ACT GGA |
| 17      | 1949–2020     | AAC AGT TTC   | gtagCtttaa      | ctattgtag ACT GGT AAA |
| 18      | 2021–2113     | CAA TCA CTG   | gtCacagtCt       | tttttgtag CCC CCA GGA |
| 19      | 2114–2167     | GGA TTG TTG   | gtaggggtt       | tcttgccag AGC ACT CTA |
| 20      | 2168–2248     | ATT ATT GAG   | gtagCcttt       | gtcttttcag GAG AGA GTA |
| 21      | 2249–2317     | GAG TTC TCT   | gtaagtagt       | tcttttcag GAA ACT AAG |
| 22      | 2318–2410     | GAC CCC TTG   | gtagaattt       | cccccctcag ATC AGT GAG |
| 23      | 2411–2526     | GTA CGA TGA   | gtaagttatg      | tttttttcag AAT TGG TTT |
| 24      | 2527–2550     | CAT GAA AAA   | gtagCcttt       | atttttag TGT TGA AAC |
| 25      | 2551–2619     | TAT GAC AAG   | gtaagCcaa       | ctgCttcag TAT GGA AAA |
| 26      | 2620–2659     | CTG CTT GAG   | gtagtttgg       | gtttttttag CCC ATC ACA |
| 27      | 2660–3794     | CCA GTG CAG   | gtagtttgg       | tcattttcag CAT CAG GAT |

*Sequence of exon 1 and 27 have not completely determined.

The table summarizes BLAST analyses (http://www.ncbi.nlm.nih.gov/BLAST) of AC133-1 cDNA sequence (AF027208) against genomic sequence of human chromosome 4 clone C0024K08 (AC005598) and RP11-45J21 (AC067999).
