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Kinetic Expression of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1/CD31) During Embryonic Stem Cell Differentiation

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Abstract Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is widely used as a marker during vasculogenesis and angiogenesis from embryonic stem (ES) cells. However, the expression of PECAM-1 isoforms in ES cells has not been determined. The present study was designed to determine the role of PECAM-1 isoforms during in vitro endothelial differentiation of ES cells. It was found that undifferentiated ES cells expressed high level of PECAM-1, which primarily located at cell–cell junction, but the expression of PECAM-1 was sharply down-regulated during early ES cell differentiation. In addition, undifferentiated ES cells were found the expressed all eight known alternatively spliced PECAM-1 isoforms, among them the expression of PECAM-1 isoforms lacking exon 15 or 14&15 was predominant. Quantitative analysis revealed a significant increase in the expression of PECAM-1 isoform lacking exon 12&14&15 as vascular development of ES cells. These results indicate a constitutive expression of PECAM-1 in undifferentiated murine ES cells and suggest a developmental role of PECAM-1 isoform changes during vasculogenesis and angiogenesis. J. Cell. Biochem. 95: 559–570, 2005.

Key words: PECAM-1/CD31; embryonic stem cells; alternative splicing; vasculogenesis; angiogenesis; endothelial differentiation

Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), a 130-kd cell surface molecule belonging to the immunoglobulin (Ig) superfamily [DeLisser et al., 1994], is expressed by the entire vascular endothelium in adult [Baldwin et al., 1994; Garlanda and Dejana, 1997]. PECAM-1 has been thought not only as a marker of endothelial cells but also as a modulator of endothelial cell migration, cell–cell adhesion, in vitro and in vivo angiogenesis [Kim et al., 1998; Pinter et al., 1999; Yang et al., 1999; RayChaudhury et al., 2001]. Recent studies have suggested that PECAM-1 may engage in both homophilic (PECAM–PECAM) and heterophilic (PECAM-X) bindings to other cell surface or matrix proteins [Deaglio et al., 1998; Righi et al., 2003]. Ligand-receptor binding results in lymphocyte rolling, adhesion, and extravasation, as well as in the implementation of a signaling pathway [Deaglio et al., 1998; Righi et al., 2003]. Some studies have shown that mouse inner cell mass (ICM) of blastocyst expressed PECAM-1 where embryonic stem (ES) cells derived from [Redick and Bautch, 1999; Robson et al., 2001]. However, the expression of PECAM-1 in ES cells has not been well studied.

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Mature PECAM-1 consists of a 574-amino acid extracellular domain comprised of six Ig-like homology domains (exons 3–8), a 19-residue hydrophobic transmembrane domain (exon 9), and a 118-amino acid cytoplasmic tail (exon 10–16) [Newman, 1997; Wang and Sheibani, 2002]. The cytoplasmic domain is complex and encoded by eight short exons, which are differentially susceptible to alternative splicing, resulting in generation of mRNA species that encode distinct PECAM-1 isoforms [Sheibani et al., 1999]. In the ICM of the blastocyst [Robson et al., 2001] and during embryonic development [Yan et al., 1995], three of the eight exons in the cytoplasmic domain are alternatively spliced (exons 12, 14, and 15) encoding eight isoforms, including the full-length, Δ12, Δ14, Δ15, Δ12&15, Δ14&15, Δ12&14, and Δ12&14&15 isoforms. The role of PECAM-1 during early development prior to vasculogenesis remains to be unclear [Furusawa et al., 2004].

ES cells, derived from the ICM/epiblast of the blastocyst [Martin, 1981], remain undifferentiated in vitro in culture on feeder layers of mouse embryonic fibroblast (MEF) cells or adding leukemia inhibitory factor (LIF) to the growth medium. Mouse ES cells express proteins including SSEA-1 (stage-specific embryonic antigen), alkaline phosphatase (ALP), and Oct-4 (a homeo domain containing octamer binding protein) [Fox et al., 1981; Pesce and Schöler, 2000]. During the endothelium differentiation of ES cells, it was previously reported that undifferentiated ES cells express PECAM-1 [Vittet et al., 1996; Redick and Bautch, 1999]. The expression pattern of PECAM-1 isoforms is known to be developmentally regulated [Baldwin et al., 1994]. However, the expression and distribution of PECAM-1 isoforms in ES cells have not been previously examined. Therefore, a better understanding of how the expression of PECAM-1 isoforms relates to the endothelium differentiation of ES cells may provide further insight of PECAM-1 in vascular development and angiogenesis.

In the present study, we examined PECAM-1 expression in ES cells during a time course of differentiation and further studied the expression pattern of PECAM-1 isoforms in ES and EB cells at specific time points. We demonstrate that PECAM-1 is a constitutive feature of undifferentiated murine ES cells but may express on cells in different isoforms as vascular development. The isoforms that lack exons 15 and 14&15 are predominant in ES cells. Moreover, the expression pattern of PECAM-1 isoforms changed during ES cells in vitro differentiation, lacking exon 12&14&15 was significantly increased as vascular development of ES cells.

MATERIALS AND METHODS

ES Cell Culture

The murine ES cell line J1 was kindly provided by Dr. En Li (MGH, Boston). Undifferentiated ES cells were maintained on irradiated SNL feeder cells on gelatin-coated dishes in culture medium consisting of Dulbecco's modified Eagle medium (DMEM; Hyclone, Logan, UT) supplemented with 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids (Hyclone), 100 μM monothioglyceral (MTG; Sigma, Saint Louis, Mo), 1 mM sodium pyruvate (Hyclone), 10 ng/ml LIF (Chemicon International, Temecula, CA), and 15% fetal calf serum (FCS; Hyclone) as described [Wang et al., 2004].

Differentiation of ES cells into EBs was induced as described [Kalberer et al., 2000]. Briefly, ES cells were seeded into 35 mm Petri dishes in medium containing Iscove modified Dulbecco medium (IMDM) supplemented with 1% methylcellulose (Stem Cell Technologies, Vancouver, Canada), 15% FCS (Hyclone), 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 100 μM MTG, $1 \times$ BIT (BSA, insulin, transferring; Stem Cell Technologies) in a final volume of 2 ml at a concentration of 1.5×10^3 cells/ml for 11 days without changing medium. To obtain large quantities of EBs, suspension cultures were carried out in a volume of 10 ml medium same to semi-solid culture at 2×10^5 cells per $100 \times$ 15 mm Petri dishes for 11 days, changing medium twice per week. To optimize endothelial differentiation, ES cells were cultured in the presence of 50 ng/ml VEGF (Pepro Tech EC, London), 100 ng/ml bFGF (Pepro Tech EC), 2 U/ml EPO (Kirin Brewery, Tokyo, Japan), and 10 ng/ml mIL-6 (PeproTech EC), as described previously [Vittet et al., 1996].

Sprouting EB Induction

ES cell differentiation into endothelial cells was performed as described [Feraud et al., 2001; Wang et al., 2004], with minor modifications. Briefly, 11 days-old EBs were harvested from methylcellulose semi-solid culture, and then

suspended into rat tail collagen type I (Becton Dickinson, San Jose, CA) culture medium at a final concentration of 50 EBs/ml and 2 mg/ml collagen. After thoroughly mixing EBs into collagen, 1.5 ml of mixture was added into 35 mm Petri dishes. The dishes were incubated at 37°C for 30 min, allowing gel polymerization prior to addition of medium. After gel formation, each dish was supplemented with IMDM containing 15% FCS, 450 μM MTG, 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10 μg/ml insulin. The cultures were then incubated for 72 h without medium change in the presence of 50 ng/ml hVEGF, 100 ng/ml bFGF, 2 U/ml EPO and 10 ng/ml mIL-6.

Immunofluorescence and Immunostaining ES Cells

ES cells were grown on gelatin-coated four Chamber Polystyrene Vessel Tissue Culture Treated Glass Slide (Becton Dickinson Labware, Franklin Lakes, NJ), then fixed with methanol for 3 min at −20°C and processed for immunofluorescence with antibodies to PECAM-1 in situ. Briefly, cells were incubated with FITC-conjugated rat anti-mouse PECAM-1 (MEC 13.3, 1:100 dilution; Becton Dickinson, San Jose, CA) for 1 h at room temperature. Cells were viewed on an Olympus confocal microscope (Fluoview FV300).

Day-11 EBs were embedded into OCT compound (Miles Scientific, Elkhart, IN) after fixation in methanol and DMSO (4:1) overnight at 4°C. Frozen sections (5 μm thick) were mounted onto poly-L-lysine coated slides, air dried overnight, and processed for immunostaining PECAM-1. For immunostaining PECAM-1, sections were incubated with primary monoclonal antibody (MoAb) followed by incubation with secondary MoAb and biotin-conjugated mouse anti-rat IgG_{2a} (G28-5, Becton Dickinson) and horseradish peroxidase streptavtin (HRP), then stained with diaminobenzidine (DAB) and counter stained with Mayer hematoxylin.

Whole-mount immunohistostaining of EBs was performed as previously described [Yoshida et al., 1996; Feraud et al., 2001] with minor modifications. The EBs and sprouting EBs were fixed in methanol and DMSO (4:1) at 4°C overnight. To block endogenous peroxidase, the fixed EBs or sprouting EBs were soaked in methanol-DMSO-30% H_2O_2 (4:1:1) for 1.5–2 h at room temperature. For staining, the rehydrated EBs or EB sprouting were first blocked by two incubations in PBSBT (2% BSA and 0.2% Tween-20 in PBS), then with PBSBT containing rat anti-mouse MoAb PECAM-1 (MEC 13.3, 1:100 dilution; Becton Dickinson) overnight at 4°C.The EBs were washed five times in PBSBT each for 1 h at 4°C for the initial three washes and at room temperature for final two. The primary antibody was labeled by incubating the EBs or sprouting EBs with biotin-conjugated mouse anti-rat IgG_{2a} (G28-5, Becton Dickinson) in PBSBT overnight at 4°C. After washing as above, the EBs or sprouting EBs were incubated with HRP for 2 h in PBS at room temperature. After washing in PBS three times for 30 min each, the antigens were detected by the color reaction with DAB and nickel chloride. The EBs or sprouting EBs were dehydrated with ethanol, clear with benzyl benzoate and benzalcohol (3:1) at least 3 h, then transferred onto poly-L-lysine coated slides with Canada balsam in methylsalycilate cover. The slides were observed with Olympus photomicroscope equipped for SPOT digital camera.

Flow Cytometric Analysis

Cell surface antigen expression was studied by a flow cytometry (Becton-Dickinson) with the following MoAbs, namely FITC-conjugated rat anti-mouse PECAM-1 and anti-mouse SSEA-1 IgM (Chemicon International), and FITC-conjugated anti-rat IgM. ES cells free of feeder cells were detached by trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Invitrogen, Carlsbad, CA). EBs were harvested and disrupted with 0.05% trypsin and 0.53 mM EDTA for 3 min at room temperature with frequent shaking, then gently passed through a 21 G needle on a 3 cc syringe three times to generate a single cell suspension from up to EBs 8 days; EBs 9 or more days were disrupted with 0.2% collagenase type I (Invitrogen, Carlsbad, CA) and incubated at 37°C for 2 h with frequent shaking, then passed through a 21-G needle on a 3 cc syringe three times. The experiments were performed at the condition of cell viability more than 90%.

RT-PCR Analysis and DNA Sequencing

Total RNAs were purified from undifferentiated ES cells, Ebs, and sprouting EBs in collagen at the time indicated, using Trizol (Invitrogen). First-strand cDNAs were generated using M-MLV Reverse Transcriptase (Invitrogen). For the PCR reaction, first-strand

cDNAs were amplified at a final volume of 50 μl with 1 U Taq DNA polymerase (TaKaRa, Dalian). In addition, all cDNA samples were adjusted to yield equal amplification of β-actin as an internal standard. Specific primers used for PCR are listed in Table I. All the genes were analyzed on more than one occasion using RNA from independently derived samples. The amplification parameters for PECAM-1 extracelluar domain, cytoplasmic domain, Oct-4, and β-actin were 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min for 30 cycles; For cytoplasmic domain isoforms of PECAM-1 were 94°C denaturation for 45 s, 50°C annealing for 30 s for 32 cycles.

The primers for PECAM-1 extracellular domain resulted in a single 260 bp amplification product. To delineate the alternatively spliced cytoplasmic domain isoforms of PECAM-1, primers were designed to span exon–exon junctions in the region of alternative splicing. The sequences of these primers were followed by numbers in parentheses referring to the corresponding exon junction. The primers sets (ps) and yielded products are listed in Table I. To amplify the cytoplasmic domains of all possible PECAM-1 isoforms from this cDNA, a sense primer spanning the border of exon 9 and 10 within the cytoplasmic domain, and an antisense primer spanning the border of exon 16 and 3′-untranslated region (Table I, gene of cytoplasmic domain) were used. The primers carry a *Bam*HI and an *Eco*RI recognition sequence to facilitate subsequent cloning. PCR products were examined on 2% agarose gels to assess their integrity and excepted size.

The cloning was performed as described previously [Wang and Sheibani, 2002]. Briefly, PCR products of cytoplasmic domain were purified, digested with *Bam*HI and *Eco*RI and then ligated into the pUC18 vector, cut with same enzymes, and transformed into *Escherichia coli* DH5$_\alpha$. Bacterial colonies were screened by *Bam*HI and *Eco*RI digestion of plasmid DNA, and inserts were sequenced by BioAsia Company (Shanghai, China).

Identification of PECAM-1 Isoforms

Identification of PECAM-1 isoforms was performed as described [Wang et al., 2003]. Briefly, the exonic mutation sites of PECAM-1 cDNA molecules were identified by comparison of the mutant sequences with that of the full length [Newman et al., 1990]. The isoform with

TABLE I. Oligonucleotide Primers Used for RT-PCR

Gene	Primer sequence	Size (bp)	Notes
β-actin	Sense 5′-ATGAAGATCCTGACCGAGCG-3′	445	
	Antisense 5′-TACTTGCGCTGAGGAGGAGC-3′		
OCT-4	Sense 5′-TGGAGACTTTGCAGCCTGAG-3′	469	
	Antisense 5′-ATGGTCTCCAGACTCCACCT-3′		
PECAM-1 extracellular domain	Sense 5′-GTCATGGCCATGGTCGAGTA-3′	260	
	Antisense 5′-CTCCTCGGCGATCTTGCTGAA-3′		
PECAM-1 cytoplasmic domain	Sense 5′-atggatccAGGAAAGCCAAGGCCAAA-3′	232,255,286,289,	Span exon 9 and 16, carrying *Bam*HI and *Eco*RI
	Antisense 5′-cggaattcTTGACTGTCTTAAGTTCC-3′	309,312,343,366	recognition sequence (lowercase letters)
ps1	Sense 5′-ACAGCCATTACGGTTATG-3′	220, 163	Span exon 11 and 12
	Antisense 5′-CCTTCCGTTCTAGAGTATC-3′		Span exon 15 and 16, recognize full length and Δ14
ps2	Sense 5′-AACAGCCATTACGACCC-3′	167, 110	Span exon 11 and 13
	Antisense 5′-CCTTCCGTTCTAGAGTATC-3′		Span exon 15 and 16, recognize Δ12 and Δ12&14
ps3	Sense 5′-ACAGCCATTACGGTTATG-3′	196	Span exon 11 and 12
	Antisense 5′-CTTCCGTTCTTAGGGTCGA-3′		Span exon 14 and 16, recognize Δ15
ps4	Sense 5′-AACAGCCATTACGACCC-3′	143	Span exon 11 and 13
	Antisense 5′-CTTCCGTTCTTAGGGTCGA-3′		Span exon 14 and 16, recognize Δ12&15
ps5	Sense 5′-ACAGCCATTACGGTTATG-3′	139	Span exon 11 and 12
	Antisense 5′-CTTCCGTTCTCTTGGTGA-3′		Span exon 13 and 16, recognize Δ14&15
ps6	Sense 5′-AACAGCCATTACGACCC-3′	86	Span exon 11 and 13
	Antisense 5′-CTTCCGTTCTCTTGGTGA-3′		Span exon 13 and 16, recognize Δ12&14&15

a new junction in the cDNA sequence that lacks exon 12 (Δ12) was identified at G^{2057}-G^{2110} (loss of 54 bp), the isoform that lacks exon 14 (Δ14) at C^{2173}-A^{2229} (loss of 57 bp), and the isoform that lacks exon 15 (Δ15) at A^{2230}-T^{2352} (loss of 23 bp). The expected size of PCR for the wild-type form (full-length PECAM-1 cytoplasmic domain) is 350 bp according to the PECAM-1 cDNA sequence. However, the alternatively spliced isoforms have variable sizes, smaller than the wild type. Absence of exon 15 in the Δ15, Δ12&15, Δ14&15, and Δ12&14&15 isoforms changes the reading frame terminating upstream of the commonly utilized termination codon (Fig. 6).

RESULTS

Expression of PECAM-1 on Undifferentiated ES Cells

In embryonic development, the preimplantation blastocyst contains PECAM-1+ cells [Redick and Bautch, 1999; Robson et al., 2001]. A study of the kinetics of endothelial marker expression during in vitro differentiation of ES cells has suggested the distinction of successive maturation steps, in which PECAM-1 was expressed after Flk-1 but before VE-cadherin [Vittet et al., 1996]. To verify whether undifferentiated ES cells express PECAM-1, we examined ES cells cultured on gelatin-coated plates. As shown in Figure 1B, PECAM-1 was detected on most of undifferentiated ES cells and localized at cell–cell border.

To evaluate the temporal switch of PECAM-1 gene expression during ES cell differentiation, we analyzed RNA samples from EBs at different time points by RT-PCR. As show in Figure 2, PECAM-1 was found to express in undifferentiated cells, which was in line with our immunostaining and FCM results (Figs. 1 and 4). Also rapidly down-regulated during first a couple of days of differentiation, the expression of PECAM-1 gene increased as EB differentiation (Fig. 2A). However, when ES cells were cultured in the absence of angiogenic growth factors, PECAM-1 expression was rapidly down-regulated and maintained at low levels thereafter (Fig. 2B). As expected, Oct-4 gene was highly expressed in undifferentiated ES cells. After treatment of ES cells with angiogenic growth factors, Oct-4 gene was rapidly down-regulated (Fig. 2), suggesting that exogenous factors promote in vitro ES cells differentiation.

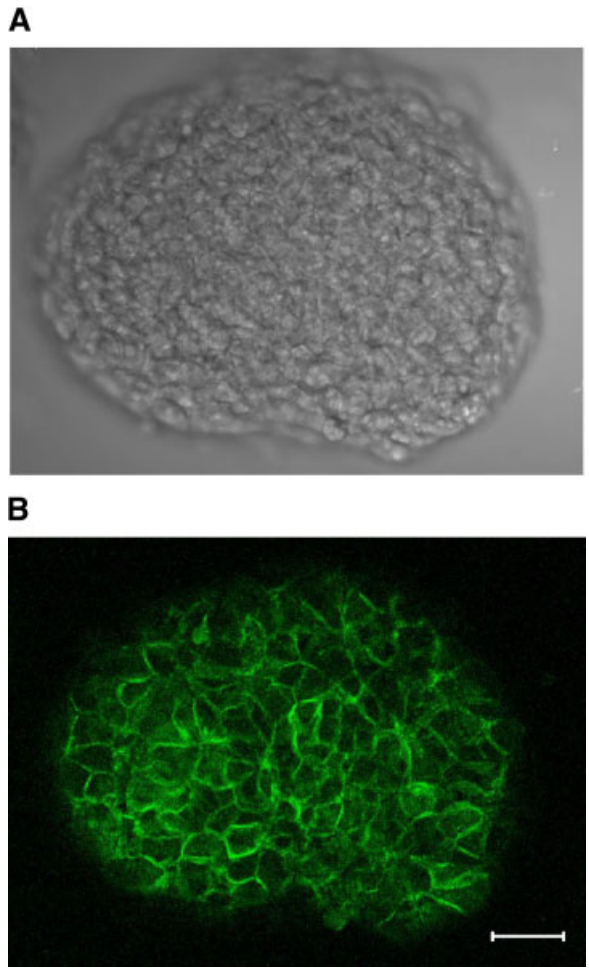

Fig. 1. Immunofluorescence staining and confocal imaging of platelet endothelial cell adhesion molecule-1 (PECAM-1) on undifferentiated embryonic stem (ES) cells. **A**: Bright view of ES cells colony. **B**: Confocal microscopy imaging of undifferentiated ES cells showing localization of PECAM-1 primarily at cell–cell junctions. Scale bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PECAM-1 Protein Expression Pattern During Differentiation of ES Cells

We next investigated PECAM-1 protein expression on differentiated ES cells in more detail by using previous established an in vitro model of vasculogenesis and angiogenesis [Feraud et al., 2001]. ES cells grown in methylcellulose or suspension culture were found to be able to differentiate spontaneously into EBs. After 11 days of EB differentiation, the EBs embedded into collagen in the presence of growth factors rapidly developed into vascular sprouting within 3–4 days. To monitor the differentiation of endothelial cells and morphogenesis of

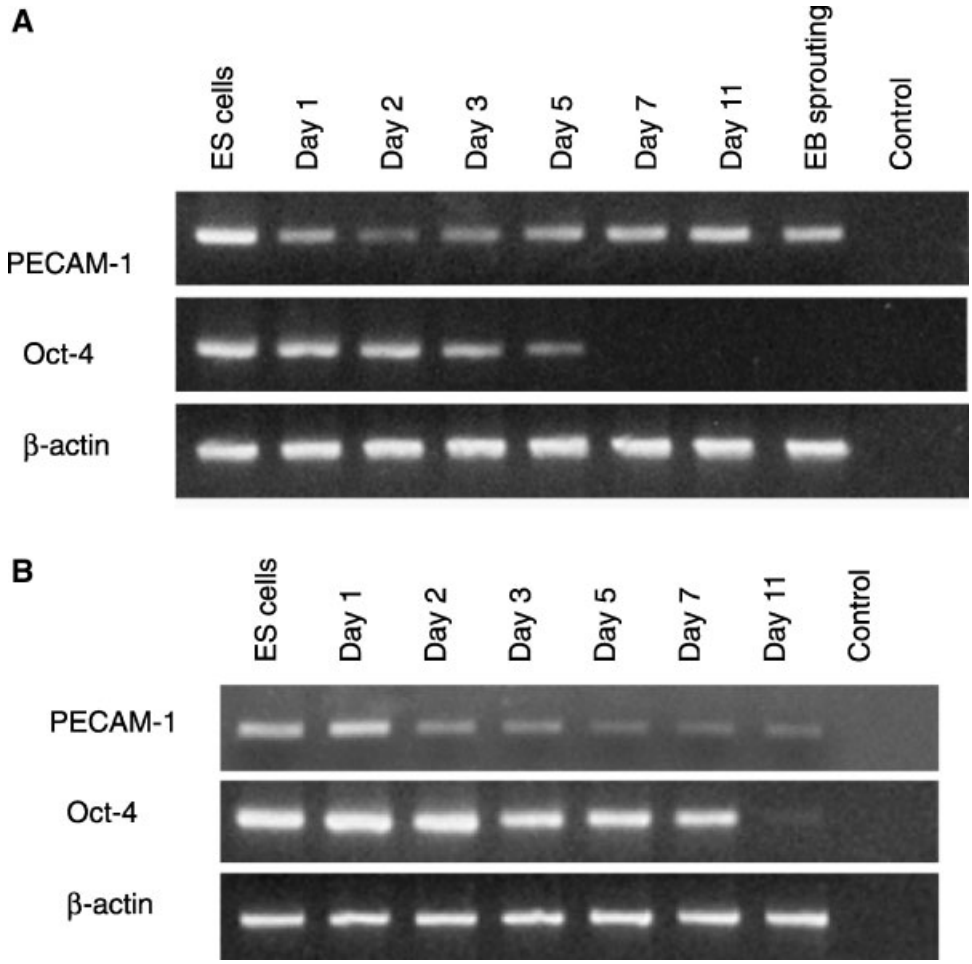

Fig. 2. PECAM-1 gene expression during ES cell differentiation. ES cells were induced for differentiation to form EBs, which were further induced sprouting in collagen. Total RNA was isolated from undifferentiated ES cells (day 0), differentiated ES cells (days 1–11) and EB sprouting cultured with or without factors. RNA was subjected to RT-PCR analysis. **A**: differentiation with angiogenic growth factors. **B**: Differentiation without factors.

vascular structures, the day-11 EBs and sprouting EBs were whole-mount immunostained with a MoAb against PECAM-1. Vascular structures organized into networks were observed in approximately 70% of EBs and sprouting EBs (Fig. 3A–D) in the presence of angiogenic growth factors. Further analysis showed that the walls of tubular channel structures in EBs, were PECAM-1 positive (Fig. 3B). In the absence of angiogenic growth factors, some of cells from day 11 EBs expressed PECAM-1. However, these PECAM-1 positive cells did not organize into vascular structures (Fig. 3E), suggesting that angiogenic growth factors were critical for vasculogenesis during EB formation.

To further determine the cell surface expression of PECAM-1, we measured PECAM-1 and SSEA-1 at several time points of EB differentiation using flow cytometry. As shown in Figure 4, a majority of undifferentiated ES cells (70%–95%) were PECAM-1+ cells, which were significantly reduced during first few days of EB differentiation. Consistent with our RT-PCR results, after initial decreasing, the PECAM-1+ cell populations steadily increased during EB differentiation. As expected, SSEA-1 expression continuously declined during ES cells differentiation.

Distribution of PECAM-1 Isoforms During Vasculogenesis and Angiogenesis of ES Cells

A recent study has suggested that alternative splicing of cytoplasmic domains of PECAM-1 may modulate its adhesive function [Wang and

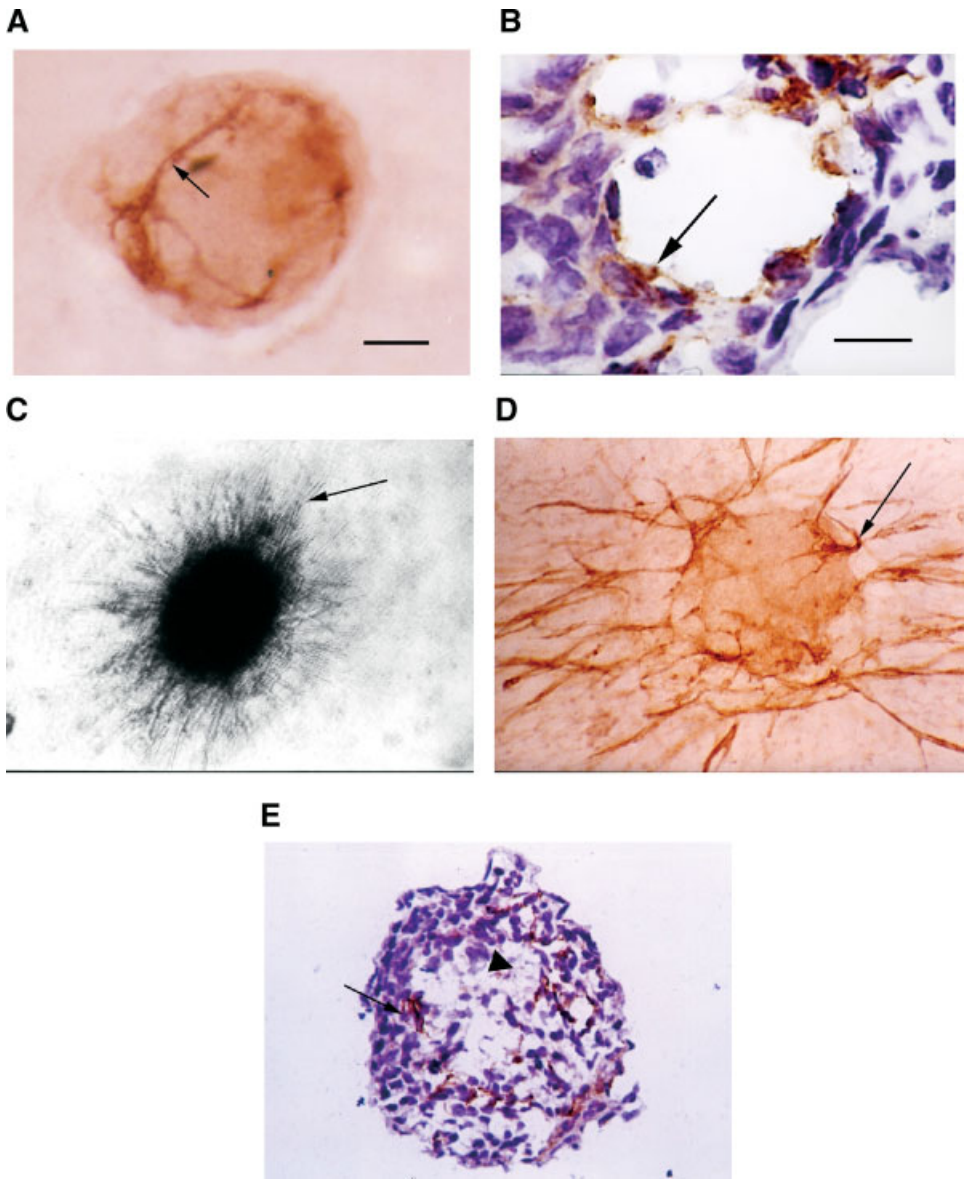

Fig. 3. PECAM-1 protein expression of ES cells-derived EBs. ES cells were differentiated to form EBs for 11 days to induce vasculogenesis with (**A**, **B**) or without (**E**) growth factors and cultured in collagen matrix for additional 3 days for angiogenesis (**C**, **D**). A: whole-mount immunochemistry of day-11 EB, the vascular structures were positive of PECAM-1, as shown with an arrow. B: PECAM-1 expression in EB was similar to vascular membrane (arrow). C: Bright view of sprouting EB. D: Whole-mount immunostaining of a sprouting EB with PECAM-1 MoAb on tubular channel structures wall (arrow). E: Frozen sections of day-11 EB cultured without angiogenic growth factors, there were only PECAM-1+ cells (arrow) and central necroses (arrowhead). EB (A) and sprouting (D) were stained with DAB. Frozen sections (B, E) were stained with DAB, and counter stained with Mayer heamatoxylin. Scale bar, A, C, D, E = 100 μm, B = 20 μm.

Sheibani, 2002]. However, the expression and distribution of different PECAM-1 isoforms in the ES cells have not been previously reported. In vitro differentiation of ES cells provides a unique opportunity to study the role of PECAM-1 isoforms in all aspects of vascular development, remodeling, and maturation. To gain a better understanding of PECAM-1 function of different isoforms, we examined the expression pattern of the isoforms during ES cell differentiation in vitro. As expected, the pair of PCR primers (for cytoplasmic domain shown in Table I), which encompassed the entire cytoplasmic domain, amplified all potential PECAM-1 isoforms. The DNA band corresponding to 350 bp was the full-length PECAM-1

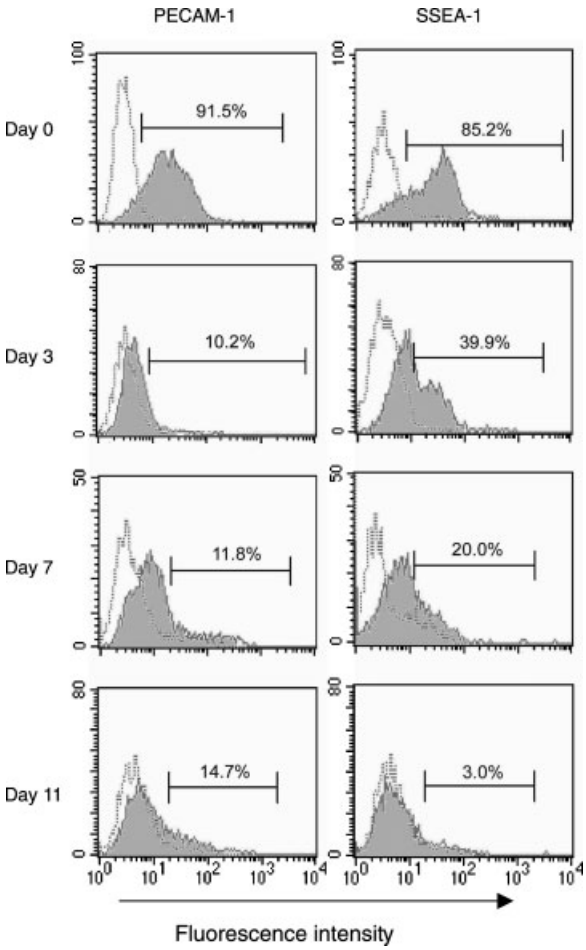

Fig. 4. Flow cytometric analysis of undifferentiated and differentiated EB cells. ES cells and the cells from differentiated EBs (day 3, 7, and 11) were stained with antibodies to PECAM-1, SSEA-1.

cytoplasmic domain. The multiple bands, which were smaller than 350 bp represented alternative spliced forms (Fig. 5A). To further determine the distribution of PECAM-1 isoforms in ES and EB cells, we did directly cloning and sequencing of the cDNAs generated in RT-PCR. Table II illustrates the different isoforms of PECAM-1 and their frequency during ES cell in vitro vasculogensis and angiogenesis. Undifferentiated ES cells expressed all of the eight known isoforms of PECAM-1, among them the isoforms Δ14&15 and Δ15 were at high level, a phenomenon in accordance with the distribution of isoforms in the ICM [Robson et al., 2001]. However, the frequencies of PECAM-1 isoforms were significantly changed as EB differentiation. The PECAM-1 isoforms of Δ14&15 and Δ12&14&15 were most frequently detected at various days of EB and EB sprouting. Expres-

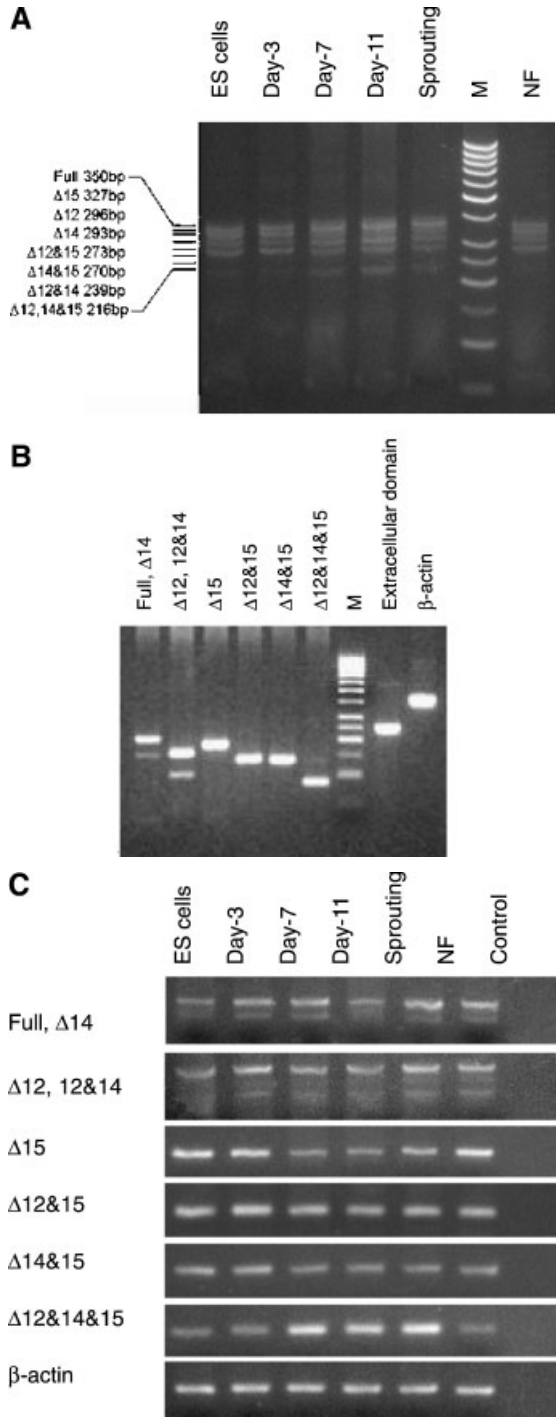

Fig. 5. Expression of PECAM-1 alternatively spliced isoforms in ES cells and EBs. **A**: RNA samples (as indicated) used for RT-PCR analysis with primers for cytoplasmic domain of all possible isoforms of PECAM-1. **B**, **C**. Six different primer sets designed to distinguish eight different isoforms of PECAM-1 were used in RT-PCR analysis. B, ES cells. C, Expression pattern of PECAM-1 isoforms during ES cell differentiation. RT-PCR was repeated with two-separate preparation of RNA with identical results. The PCR product was separated on a 2% agarose gel, M: the bands correspond to 1031, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, and 50 bp respectively.

TABLE II. Distribution of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) Isoforms in Embryonic Stem (ES) Cells During In Vitro Differentiation

		PECAM-1 isoforms							
	n	Full-length	Δ12	Δ14	Δ15	Δ12&14	Δ12&15	Δ14&15	Δ12&14&15
UD ES cells	31	10	3	10	26	3	3	35	10
NF ES cells	20	5	ND	ND	35	ND	10	35	15
D3 EB	19	5	5	ND	21	5	5	37	26
D7 EB	19	16	ND	5	ND	ND	ND	26	53[a]
D11 EB	20	12	15	6	10	5	ND	20	50[a]
Sprouting EB	20	ND	5	ND	5	15	5	25	45[b]

Values represent the percentage of PECAM-1 isoforms expression; n, total number of PECAM-1 clones examined. Isoforms of PECAM-1 were identified by cloning and sequencing RT-PCR products from mRNA isolated from ES cells at different time-point and conditions during in vitro differentiation; UD, undifferentiated; NF ES cells, ES cells were cultured for 11 days without growth factors; ND, not detected.
[a]$P < 0.01$.
[b]$P < 0.05$ versus UD ES cells, binomial proportion analysis was used.

sion pattern of PECAM-1 isoforms in EBs grown without angiogenic growth factors was same to undifferentiated ES cells.

To further explore expression of different isoforms and determine the time-specific expression of different PECAM-1 isoforms, we designed six sets of PCR primers to determine all eight isoforms based on the sequences shown in Figure 6, including (1) full-length, (2) Δ12, (3) Δ14, (4) Δ15, (5) Δ12&Δ15, (6) Δ14&Δ15, (7) Δ12&Δ14, and (8) Δ12&Δ14&Δ15. As shown in Figure 5B, undifferentiated ES cells express all eight known isoforms. Semiquantitative analysis revealed that all eight known spliced isoforms were observed in all time courses (Fig. 5C), but the relative expression levels changed as ES cell differentiation. The expression of Δ12&14&15 was up-regulated and Δ15

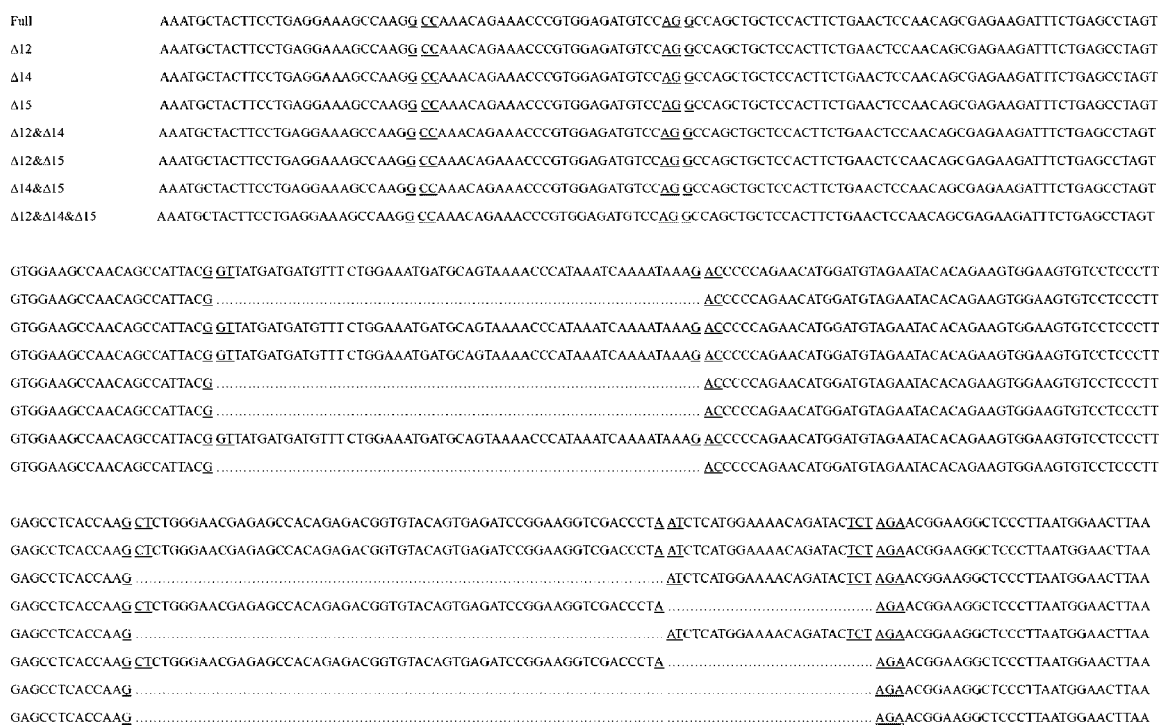

Fig. 6. cDNA sequence of cytoplasmic domain of murine PECAM-1 isoform. Nucleotide encoding cytoplasmic domain of isoforms lacking exon 12, 14, 15, 12&14, 12&15, 14&15, and 12&14&15 are compared with sequence for full-length PECAM-1 isoform. Deleted sequences are indicated by dashes. The predicted exon sequences are separated by a space as indicated by Yan et al. [1995].

was down-regulated from day 7 to EB sprouting. In contrast, the expression of other isoforms including full, Δ12, Δ14, Δ12&14 and Δ12&15 was not significantly changed through the time course of differentiation. Interestingly, the ES cells cultured without factors for 11 days showed similar expression patterns with undifferentiated ES cells. The results were consistent with cloning method. Although most of isoforms were detected by RT-PCR during ES cells in vitro differentiation (Fig. 5B,C), some isoforms were not detected by cloning method (Table II), possibly due to low levels of expression.

DISCUSSION

In the present study, we have shown a constitutive expression of PECAM-1 on undifferentiated murine ES cells through several approaches including immunofluorescent, RT-PCR and flow cytometric analysis. The expression of PECAM-1 was constant in various levels during EB differentiation and all known eight PECAM-1 isoforms could be detected in murine ES cells. These results confirm the finding that undifferentiated ES cells express PECAM-1 [Vittet et al., 1996; Redick and Bautch, 1999] and further demonstrate an expression of multiple isoforms of PECAM-1 in ES and EB cells in a developmentally regulated pattern.

PECAM-1 expression has been reported to be first detected in the blastocyst at 3.5 days post coitum (dpc) and its expression was restricted to ICM cells. At 4.5 dpc, its expression was limited to the epiblast, and by 5.5 dpc, was disappeared in the epiblast by the early egg cylinder. At 7.0 dpc, the expression was re-detected at the location of yolk sac and allantois during the vasculogenesis in embryo [Drake and Fleming, 2000; Robson et al., 2001]. An intrinsic molecular clock controlling PECAM-1 expression [Robson et al., 2001] may exist and determine cellular differentiation of undifferentiated ES cells. This may be crucial for most normal developmental processes including gastrulation, neural crest formation, and cell migration.

During the murine embryogenesis, PECAM-1 expression was initially detected on the entire cell surface and was later localized at sites of cell–cell contact [Drake and Fleming, 2000]. The molecules involved in cell–cell and cell-matrix interactions are presumably important in defining and maintaining the properties of the ICM and directing its future differentiation [Robson et al., 2001]. It has been shown that PECAM-1 is expressed by the entire vascular endothelium in adult [Baldwin et al., 1994]. However, the present study observed an expression of PECAM-1 on ES cells despite of lacking vascular structures in the absence of angiogenic factors. The addition of angiogenic growth factors into culture promoted PECAM-1+ cells to form vascular structures. PECAM-1 was distributed on cell–cell border of undifferentiated ES cells, suggesting a possible role of PECAM-1 in cell adhesion or transmembrane signaling. Similar results were obtained by others from F9 EB [Tang and Honn, 1995]. In addition, PECAM-1-deficient mice are viable and undergo normal vascular development [Duncan et al., 1999]. These results suggest that PECAM-1 is not essential for vasculogenesis and embryo viability, and its expression alone may not be sufficient to represent real vasculogenesis or angiogenesis.

The cytoplasmic domain PECAM-1 has been shown to be an important scaffolding molecule involve in several signaling pathways, suggesting an interesting possibility that PECAM-1 isoform switching may play an important role during developmental and reparative angiogenesis in a number of situations [Ilan and Madri, 2003]. The cytoplasmic domain of murine PECAM-1 is encoded by eight exons and exon 12, exon 14, and exon 15, and are susceptible to alternative splicing, resulting in generation of mRNA species that encode eight isoforms [Ilan and Madri, 2003; Newman and Newman, 2003]. The signaling properties of PECAM-1 are mediated by a series of interactions with adaptor molecules, mainly through phosphorylation of specific tyrosine-based residues located in an immunoregulatory tyrosine-based activation motif (ITAM) and immunoreceptor tyrosine-based inhibition motif (ITIM) domain in the cytoplasmic tail [Cao et al., 1998]. The PECAM-1's ITAM domain is encoded by exon 13 and exon 14 and has two consensus sites, the tyrosine core residues 663 and 686, for binding SH2 domains [Lu et al., 1997]. The cytoplasmic domains of PECAM-1 isoforms lacking exon 14 miss the ITAM and fail to associate with SHP-2 even though other tyrosines are phosphorylated [Sheibani et al., 2000]. The exon 15 encodes the binding site for tyrosine-phosphorylated β-catenin (β-cat), STAT3, and STAT5 [Ilan and Madri, 2003].

To further understand the function of PECAM-1 in ES cell differentiation, we deter-

mined the distribution of PECAM-1 isoforms in ES cells and EBs. We have first shown that undifferentiated and endothelial lineage differentiated ES cells and EB sprouting express all eight known isoforms of PECAM-1, although the expression patterns varied during differentiation. In addition, the Δ12&14 isoform of PECAM-1, whose expression was not reported in the early mouse embryo [Yan et al., 1995] and blastocyst [Robson et al., 2001], was detected in ES cells. Secondly, undifferentiated ES cells were found to express abundant Δ15 and Δ14&15 isoforms of PECAM-1. However, in the presence of angiogenic factors, expression of Δ14&15 and Δ12&14&15 isoforms became evident during vasculogenesis and angiogenesis from 11-days EBs, suggesting Δ12&14&15 may play a role in endothelial differentiation of ES cells.

Self-renewal of murine ES cells involves the binding of LIF to the cell surface receptor gp130 and activation of STAT3 [Robson et al., 2001]. The exon 13, exon 14, and exon 15 of PECAM-1, with its ability to activate STAT3, may play a role in LIF-mediated maintenance of undifferentiated ES cells [Sheibani et al., 2000; Robson et al., 2001; Ilan and Madri, 2003; Newman and Newman, 2003]. The abundant expression of Δ15 in the undifferentiated ES cells is intriguing given that an apparent dedifferentiation has been observed when the Δ15 isoform is stably transfected into an epithelial cell line [Sheibani et al., 1999]. PECAM-1 isoforms lacking exon 15 end with a different sequence, but the consequence of this variation is not known [Newman and Newman, 2003].

In conclusion, our results have demonstrated both spatially and temporally that PECAM-1 expression is a feature of undifferentiated murine ES cells, and that murine ES cells express all eight known alternatively spliced isoforms of PECAM-1 but dominant isoform varied during in vitro endothelial differentiation. The changes in the expression of cytoplasmic domain of PECAM-1 may affect its function during ES cell maintenance, vasculogenesis, and angiogenesis.

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