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# Beyond Mere Markers

## *Functions for CD34 Family of Sialomucins in Hematopoiesis?*

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## Abstract

CD34, podocalyxin, and endoglycan are members of a family of single-pass transmembrane proteins that show distinct expression on early hematopoietic precursors and vascular-associated tissue. In spite of the fact that the expression of CD34 on these early progenitors has been known for over 20 yr and used clinically in hematopoietic stem cell transplantation for more than 15 yr, little is known about its exact role or function. More recently, CD34 expression has been shown to distinguish activated early progenitors from quiescent cells. With the subsequent identification of podocalyxin and endoglycan as related family members also expressed on early progenitor cells, attention is slowly shifting toward understanding how these molecules might contribute to progenitor function and behavior. In this review we examine the existing evidence and propose testable models to reveal the importance of these molecules for stem and progenitor cell function.

## Key Words

CD34
Podocalyxin
Endoglycan
Sialomucin
Hematopoiesis
Hematopoietic stem cells
Adhesion
Migration
NHERF-1
NHERF-2

## Introduction

CD34, podocalyxin and endoglycan are grouped as a protein family (henceforth termed the CD34 family) based on conservation of domain organization (Figs. 1A, C, and 2)*(1–3)* and their conserved genomic organization (Fig. 1A) *(2–4)*. At the genomic level all members are encoded by eight exons, with paralogous exons encoding paralogous protein domains, which is the strongest evidence for shared descent (Fig. 1A) *(2–4)*. This is important in establishing this group as a family because they do not naturally form a clade based solely on amino acid sequence similarity (Fig. 1B). To date, alternatively spliced variants of CD34 and podocalyxin have been identified, both of which make use of an alternative eighth exon resulting in mRNAs encoding proteins with a truncated cytoplasmic domain *(4,5)*. The structures of the three family members, based on biochemical and sequence information, consist of an extracellular N-terminal mucin domain followed by a

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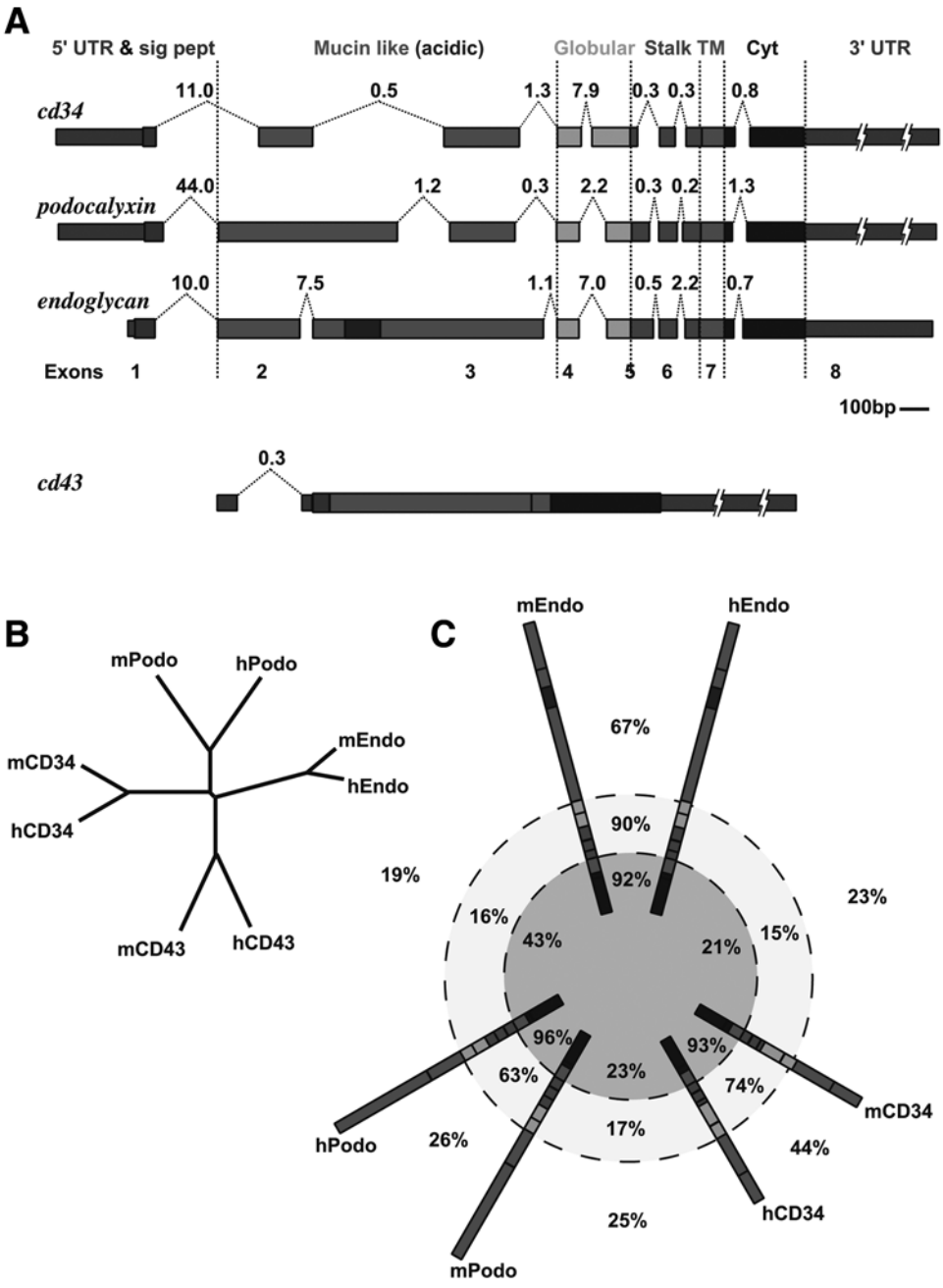


globular domain and stalk, with a single-pass transmembrane region and a charged cytoplasmic tail (Fig. 2). CD34 family members have been identified in a variety of vertebrates, including mouse, human, dog, and chicken and gene predictions have been made for zebrafish. No homologous proteins have been identified in urochordates, invertebrates, or

**Fig. 1.** Genomic structure and relationship based on primary amino acid sequence of CD34 family members. **(A)** Schematic representation of the genomic structure of human *CD34, podocalyxin, endoglycan,* and *CD43* (used as an outgroup) based on sequence contigs and genomic mapping available in the NCBI human sequence database. Numbers above dashed lines indicate intron size in kilobases, exon numbers are indicated below (and marked). The relative sizes of exons are shown to scale (see scale bar), with the exception of the 3′ UTRs of *CD34, podocalyxin,* and *CD43.* The location of coding sequences is indicated: signal peptide (sig pept–red), mucin-like domain (orange), globular domain (yellow), stalk (green), transmembrane (TM–cyan), and cytoplasmic tail (Cyt–dark blue). **(B)** An unrooted phylogram showing the relationship of human and mouse CD34, podocalyxin, endoglycan, and CD43 (used as an outgroup) proteins indicating the difficulty in assigning CD34, podocalyxin, and endoglycan to a single family based solely on amino acid sequence. Branch lengths are proportional to calculated distance. **(C)** A schematic representation of the relationship of CD34, podocalyxin, and endoglycan deduced amino acid sequences highlighting the sequence conservation differences between the various domains and family members. Color scheme follows that of **A,** numbers indicate % identity between neighboring proteins, and domains have been grouped: mucin like, globular + stalk domain, transmembrane + cytoplasmic tail.

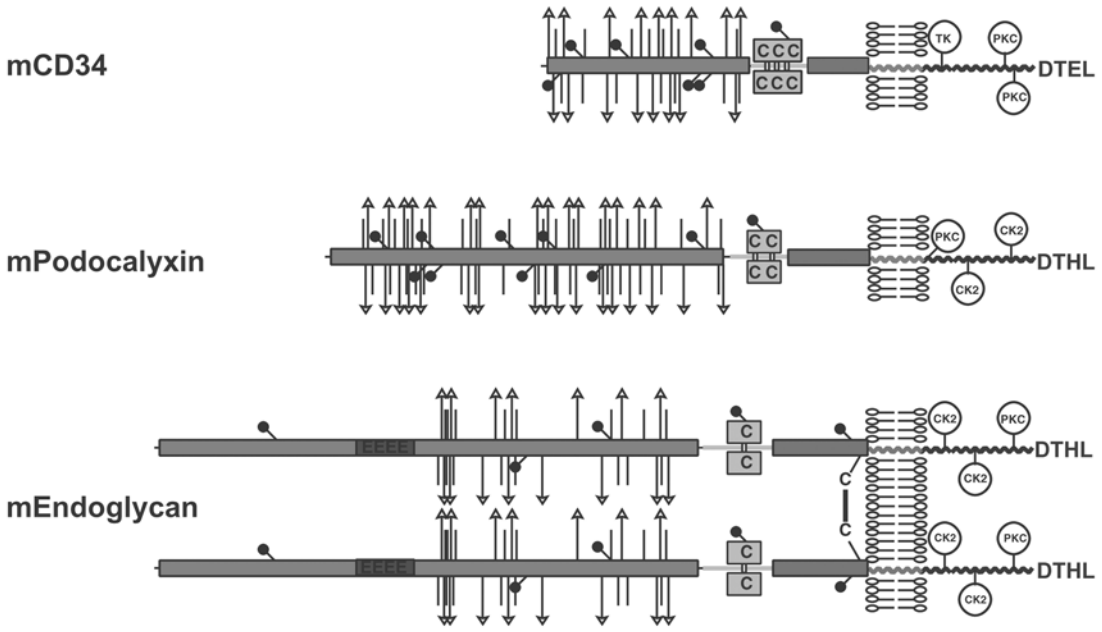


**Fig. 2.** Schematic of primary protein structure and proposed models for family function. Schematic representation of predicted secondary protein structure of mouse CD34, podocalyxin, and endoglycan. Color scheme follows that of Fig. 1A [mucin-like domain (orange), globular domain (yellow), stalk (green), transmembrane (cyan), and cytoplasmic tail (dark blue)] with the poly acidic stretch of endoglycan indicated in purple. Angled lines with filled circles represent potential $NH_2$-linked carbohydrates, horizontal bars with and without arrows indicate potential O-linked glycosylations, arrows are potential sialic acid modified O-linked glycosylations. Cyteines available for disulfide bond formation are indicated by C, consensus phosphorylation sites for Tyrosine Kinase (TK), Protein Kinase C (PKC), and Casein Kinase 2 (CK2) are indicated as is the C-terminal PDZ docking motif (DTE/HL).

other eukaryotes and thus it seems likely that the parental gene arose among the first vertebrates, followed closely by gene duplication. These proteins, therefore, appear to have arisen to accommodate some aspect of vertebrate body plan not shared by other chordates.

Mucin domains are common to a wide range of extracellular and transmembrane proteins including the mucins, sialomucins (to which the CD34 family belongs), lectin ligands, as well as some transmembrane receptors. Mucin domains are heavily modified by O-linked carbohydrate moieties and are usually coded by one exon only. They are characterized as being rich in serine, threonine, and proline and are generally poorly conserved at the primary amino acid level. In the case of the CD34 family, the mucin domains are sparsely modified with N-linked and heavily modified with O-linked glycosylation *(1,6,7).* Much of the O-linked glycosylation is decorated with sialic acid and this, combined with sulfation *(8–10),* contributes to a high net negative charge. The mucin domain of CD34 family members is capable of binding the leukocyte homing receptor, L-selectin *(10–12)* (Table 2). However, this interaction is highly dependent on a particular pattern of glycosylation *(9,10)* that has only been observed in specialized lymph node endothelial cells known as high endothelial venules (HEV) *(13–15).* To date, despite their conservation (Fig. 1C), no specific function has been attributed to either the globular or stalk domains of any family member. The cytoplasmic tail is presumed to have important functions, based on its high sequence conservation and has been shown to be subject to signal-regulated phosphorylation *(16–18)* and to bind several intracellular ligands (Table 1, and see below) *(19–25).* The transmembrane region and cytoplasmic tail are the most conserved motifs between family members and are extremely well conserved orthologously (Fig. 1C).

## CD34

### CD34 Identification and Expression

CD34 was first identified as an antigen expressed on hematopoietic progenitors in screens of monoclonal antibodies generated against human hematopoietic precursors *(26–28).* The subsequent demonstration that the CD34 positive (CD34+) cell fraction of bone marrow could successfully engraft both baboons *(29)* and humans *(30)* has led to it becoming one of the most widely used markers of hematopoietic stem cells, with well over 12,000 Medline citations. In spite of the vast amount of literature on CD34 as a marker, there is only limited and conflicting information regarding its function.

### CD34 Endothelial Expression

Beyond its expression on HSCs (see below), CD34 is expressed on vascular endothelia, both during development and in the adult *(31–35).* During development, too, CD34 is on all vascular endothelia with highest expression in small vessels and filopodia of angiogenic sprouts *(35,36).* This expression includes those endothelial cells surrounding the blood islands of the yolk sac *(35–37)* as well as endothelia of the aorta–gonad–mesonephros (AGM) region *(36,38,39).* These CD34+ endothelial and stromal cells from the AGM have been used, in vitro, for long-term expansion of HSC populations *(39,40).* In the adult, high expression has been reported on the endothelia of vessels in all organs, primarily in small vessels *(31, 33,41,42).*

### CD34 Expression on Stromal and Mesenchymal Stem Cells

Mesenchymal stem cells are nonhematopoietic cells that reside in the bone marrow and are capable of giving rise to various cells involved in the maintenance of bone and bone marrow, including osteoblasts, chondrocytes,

adipocytes, and stromal cells. CD34 has been reported to be expressed on a subset of stromal cells of bone marrow origin *(43–45),* a subset of muscle-derived progenitor cells that are distinct form satellite cells *(46),* as well as placental stromal cells *(47).* Both of these bone marrow– and muscle-derived progenitor cell populations appear to represent an early progenitor of stromal cells that may be functionally equivalent to the proposed mesenchymal stem cell (MSC). There is broad evidence supporting the model that the CD34+ subset of stromal cells might represent a type of MSC. In vitro, these cells have high fibroblast colony forming unit potential (CFU-F) *(45)* and, when isolated from muscle, are capable of contributing to regeneration of damaged muscle and bone *(46).* Most importantly, human CD34+ stromal cells are capable of reconstituting stroma of sublethally irradiated NOD/SCID mice *(48)* suggesting that the CD34 population does contain MSCs. However, there are also reports that both human *(49)* and mouse *(44)* mesenchymal stem cells are not CD34+. In the case of Hung et al. *(49)* the absence of CD34 may be an effect of long-term culture, prior to analysis, or may be a reflection that there is more than one type of MSC. Peister et al. *(44)* analyzed CD34 expression on MSCs from a variety of inbred mouse strains after long-term culture and found expression in two mouse strains and lack of expression in two others. Again, it is unclear whether this real difference between strains, in terms of endogenous MSCs, is an effect of long-term culture or is due to epitope differences between mouse strains (insufficient information is provided about the antibody used to make a judgement in this regard). The balance of evidence supports the idea that an early multipotential stromal precursor exists and this precursor can be CD34+, a concept that will be discussed further.

### *CD34 Expression in the Hematopoietic System*

The first evidence of CD34 as a marker of hematopoietic stem/progenitor cells came from FACS analysis demonstrating that its expression is restricted to a subset of bone marrow cells and absent from terminally differentiated hematopoietic lineages *(26,28).* Moreover, sorting of CD34+ cells greatly enriched for colony forming units (CFUs) of a variety of lineages *(26–28).* The subsequent demonstration that the CD34+ fraction of bone marrow can fully reconstitute the hematopoietic system of baboons and humans *(29,30)* confirmed that these cells had properties of HSCs. Owing to the clinical utility, in terms of improved hematopoietic engraftment, achieved using sorted CD34+ human bone marrow or mobilized progenitor donor cells, CD34 became widely accepted as a marker of HSCs *(50).* The fact that CD34+ cells from the bone marrow and mobilized peripheral blood display HSC activity does not exclude the possibility that CD34– cells also display this activity. In fact a number of groups have reported that CD34– cells are capable of long-term reconstitution of the hematopoietic system *(51–56).* Hematopoietic reconstitution by CD34– stem cells was demonstrated first in mice *(51–53)* and subsequently in primates, including humans, using a xenotransplantation model *(54–56).* However, definition of these HSCs as CD34– relies on absence of staining with monoclonal antibodies whose epitope recognition is unchanged by neuraminidase and glycoprotease cleavage *(36,57)* (implying recognition of the globular or stalk region) to analyse cell surface expression. Since Jones et al. *(51)* reported very low levels of CD34 mRNA in CD34– HSCs, it is formally possible that CD34 is expressed on these cells but the epitope is unavailable for recognition by the antibodies, or CD34 expression is being posttranslationally regulated, for example, by

being maintained in intracellular stores. Independent studies in mice, using the same type of antibody, demonstrate that HSCs are present in both CD34+ and negative cell populations *(58–60).* At the time of publication, these reports were controversial because CD34 expression had been widely used as a key clinical method for separating HSCs for transplantation and as a prognostic indicator of reconstitution efficiency *(61).* Some resolution has been forthcoming with in vivo data from mice providing evidence that CD34 expression is reversible and dependent on stem cell activation status *(62).* Further to this, it has been shown for both human *(63)* (in a xenotransplantation model) and mouse *(64)* that CD34 expression on HSCs is reversible and that both CD34– and CD34+ stem cells are competent for serial transplantation and reconstitution.

Whether a particular stem cell expresses CD34 or not may correspond to the activation status of the cell *(65–67).* Quiescent HSCs are CD34–, whereas G-CSF mobilized cells are CD34+ *(65).* Quiescent HSCs are largely in $G_0$ and granulocyte colony-stimulating factor (G-CSF) mobilized stem cells have been also reported to be in $G_0G_1$ *(68,69).* This suggests that CD34 expression is not strictly cell cycle dependent and does not require stem cell division, merely activation. Activation and cell cycle regulated expression in HSCs may reflect an aspect of CD34 regulation that is true also for stromal precursors and may, thus, resolve the anomalies between reports regarding its expression in this cell type. This raises the possibility that CD34 may play an important role on a variety of activated precursors.

This recent evidence that CD34 expression on HSCs is related to activation status, dovetails well with the observations concerning hematopoietic CD34 expression during development. The earliest sites of hematopoiesis during development are in the blood islands of the yolk sac *(70)* and the AGM region, which is the first site of definitive hematopoiesis *(71).* In both regions, hematopoietic progenitors show CD34 expression *(34–36,72).* Shortly after the development of the liver primordial, the liver is colonized by hematopoietic progenitors and for the rest of embryogenesis this becomes the principal site for definitive hematopoiesis *(73).* Again, hematopoietic progenitors in the fetal liver express CD34 *(34,35,71,74,75).* In all these developmental sites for hematopoiesis there is massive expansion of the cell population as well as repeated mobilization in order to seed new compartments. The expression of CD34 in these developmental compartments provides further support for the idea that CD34 may have some important function on activated or mobilized HSCs.

It is important, having discussed CD34 expression on HSCs, to note that CD34 appears not to be expressed on any mature hematopoietic cells with the exception of murine mast cells *(76).* Although the current evidence suggests that expression on *mature* mast cells is a feature specific to the murine hematopoietic system *(77–80),* in both humans and mice the data suggests committed mast cell *precursors* express CD34 *(76,77,81).* Bone marrow mast cell progenitors are somewhat elusive because they appear morphologically and phenotypically indistinguishable from CD34+ HSCs *(76,81)* and are also present at the same frequency as HSCs *(82).* The similarity between mast cell progenitors and HSCs may also present the converse problem, that various types of analyses in which HSC cell surface phenotype is used could be confounded by the presence of mast cell precursors.

Although CD34 does appear to be absent from most mature hematopoietic lineages, it is expressed on a subset of bone marrow multipotent precursors of the thrombocytic, myeloid,

and a very small fraction of thymic precursors *(83,84).* Because these precursors are difficult to identify, either by morphology or by cell surface antigens, the assessment that these are in fact precursors of particular lineages depends on performing colony assays, or limiting dilution reconstitution assays. This leaves open the question of how early a precursor is really marked by the presence of CD34. In any case, the fact that these precursors express CD34, while it is not expressed by mature lineages, hints that this molecule may be important for a precursor function, not present in terminally differentiated hematopoietic cells.

### *CD34 Function*

In light of the above discussion on CD34 expression, particularly that it is associated with HSC activation and mobilization, the obvious question is what function does this protein serve? CD34 has been proposed to function as (1) an inhibitor of differentiation and promoter of proliferation in HSCs, (2) an adhesion molecule, possibly capable of signaling, and (3) an anti-adhesion molecule. Thus far, the only ligand or counterreceptor identified for CD34 is L-selectin and the ability of these two molecules to interact is exquisitely dependent on a particular glycosylation pattern on CD34, only occurring in the specialized cells of HEVs. Here we will evaluate the evidence for each functional model.

### CD34 and Proliferation/Differentiation

As discussed in the section dealing with hematopoietic expression of CD34, its expression is limited to non-quiescent or activated hematopoietic precursors and is absent from differentiated hematopoietic lineages. This correlates with the idea that it may have roles in inhibition of differentiation and promotion of proliferation. The main confirmatory evidence that CD34 might play a role in inhibition of differentiation comes from overexpression studies in a hematopoietic progenitor cell line. Fackler et al *(85),* showed that overexpression of full-length CD34, but not the naturally occurring form with a truncated cytoplasmic domain, inhibits in vitro differentiation of the myelomonocytic M1 cell line. They interpreted this as evidence that the cytoplasmic tail of CD34 provides signals that inhibit differentiation. Because the overexpression construct used resulted in protein levels far beyond endogenous, it is difficult to say whether this reflects a normal function of CD34. It is possible, for example, that this overexpression may have resulted in either constitutive signaling or squelching of an alternative pathway by a molecule that may or may not normally signal during hematopoietic differentiation.

The only other data linking CD34 to maturation and proliferation focused on a strain of CD34– null mice. In these studies CD34– null embryonic stem (ES) cells and cells derived from CD34– null yolk sac, fetal liver, adult bone marrow, and spleen exhibited lower CFU activity in vitro, when compared with wild-type–derived counterparts *(86).* In ES cells, this phenotype can be rescued by transfection of full-length CD34, or the naturally occurring splice variant encoding CD34 with a truncated cytoplasmic domain *(86).* The simplest interpretation here is that CD34 is required, at normal expression levels, for natural proliferation or differentiation and maturation of progenitor cells. However, in the light of subsequent evidence that CD34 is a regulator of adhesion, it is possible that these maturation defects are actually downstream of an adhesion defect (see below).

### CD34 as a Pro-Adhesive Molecule

There is one line of evidence supporting a direct adhesive role for CD34; it has been shown that CD34, when expressed on HEVs,

is suitably modified for binding L-selectin *(11)* and thus serves a direct adhesive role in leukocyte homing. On HEVs a number of glycoproteins are reported to be modified with a HEV-specific glycosylation, sulfated sialyl-Lewis x *(13),* and this modification serves as a specific ligand for L-selectin and is required for leukocyte homing (8). CD34 is one of at least four glycoproteins that carries this unusual HEV-specific modification *(11,87),* is thus capable of supporting L-selectin–dependent lymphocyte rolling in in vitro *(88)* and is responsible for up to 50% of L-selectin–dependent tethering *(88).* In spite of this, in vivo, L-selectin–dependent lymphocyte rolling is unaffected in CD34– null mice *(89)* and lymphocyte homing appears normal *(89).* CD34–null mice do, however, show a defect in allergen-induced eosinophil recruitment to the lung *(89),* a defect not reported in an independent CD34 knockout strain *(86).* It seems likely there is functional compensation by other L-selectin ligands on HEVs, and this accounts for the discrepancy between the in vitro and in vivo data.

## CD34 and Signaling

There is some evidence for signal-regulated phosphorylation of CD34, with Fackler et al *(17)* showing that PKC could serine phosphorylate CD34 resulting in upregulated surface expression *(18).* The site of PKC phosphorylation has not been identified; however, there are seven conserved serines in the cytoplasmic tail of CD34; six of which give high scores for predicted phosphorylation, but only one is a predicted PKC site *(90,91).* It would be useful to have this site formally demonstrated because phosphorylation is a common modification for regulation of protein–protein interactions. It has also been shown that CD34 is subject to tyrosine phosphorylation *(92),* although the kinase responsible is unknown. CD34 is thus subject to a number of regulatory events, suggesting that its function might be tightly controlled. They also hint that CD34 might be a signaling molecule. In the absence of a known ligand for CD34, as it is post-translationally modified on most cells, several groups have evaluated potential outside-in signaling capacity of CD34 by crosslinking with antibodies. A variety of anti-CD34 monoclonal antibodies exist and have been classified according to their epitope sensitivity to neuraminidase and glycopeptidase *(93).* Those antibodies sensitive to either treatment recognize the mucin-like domain of CD34, whereas those that are resistant to both recognize the globular, or stalk, domain. Crosslinking using antibodies of all classes leads to clustering of CD34 *(94)* in hematopoietic precursors; however, only antibodies recognizing the mucin domain can induce capping and tyrosine kinase activation *(94).* CD34 capping, induced mucin-directed antibodies, results in actin polymerization and tyrosine phosphorylation of both Lyn and Syk membrane-associated tyrosine kinases *(94).* Similarly, it is antibodies of this class that are capable of inducing both homotypic *(95,96)* and heterotypic *(95,97)* adhesion of hematopoietic cells. These data support the idea that CD34 has a role in blocking adhesion when uniformly distributed. The antibody-induced capping of CD34 and subsequent homotypic adhesion are ATP, calcium, and tyrosine kinase dependent, and are at least partially mediated by activation of β2 integrin and ICAM-1 *(95,96).* These data argue that CD34 is actively involved in cell signaling cascades that regulate cell–cell adhesion and that CD34 cell surface expression and localization are regulated by intracellular signals. The limitations of the data relating to CD34's ability to induce intracellular signaling cascades are that neither the intracellular mediators of this signaling nor a ligand or counterreceptor have been identified. Thus, there is no information on the mechanism that may shed light on a function for CD34. The

intracellular SH2 family adaptor CrkL has been identified as a potential interactor with CD34 *(19)* (table 1); however, there is not yet any information about the conditions under which this interaction might occur and whether this adaptor is responsible for signal transduction through CD34.

## CD34 as an Antiadhesive Molecule

Recently we showed that, in mouse, CD34 is a selective marker of mast cells *(76).* We have subsequently exploited this observation, and the ability to culture pure populations of primary mast cells, as a means to address CD34 function *(98).* Bone marrow mast cells (BMMC) from CD34 knockout mice display higher homotypic adhesion compared with mast cells from wild-type animals, a phenotype that can be reversed by transfection of full-length or naturally occurring truncated CD34 *(98).* The version with a truncated cytoplasmic domain appears to function as a more potent inhibitor of adhesion than the full-length molecule *(98).* This supports a model of CD34 being an antiadhesion molecule and suggests that the cytoplasmic tail is important to allow proper regulation of this antiadhesive property. When used in competitive reconstitution assays, CD34– null cells show a significant defect in their ability to contribute to the peritoneal mast cell population and to reconstitute bone marrow when compared with wild-type cells *(98).* Thus, it seems that the antiadhesive role of CD34 might be important in the ability of mobilized precursors to migrate to the bone marrow or to find the appropriate niche. In light of CD34's ability to block adhesion and yet show a crosslinking-dependent ability to induce adhesion, we speculate that CD34 may passively block adhesion via its bulky, negatively charged mucin domain, and yet actively signal this change in adhesion behavior when clustered or crosslinked (Fig. 3A).

### *CD34 So Far*

How far have we come in understanding CD34's role in the hematopoietic system? We now know that CD34 definitively marks a subset of hematopoietic progenitors and that these are non-quiescent cells involved in migration or proliferation. So far the gene deletion studies have provided little illumination, with two independent knockouts published with different but mild hematopoietic defects and no apparent defect in other tissues expressing CD34 *(86,89).* This argues for redundancy in function, which may be provided by distantly related proteins, such as CD43, or the other CD34 family members, podocalyxin and endoglycan (see below). Interpretation of the relatively small amount of molecular data has, until recently, been hampered by the absence of any clear indication of function either from primary amino acid sequence; genetic evidence, or identification of a ligand or counterreceptor. The accumulating evidence suggests that CD34 is actively involved in regulating adhesion by serving as an anti-adhesion molecule. CD34 can be uniformly distributed over the cell surface and block adhesion through stearic hindrance, via the bulky mucin domain and possibly by signaling to inhibit the activity of adhesion molecules (Fig. 3A). Under alternative conditions, either via indirect signaling or possibly by CD34 binding to a ligand or counterreceptor, active relocalization of CD34 occurs and reveals adhesion molecules (Fig. 3A). CD34 may then be involved in inside-out signaling to activate adhesion molecules and signaling cascades. Thus, we propose that CD34 has an important role in facilitating migration of various hematopoietic progenitors to their appropriate niche, for example, mast cells to the peritoneum *(98)* or eosinophils to the lung *(89).*

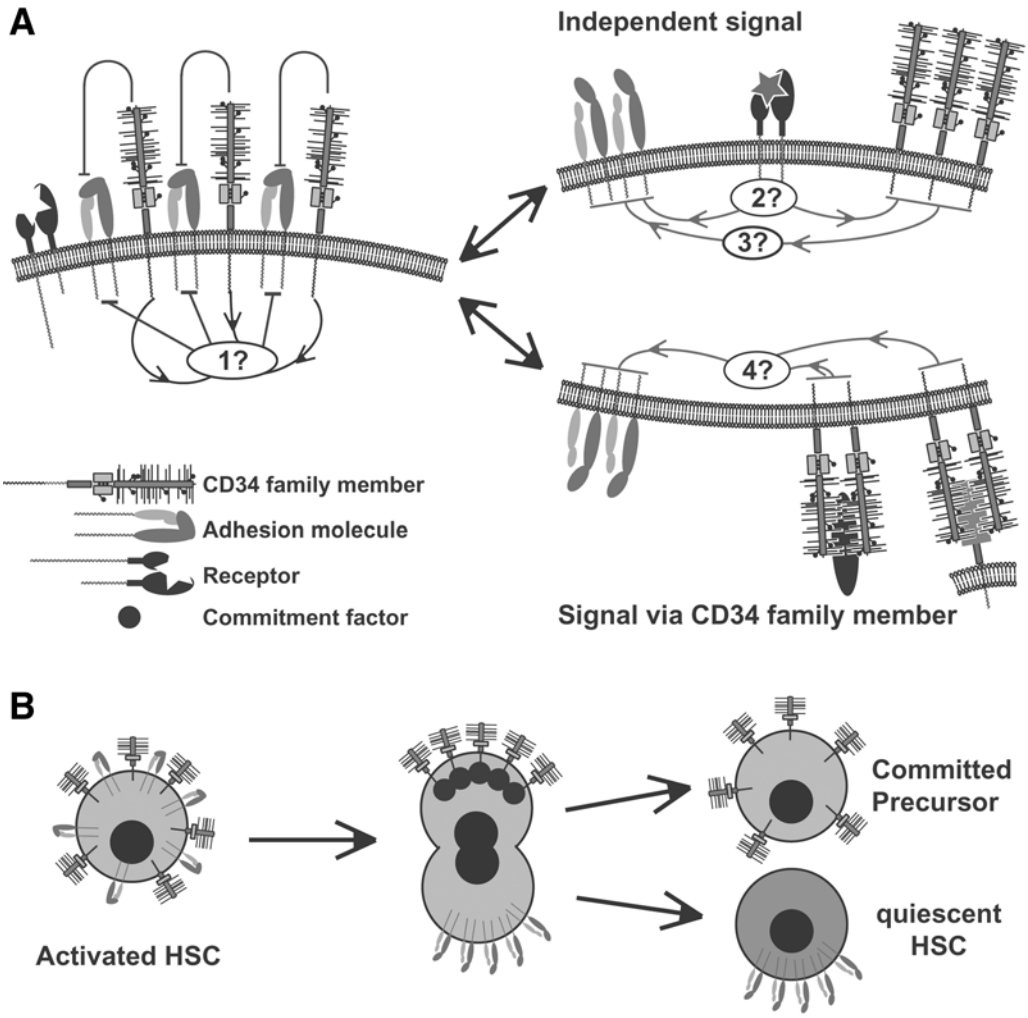


**Fig. 3** **(A)** Cartoon of the surface of a cell in non-adhesive mode is shown on the left, in which a CD34 family member prevents adhesion by stearic hindrance via the mucin-like ectodomain while also blocking inside out signaling (1?) responsible for adhesion molecule activation. On the right is shown two alternative mechanisms by which CD34 family members might be involved in upregulating cell adhesion. In the top cartoon a ligand binds a cell-surface receptor, such as a cytokine receptor triggers intracellular signaling which results in partitioning of CD34 family members (2?) away from cell adhesion molecules and either signaling from the receptor (2?), or from clustered CD34 family members (3?) activates adhesion molecules, as represented by extended structure. In the bottom cartoon either a soluble ligand (purple) or counter receptor (light teal) binds CD34 family member that transduces signal (4?) necessary for partitioning of and activation of adhesion molecules. **(B)** Cartoon showing how CD34 family members might be involved in asymmetric cell division. As a hematopoietic precursor divides the CD34 family member localizes to one pole of the dividing cell and drags with it factors involved in precursor commitment (shown in purple) (such as β-catenin) allowing maintenance of pluripotential by that daughter cell whilst also allowing the other daughter cell to differentiate.

## Podocalyxin

### Podocalyxin Identification and Expression

Podocalyxin [also known as Myb-Ets-transformed progenitor (MEP) 21, podocalyxin-like protein 1 (PCLP1), thrombomucin, and gp135] was first identified as a marker of kidney glomerular podocytes *(6).* To date the most detailed analysis of its function and activity has been conducted in kidney cells. The most obvious fundamental differences between podocalyxin's and CD34's structures are that podocalyxin's small globular domain contains two, instead of three, paired cysteines, and a much larger mucin-like domain comprising roughly 250 amino acids compared with about 120 in CD34. In terms of non-hematopoietic expression, podocalyxin is expressed on the primitive endoderm, ectoderm, and the first intraembryonic mesodermal precursors *(99).* It is also expressed by hemangioblasts of the AGM *(100,101)* and throughout development on the luminal face of vascular endothelia *(100,102),* on the kidney glomerular podocytes *(6,100,103),* neuroepithelium in the primitive ectoderm *(99),* and subsequently throughout boundary elements in the brain *(104).* It is also on other boundary elements such as the mesothelial linings and luminal faces of newly formed cavities *(99,100).* In the adult, Podocalyxin is expressed on kidney podocytes *(6,102)* and all vascular endothelia *(102).* It will be apparent to the reader that the embryonic endothelial expression appears to mimic that of CD34, although it has not been shown that podocalyxin positive (podocalyxin+) cells from the AGM are the same cells as those expressing CD34, or that these podocalyxin+ cells possess mesenchymal/stromal stem cell activity. It is tempting to think that these family members might be capable of functional compensation in these tissues (see Table 1 for a comparative overview of expression). Likewise, CD34 and podocalyxin show coincident vascular endothelial expression in the adult, and podocalyxin has been demonstrated to be modified appropriately on HEVs to enable binding of L-selectin *(12).*

### Podocalyxin Expression in the Hematopoietic System

The first description of podocalyxin on hematopoietic cells came from studies in chickens, with the identification of MEP21/thrombomucin as a marker of normal and transformed multipotent hematopoietic progenitors *(100,105,106).* Subsequently, podocalyxin expression has been reported on hematopoietic precursors in mice *(99,101)* and humans *(107)* and this expression is maintained on early hematopoietic cells throughout development *(99).* As with CD34+ HSCs, those expressing podocalyxin are capable of long-term reconstitution in mice *(99).* It has not yet been reported whether CD34 and podocalyxin are coexpressed on these HSCs, nor whether the expression of podocalyxin on these cells is activation/proliferation dependent, as is the case with CD34. This expression profile and reconstitution capacity supports the notion that these family members could provide functional compensation in the hematopoietic system.

Podocalyxin is also expressed beyond hematopoietic progenitors and is present on thrombocytes in chicken *(100)* and the functionally equivalent cells (platelets) in rats *(108)* as well as their precursors (megakaryocytes) *(108).* In resting platelets much of the podocalyxin is present in intracellular pools and it is significantly upregulated at the cell surface in response to stimulation with thrombin *(108).* This seems to mirror data regarding CD34 on HSCs, showing that it is mobilized to the cell surface in response to signaling *(18,66).* Podocalyxin is also expressed on nucleated erythroid cells and this expression

**Table 1.** Tissue Distribution of CD34 Family Memebers

| Tissue/cells | CD34 | Podocalyxin | Endoglycan |
|---|---|---|---|
| **Multipotent hematopoietic progenitors** | | | |
| Adult | + | + | + |
| Embryyonic | + | + | ? |
| **Prescursors** | | | |
| Erythroid | – | + | ? |
| Thrombocytic | + | + | ? |
| Myeloid | + | – | ? |
| Lymphoid (thymocyte subset only) | + | + | ? |
| **Mature Hematopoietic cells** | | | |
| B cells | – | – | ?/– |
| T cells | – | – | ?/– |
| Macrophages | – | – | ?/+ |
| Granulocutes | – | – | ?/+ |
| Eosionphils | – | – | ?/– |
| Mast cells | +* | – | ?/– |
| Erythrocytes | – | +† | ?/– |
| Platelets | – | + | ?/– |
| **Vascular endothelia** | + | + | + |
| **Smooth muscle** | – | – | + |
| **Podocytes** | – | + | ? |
| **Brain(neural)** | – | +¥ | + |
| **Mesothelial boundary elements** | – | + | ? |

* Murine mast cells only.

† Embryonic and anemic erythrocytes only.

¥ Ependymal layer only.

correlates closely with high rates of erythropoiesis, including anemic responses, and seeding of erythroid progenitors to new hematopoietic microenvironments *(99)*.

## Podocalyxin Function and Comparison with CD34

In the kidney glomerulus, Podocalyxin is expressed on the surface of specialized structures called podocyte foot processes *(6)*. Podocytes develop from epithelial cells that face the lumen of the glomerular capsule and their foot processes form an interdigitating network of cell extensions, with narrow filtration slits that allow the passage of filtrate into the capsule. Podocalyxin is the major sialoprotein of these foot processes *(6,102)* and systemic treatment with agents that remove or neutralize the surface negative charge from the foot processes results in collapse of these structures (effacement) leading to kidney dysfunction *(6,109–111)*. That podocyte foot processes are dependent on podocalyxin is unequivocal, because, in podocalyxin-null animals, these structures fail to form and the podocyte epithelial precursors retain tight junctions, resulting in anuria and perinatal mortality *(2)*. The widely accepted model for how the mature foot process structures are maintained by podocalyxin expression is that podocalyxin's high net negative charge maintains the filtration slit via charge repulsion. This is consistent with the reported data; however it has not been demonstrated that the treatments that neutralize or remove negative charge from podoca-

lyxin do not also affect its localization. Moreover, podocalyxin is not detected in the filtration slits, but rather on the more apical luminal face of the foot processes *(6)*. It is also possible that the decoration of the mucin domain with this high net negative charge is important for some aspect of signaling via podocalyxin to maintain these structures. This is supported by the observation that treatments resulting in the removal of this negative charge result in uncoupling of podocalyxin from the cytoskeleton *(24)*. In podocyte foot processes, podocalyxin is bound at its very C-terminus to the adaptor protein NHERF-2 (Na(+)/H(+) exchange regulatory factor 2, also known as E3KARP [Na(+)/H(+) exchanger (NHE) type 3 kinase A regulatory protein] and SLC9A3R2 (Solute carrier family 9 isoform A3 regulatory factor 2) *(20,23,24)*. NHERF-2 is a cytosolic scaffolding and signaling protein containing two tandem PDZ (PSD-95, Dlg, ZO-1) domains and a carboxyl terminal ERM (ezrin, radixin, moesin) binding domain. NHERF-2 has been shown to interact with a number of transmembrane proteins via one or other of its PDZ domains [for review see Volz et al. *(112)*]. It can link these proteins to the cytoskeleton via its own interaction with ERM family members, which then interact with the actin cytoskeleton *(113)*. Interaction of the ERM family members with both the actin cytoskeleton and their binding partners is signal-regulated *(113)*. The NHERF family of proteins have complex and incompletely understood roles in regulation of membrane protein trafficking, activity, and recruitment of signaling molecules *(112)*. In podocytes, podocalyxin, NHERF-2, and ezrin form a complex that presumably is capable of linking podocalyxin to the actin cytoskeleton *(24)*. NHERF-2 shows extremely restricted expression, and we do not find it expressed in any hematopoietic tissues (unpublished observation), although its close relative NHERF-1 is *(25)*. Given the similarity between NHERF-1 and -2, it has been assumed that both these proteins would bind podocalyxin. NHERF-1 and podocalyxin have been shown to colocalize in the canine kidney cell line MDCK *(21,22)* and both a recombinant fusion protein containing podocalyxin's cytoplasmic tail *(22)* and transiently transfected tagged NHERF-1 *(24)* can co-immunoprecipitate each other. In addition, we have shown that, in hematopoietic cells, there is an interaction of endogenous proteins *(25)*. The interaction between podocalyxin and NHERF-1 occurs via NHERF-1's second PDZ domain and the C-terminal 4 amino acids (DTHL) of podocalyxin *(24,25)*. This C-terminal has the alternate sequence DTEL in CD34 and, in vitro, although podocalyxin's tail interacts with NHERF-1, CD34's tail does not *(25)*. Moreover, we are able to immunoprecipitate only a very small fraction of NHERF-1, with anti-podocalyxin antibodies, from early hematopoietic precursors, suggesting that although the in vitro interaction is very stable, the cellular interaction is either transient or regulated dynamically by a signal we have yet to identify *(25)*. Podocalyxin also appears to be a target for phosphorylation (as has been shown for CD34) in response to phorbol esters, although we have yet to identify the kinase responsible (unpublished data). Overexpression of podocalyxin in MDCK cells leads to activation of RhoA (Ras homolog A) *(22)* and enhanced phosphorylation of NHERF-1 *(22)*. Schmieder et al. *(22)* argue that this is due to a interaction between podocalyxin and ezrin (via NHERF-1) displacing RhoGDI (Ras homolog Guanine nucleotide Dissociation Inhibitors) and thus activating RhoA *(22)*. However, because RhoA signaling can operate upstream or downstream of ezrin [for review see Ivetic and Ridley *(114)*], it is possible that podocalyxin overexpression results in a signaling cascade where ezrin activation is the consequence of RhoA activation rather than vice versa. In either case it is clear that

**Table 2.** CD34 Family Member Ligands

| Ligand | Family members bound | Cell type | Type of interaction |
|---|---|---|---|
| **Extracellular** | | | |
| **L-selectin** | **CD34, podocalyxin, endoglycan** | **HEV** | **sialyl lewis-x carbohydrate dependent** (refs. *10–12*) |
| **Intracellular** | | | |
| **NRERF-1** | **Podocalyxin, endoglycan** | **Hematopoietic cells** | **C-terminal PDZ interaction (DTHL)** (refs. *22,24,25*) |
| | | **Breast cancer** | |
| **NHERF-2** | **Podocalyxin** | **Podocyte** | **C-terminal PDZ interaction (DTHL)** (refs. *20,21,23*) |
| **ERM** | **Podocalyxin** | **MDCK** | **Juxtamembrane (HQRIS**QRKDQQ**R**) (ref. *22*) |
| **CrkL** | **CD34** | **Hematopoietic cells** | **Juxtamembrane** (RRSWSPTGER*) (ref. *19*) |

Amino acid sequences of interaction motifs are given, with critical residues in bold. *In the case of CD34–CrkL the critical residues within the sequence habe not been mapped. References (see main text) are given.

forced overexpression of podocalyxin allows it to direct, or participate in, signaling events.

CD34 does not appear to associate with NHERF-1 (Table 2), suggesting that if functional compensation exists between these two family members in HSCs, NHERF-1 does not participate in the shared pathway. By the same analogy, the reported binding site for CrkL on CD34 is completely absent from podocalyxin's cytoplasmic tail, implying that CrkL is not participating in a shared hematopoietic pathway.

## Podocalyxin So Far

Podocalyxin was first identified as the major sialoprotein of the glomerular foot processes and it is clear that it has an important role in maintaining these structures. Experiments conducted so far do not address the mechanism by which abrogation of podocalyxin's negative charge results in collapse of these structures. The model in which charge repulsion alone maintains the filtration slits does not fully address the subtleties involved in maintenance of these structures. First, it appears that podocalyxin is localized in a manner inconsistent with maintenance of slits by charge repulsion alone, being restricted to the apical domain of the foot processes, which faces the lumen of the glomerulus. Second, effacement of the foot processes involves loss of adhesion of these structures to the basal lamina rather than the formation of tight junctions seen in podocalyxin-null animals. Furthermore, podocalyxin's localization after treatment to abrogate the negative charge has never been assessed, nor has the effect of this treatment on signaling to various junctional complexes.

In terms of hematopoiesis, it is clear that podocalyxin expression marks a subset of HSCs, but further research needs to be conducted to analyze whether this represents the same, distinct, or overlapping subsets compared with CD34. The genetic evidence for podocalyxin function in hematopoiesis is difficult to interpret, because null mice are not viable. Delineation of podocalyxin function would thus benefit from a conditional knockout. As for CD34, the functional data from

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gene targeting and overexpression of podocalyxin support the notion that podocalyxin is involved in signal-regulated loss of cell adhesion (see Fig. 3A).

## Endoglycan

### Endoglycan Identification, Expression, and Function

Endoglycan is the most recently identified member of the CD34 family *(1)*. The most notable difference from CD34 and podocalyxin is endoglycan's mucin domain, which is predicted to be approx 350 amino acids and also contains a poly glutamic acid stretch of almost 30 uninterrupted residues. In addition, endoglycan has only a single pair of cysteines in the globular domain and an unpaired cysteine in the juxtamembrane region, which is presumed to allow homodimerization (Fig. 2). Endoglycan mRNA is highly expressed in the brain, pancreas, and kidney with very low expression in the liver *(1)*. Preliminary data suggest expression on vascular smooth muscle (unpublished). All hematopoietic tissues have low levels of mRNA with highest expression in the lymph nodes *(1)*. FACS analysis also shows expression of endoglycan on the CD34+ fraction of bone marrow. Beyond its expression on CD34+ cells from the bone marrow and on HEVs, nothing is known of the exact cell types that normally express endoglycan. Based on staining data it is assumed (but not shown) that endoglycan, like podocalyxin and CD34, is expressed on a subset of hematopoietic precursors and endothelia. As with the other members of the CD34 family, endoglycan can function as a ligand for L-selectin *(10,115)*.

## CD34 family

We are starting to see some of the important roles that the CD34 family of proteins have in various aspects of hematopoiesis. Presently, we are at a point where the field needs some thorough molecular analysis and side-by-side comparison of the expression of the three members. It will be important to establish under what conditions early progenitors express these proteins and whether their expression is regulated in a coordinate manner, as would be necessary for any functional compensation. A summary of what is presently known about their expression is given in table 1. Promoter analysis would be another avenue of research that may provide clues to the roles of these proteins, especially because the expression of CD34 (at least) is upregulated as HSCs are activated/start to proliferate. Basic information about the nature of these proteins in signaling should be established. All members have consensus phosphorylation sites for (at least) CK2 (Casein Kinase 2) and PKC and, based on CD34, are probably also targets for phosphorylation by tyrosine kinases (TK). It would be valuable to establish which kinases are capable of signal-regulated phosphorylation. Although sequence analysis would indicate that endoglycan is capable of covalent homodimerization, analysis of the function of all members would benefit greatly from data regarding oligomerization. One of the current models for regulation of adhesion by CD34 family members is that they act as an umbrella to stearically hinder adhesion molecule function; inactive integrins extend approx 23 nm above the lipid bilayer and, based on heights of classical mucins, CD34, podocalyxin, and endoglycan would be expected to project 33, 55, and 77 nm, respectively. Experimental confirmation of this would add weight to this model but would still not address what signaling mechanisms, normally associated with integrin function, are activated in response to capping of CD34 family members. Lastly the function of the globular and stalk domains needs to be addressed.

Obviously we have put forward a strong case for the CD34 family playing an active role as anti-adhesion molecules in regulating cell–cell and cell–substrate adhesion (see Fig. 3A). The expression of both CD34 and podocalyxin (an probably endoglycan) are restricted to subsets of hematopoietic precursors, and both are capable of being localized to one pole of these cells. Therefore, there may be instances in which CD34 family members partition to one daughter cell. With the identification of NHERF-1 as a ligand for podocalyxin there are even more intriguing possibilities. NHERF-1 (and a number of other PDZ-containing proteins) interacts with β-catenin and so, via NHERF-1 (or alternative PDZ protein), CD34 family members may have a role in asymmetric cell division by dragging important transcription factors, such as β-catenin, to only one daughter cell (see Fig. 3C).

Now is an exciting time for this family of proteins, the pressure generated due to CD34's use as a pan-HSC marker has abated. With this encumbrance gone, research focus can now shift to understanding the essence of what these proteins actually do!

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