Article

A Novel Anti-Human Syndecan-1(CD138) Monoclonal Antibody 4B3: Characterization and Application

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Syndecan-1 (CD138), a member of integral membrane heparin sulfate proteoglycans, is an essential matrix receptor for maintaining the normal morphological phenotypes. In this study, we generated a specific mouse anti-human syndecan-1 monoclonal antibody (mAb) 4B3 and identified it by competition assay with the available syndecan-1 mAb (BB4). Stained by 4B3, the expression of syndecan-1 was detected on tumor cell lines, such as 8226, U266, XG-1, XG-2, Daudi and Jurkat. The expression was also found on neuron stem cells. It was established that 4B3 mAb could inhibit XG-1 and XG-2 proliferation. The data not only determined that 4B3 mAb was a functional anti-human syndecan-1 mAb, but also indicated that syndecan-1 might be a valuable surface antigen and play an important role in regulation of tumor pathology and differentiation of neural stem cells. This novel antibody 4B3 may be value of study of tumor proliferation/survival mechanism and contributes to diagnosis and treatment of diverse diseases. *Cellular & Molecular Immunology*. 2007;4(3):209-214.

Key Words: syndecan-1(CD138), monoclonal antibody, multiple myeloma

Introduction

Syndecan-1 is a member of the family of integral membrane heparin sulfate proteoglycans (HSPGs). It consists of a core protein with five putative glycoaminoglycan (GAG) bearing three heparan sulfate chains (HS) and two chondroitin sulfate chains (CS) attachment sites which are located in the ectodomain. HS binds a variety of extra cellular ligands such as matrix components, cell-cell adhesion molecules, heparin binding factors, degradative enzymes, protease inhibitors and some serum proteins through their covalent attachment with heparin sulfate chains (1-3). Syndecan-1 is known to participate in the regulation of the interaction between cells and their environment as cell co-receptor, and to take part in organ development, vessel formation and tissue regeneration (4, 5). Myeloma functional studies reveal that syndecan-1 may act as a multifunctional regulator of cell behavior in the tumor microenvironment. It mediates cell-cell adhesion, binding of myeloma cells to type I collagen, and inhibits tumor cell invasion into collagen gels (6). In view of its important effects on tumor cell growth, survival, adhesion and invasion and the prognostic value in some human cancers (7-11), syndecan-1 may be an important potentially beneficial regulator of tumor pathobiology.

Differentiating cells undergo many changes in cell-cell and cell-matrix adhesive interactions that modulate their migration through a series of distinct microenvironments. They also influence cell phenotype and physiology. HSPGs are premier candidates for molecules involved in these regulatory processes (12). Expression of syndecan-1 is gradually decreased from early progenitors to mature oligodendrocytes as cells differentiated (13). It indicates that syndecan-1 is predominantly expressed in the neonatal brain, while there is no direct evidence to support the expression of syndecan-1 in human fetus nervous system.

Physiological and pathological functions of syndecan-1 were explored by generating a mouse anti-human syndecan-1 mAb (clone 4B3). The biological characterization of 4B3 was studied. By competition assay, 4B3 was found to bind the samiler epitope of syndecan-1 as an available syndecan-1 mAb BB4. 4B3 mAb could also inhibit the proliferation of XG-1 and XG-2. This novel antibody 4B3 may contribute to an evaluation of tumor proliferation/survival mechanism. Using the novel antibody 4B3, syndecan-1 was found to

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coexpress with AC133 in the human fetus brain cortex stem cells.

Materials and Methods

Cell preparation and culture

Mouse plasmocytoma cell line SP2/0 and all the human tumor cell lines (Raji, Daudi, U266, 8226) were obtained from American Type Culture Collection (Manassas, VA, USA). XG1, XG2 and XG7 were gifts by Dr. B Klein (Center Hospital University of Montpellier, Montpellier Medical University, France). Cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA) or standard DMEM (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% FCS (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and kept in 37°C incubator with 5% CO_2. A series of anti-human mAbs including phycoerythrin (PE) conjugated anti-CD138 (clone BB4), anti-AC133 mAb, biotin-conjugated goat anti-mouse IgG (H + L) Ab, and mouse IgG1 were all purchased from Immunotech Company (Immunotech, Marseilles, France). BALB/c mice were purchased from the Department of Experimental Animals, Shanghai Institute of Biological Products, Ministry of Health of China. Tissue was obtained from randomly selected tumor cases subjected to surgery at the No.1 Affiliated Hospital of Soochow University. Samples were collected from patients who provided consent according to Chinese regulations.

Isolation and culture of human neural stem cells (NSCs)

Human fetus brain cortex samples and neural stem cells were obtained from a 10-week-old fetus during routine legal terminations performed at the No.1 Affiliated Hospital of Soochow University. Fetal brain tissue samples were mechanically dissected in DMEM/Ham's F-12 (1:1) and tissue samples were enzymatically digested with 0.05% trypsin/0.53 mM EDTA. Tissue samples were washed three times in DMEM/Ham's F-12 (1:1) and triturated using a fine polished Pasteur pipette and passed through a 40 μm nylon mesh to achieve single-cell suspensions. Cell suspensions were grown in a flask with 40 ml culture medium DMEM/F-12 (1:1) supplemented with hr-EGF (20 ng/ml), hr-FGF2 (20 ng/ml), hr-LIF (10 ng/ml), heparin (5 mg/ml), B27 supplement, HEPES (15 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml). Cell suspensions were seeded 2×10^5 cells/ml and incubated for 7 days in 5% CO_2 at 37°C. After one week, neurospheres formed and selected were for immunocytochemistry. Aborted fetuses were used according to Chinese regulations.

Production of anti-syndecan-1 monoclonal antibodies

Six-week-old BALB/c mice were immunized by using four injections of 1×10^7 mitomycin-treated 8226 cells in 0.5 ml PBS per mouse at 21 days interval. The first subcutaneous injection was accompanied with incomplete Freund's adjuvant (Sigma, St Louis, MO, USA). Each of the following two abdominal cavity injections was performed for 3 weeks without adjuvant. Four days after the final injection, the immunized mouse splenocytes were fused with murine myeloma SP2/0 cells according to Groth and Scheidegger (14, 15). Flow cytometry (Altra; Beckman Coulter, Miami, FL, USA) was performed to identify the antigen by these mAbs. Finally, the mAb that recognized the syndecan-1 was purified from ascites of BALB/c mouse using Protein G-sepharose CL4B affinity columns (Amersham Pharmacia, Uppsala, Sweden).

Characterization of monoclonal antibodies

The Ig isotypes were identified with the rapid test paper (Roche Co., Varilhes, France) according to the manufacturer's instruction. The competition assay determined the antigen epitopes recognized by 4B3 and BB4 mAbs. The XG-2 cells (1×10^6) were incubated with 4B3 (2 mg/20 ml, 10 mg/20 ml, 20 mg/20 ml, 40 mg/20 ml, 100 mg/20 ml) for 45 min at 4°C. The cells were washed with PBS, incubated with PE-mouse anti-human syndecan-1 mAb (BB4) for another 30 min at 4°C, washed again and analyzed by flow cytometry.

Flow cytometric analysis

The above tumor cells (1×10^7/ml) were incubated with BB4 for 30 min at 4°C and washed. For indirect staining, PE- or fluorescein isothiocyanate-labeled goat anti-mouse antibody as secondary antibody was incubated for another 30 min and washed. Results were analyzed by Beckman-Coulter's Expo32 MultiComp software.

Western blotting analysis

The binding capacity of 4B3 mAb to syndecan-1 was identified by Western blotting analysis. U266 and BB4 mAb was set as a positive control. The U266 cell lysis was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%), transferred onto nitrocellulose filters, and stained with BB4 and 4B3 mAb. The protein band was visualized by using the BM Chemiluminescence. Western Blotting Kit (Boehringer, Mannheim, Germany) was performed according to manufacturer's instructions.

Tissue processing and immunohistochemical staining

Surgically dissected specimens were fixed with 10% neutral formalin, embedded with paraffin, and serially sectioned at 5 μm. Sections were mounted onto the histostick-coated slides. Four or five adjacent ribbons were collected for immunohistochemical staining. 4B3 is a monoclonal mouse antiserum against human syndecan-1. The biotin-streptavidin complex method was used for the immunostaining of syndecan-1. Negative controls were established by replacing the primary antibody (4B3) with PBS or normal mouse IgG1 (BD PharMingen). Specific staining for syndecan-1 was categorized as either positive or negative based on the presence or absence of brown-color staining. When more than 10% cells were stained, the result was regarded as positive. Clear staining for both the cytoplasm and cell membrane was the criterion for a positive reaction.

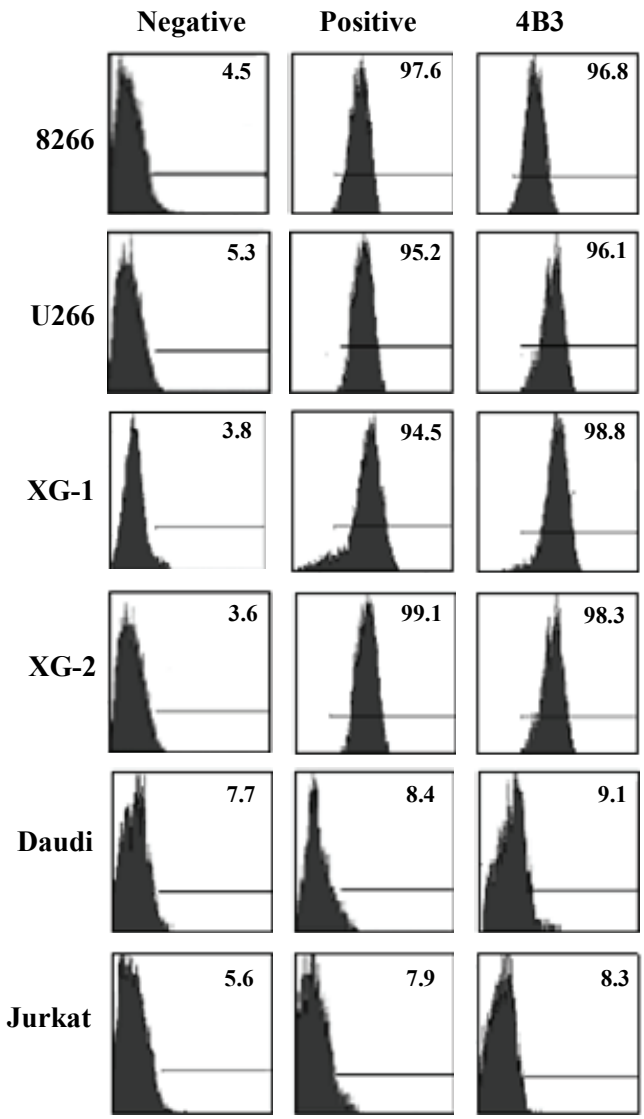

Figure 1. Expression of syndecan-1 on 8226, U266, XG-1, XG-2, Jurkat and Daudi cell lines detected by 4B3 mAb. An available syndecan-1 mAb BB4 was used as positive control.

Neurospheres were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20 min at room temperature. After fixation, the neurospheres were dipped in 30% sucrose PBS with for 30 min at room temperature. The fixed neurosphere on glass disks were washed in PBS, blocked with 10% goat serum for 1 h at room temperature and incubated overnight with anti-nestin monoclonal antibody (1:500) in PBS containing 10% normal goat serum at 4°C. After washing, the neurospheres were incubated with secondary antibodies at room temperature for 1 h. Fluorescent signals were detected with a fluorescent microscope.

Cell proliferation

XG-1 or XG-2 cells (2×10^5/ml), two multiple myeloma cell lines, were seeded (1 ml/well) into 24-well flat-bottom plate

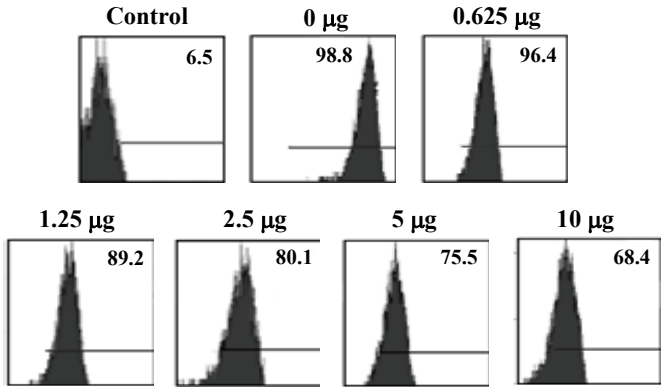

Figure 2. Competitive inhibition assay of epitope recognized. XG-2 cells were pre-incubated at 4°C for 45 min with different concentrations of 4B3 mAb (0.625 μg, 1.25 μg, 2.5 μg, 5 μg, 10 μg respectively). Expression of syndecan-1 was detected by flow cytometry stained with BB4-PE.

cocultured with anti-CD138 mAb 4B3. 4B3 mAb final concentration was 2.5 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml. Each different 4B3 mAb concentration group had three wells. Trypan blue staining and cell counting from days 1-5 were used to get the cell concentration in each well that was added different 4B3 mAb concentration. Mouse IgG and PBS were used as negative controls.

Results

Establishment of one novel anti-human syndecan-1 monoclonal antibodies

Mouse splenocytes were immunized by 8226 cells and fused with myeloma SP2/0 cells. After repeated screening and cloning, one mAbs, named 4B3 was obtained. 4B3 mAb could recognize syndecan-1 expressing on 8226, U266, XG-1, XG-2 cells, but not on Jurkat and Daudi cells. Results were similar to the positive control and indicated that 4B3 mAb was specific for syndecan-1 (Figure 1).

4B3 mAb recognized similar epitope with BB4

To determine the syndecan-1 epitope recognized by 4B3, a competition assay with BB4 was performed as previously described. Result showed that 4B3 mAb could occupy the

Figure 3. Western blotting analysis of the 4B3 mAb. Total protein from U266 was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by Western blotting with 4B3 (A) and BB4 (B) according to the manufacturer's protocol. 4B3 and BB4 recognized a protein with approximate molecular weight of 195 kDa.

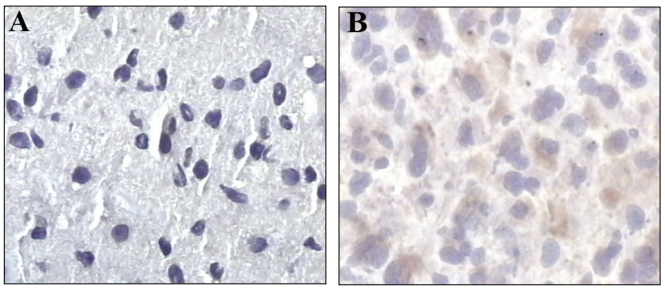

Figure 4. Immunohistochemistry analysis of syndecan-1 in glioma samples by 4b3 mAb. (A) Isotype-matched mouse IgG1 mAb was used as control. (B) Expression of syndecan-1 was found on glioma cell cytoplasm (400×).

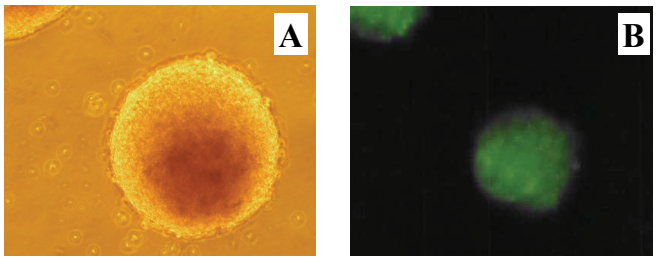

Figure 6. Neurospheres in culture and nestin immunofluorescence staining of neurospheres. (A) Phase contrast image of neurospheres (200×). (B) Nestin immunofluorescence staining of neurosphere cells (100×).

epitope of BB4 mAb and indicated that 4B3 mAb may recognize a similar epitope of BB4 mAb (Figure 2). 4B3 mAb isotype was IgG1 with κ. For further analysis, Western blotting was performed with U266 lysis. 4B3 could recognize the same molecular weight protein band as BB4 mAb (Figure 3).

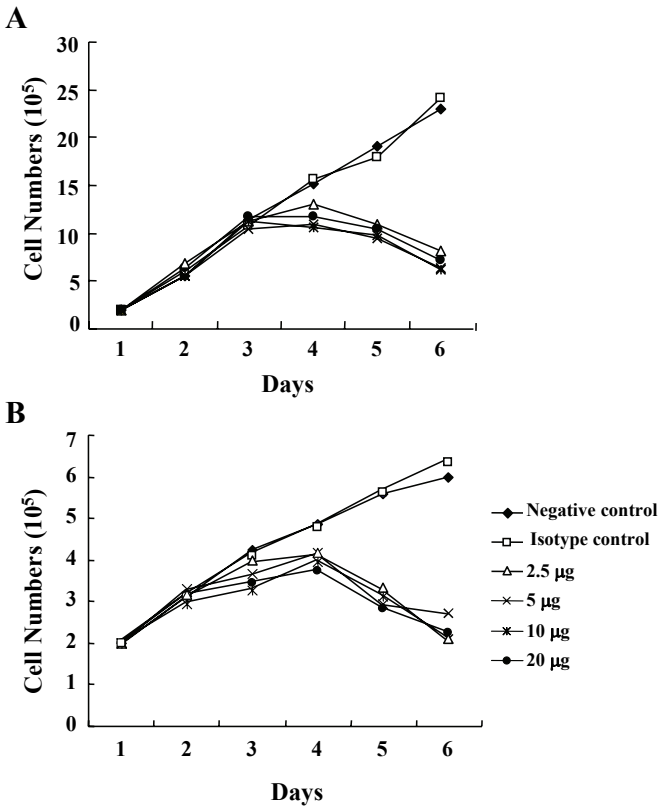

Figure 5. Inhibition of XG-1 and XG-2 proliferation by 4B3 mAb. XG-1 cells (A) and XG-2 cells (B) were cocultured with different concentrations of 4B3 mAb (2.5 μg, 5 μg, 10 μg, 20 μg, respectively). The number of cells was decreased from the second day even if the concentration of 4B3 was 2.5 μg/ml. Isotype-matched mouse IgG1 mAb and PBS were used as isotype control and negative control respectively.

Immunohistochemistry analysis of syndecan-1 in glioma samples

Expression patterns of syndecan-1 on human tumor cell lines were detected by 4B3 mAb (as shown in Figure 1) with flow cytometry. Several studies have demonstrated that syndecan-1 can be expressed in malignant glioma cells (16, 17). In immunohistochemistry examinations, positive immunostaining for 4B3 was also observed in the glioma (Figure 4B). Syndecan-1 immunoreactivity was located mainly in the cytoplasm and cell membrane.

Syndecan-1 signaling inhibited XG-1 and XG-2 proliferation

It has been reported that syndecan-1 plays a major role in regulating the pathology of myeloma. *In vitro*, soluble syndecan-1 is proved to participate in the regulation of multiple myeloma cell growth as competitive inhibitors and a negative regulator through a signal transduction that is different from IL-6. XG-1 and XG-2 proliferation assay was performed in order to study the biological functions of 4B3 *in vitro*. Results demonstrated that XG-1 and XG-2 proliferation could be well inhibited by 4B3 mAb from the second day even when the concentration of 4B3 is 2.5 μg/ml (Figure 5). These results suggested that 4B3 mAb was a functional anti-human syndecan-1 mAb and may be a valuable surface antigen with an important role in regulating tumor pathology.

Human neural stem cell culture and coexpression of CD138 and AC133 in these cells

Human NSCs isolated from a 10-week old fetus cortex were cultured and formed neurospheres in the culture medium after one week (Figure 6A). The neurosphere cells had capabilities of self-renewal and proliferation. Immunocytochemistry result showed that neurosphere cells were nestin-positive (Figure 6B). CD138 and AC133 can express in these neurosphere cells by flow cytometric analysis (11.6% and 15.7% respectively). Double stained with CD138-FITC and PE-conjugated AC133 showed more than half of the cells that expressed AC133 coexpresed CD138 (Figure 7).

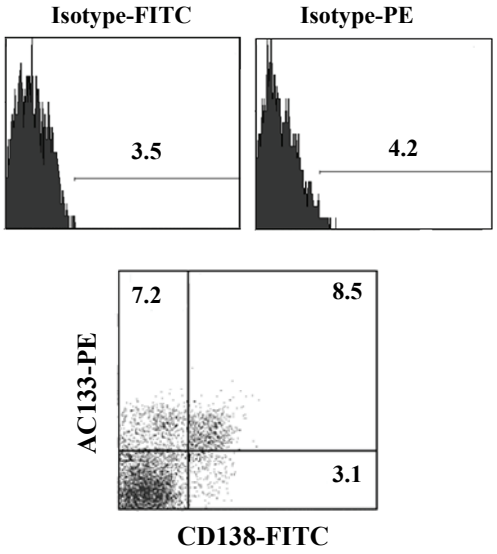

Figure 7. Flow cytometric analysis of CD138 and AC133 expression in human neuron stem cells.

Discussion

Syndecan-1 is a transmembrane proteoglycan expressed on the surface of tumor cells of various origins including myeloma, Hodgkin's disease, and certain human immunodeficiency virus associated lymphomas (18-21). Functional studies in myeloma reveal that syndecan-1 may act as a multifunctional regulator of cell behavior in the tumor microenvironment. Syndecan-1 mediates cell-cell adhesion, binding of myeloma cells to type I collagen and inhibits tumor cell invasion into collagen gels (22-24). Syndecan-1 is also released from myeloma cell surface, inhibits growth, induces apoptosis of myeloma cells and may modulate myeloma bone disease by inhibiting osteoclast formation and promoting osteoblast formation (5). In view of its effects, syndecan-1 may be an important regulator of myeloma pathobiology and a potential target for tumor therapy. In order to clarify the mechanism of syndecan-1 function, a novel anti-human syndecan-1 mAb (4B3) was prepared. Western blotting showed 4B3 mAb could recognize the same molecular weight protein band as commercialized BB4 mAb. Titration studies showed that 4B3 mAb could occupy the epitope of BB4 mAb and indicated that 4B3 mAb may recognize the same or similar epitope of BB4 mAb.

XG-1 and XG-2 cell lines can highly express syndecan-1 molecules on their cell membrane. Using different concentrations of 4B3 mAbs cocultured with XG-1 cells, we found 4B3 mAb inhibited XG-1 and XG-2 proliferation remarkably even if the concentration of 4B3 mAb is 2.5 μg/ml. Recent studies indicated that syndecan-1 was not simply mediating adhesion, it formed complexes with other molecules at the cell surface to promote signaling that alters cell behavior. However, the mechanisms of syndecan-1 action on XG-1 and XG-2 need to be defined. How does syndecan-1 inhibit growth? Is this a direct effect mediated by syndecan-1 binding to cell surface receptors, or does this occur *via* heparan sulfate interactions with XG-1 and XG-2 survival factors? Answers to these questions will provide a better understanding of the mechanisms of syndecan-1 action and may lead to new therapeutic strategies for controlling the growth of XG-1 and XG-2 cells and possibly other tumors. Thus, anti-syndecan-1 mAb may be an effective tool in cancer research and cancer therapies.

Several *in vitro* studies have demonstrated that the expression of syndecan-1 was gradually decreased from early progenitors to mature oligodendrocytes, suggesting that syndecan-1 is predominantly expressed in the neonatal brain (13). Nestin, an intermediate filament protein, is only expressed in the neural stem cells of the developing central nervous system (25, 27) and disappears as they differentiate. Nestin has been as a marker molecule of neuron stem cells. In our study, neurosphere cells not only had the ability of self-renewal and proliferation, but also were nestin positive. The result indicates the cultured fetus cerebral cortex cells may be neuron precursor cells or neuron stem cells. It was demonstrated that syndecan-1 can be expressed in these cells. Furthermore, about half of the cells that express AC133 can also coexpress syndecan-1. As we know, AC133 is expressed on several primitive cells such as hematopoietic stem and progenitor cells derived from bone marrow, fetal liver, and peripheral blood (27, 28), neural and endothelial stem cells. It is also a new marker to define early progenitor cells (29-31). Syndecan-1 can be coexpressed with AC133, which may indicated that it could be expressed on early progenitor neural cells.

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