

Developmental Platelet Endothelial Cell Adhesion Molecule Expression Suggests Multiple Roles for a Vascular Adhesion Molecule

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Platelet endothelial cell adhesion molecule (PECAM) is used extensively as a murine vascular marker. PECAM interactions have been implicated in both vasculogenesis and angiogenesis. To better understand the role of PECAM in mammalian development, PECAM expression was investigated during differentiation of murine embryonic stem (ES) cells and in early mouse embryos. Undifferentiated ES cells express PECAM, and as *in vitro* differentiation proceeds previously unidentified PECAM-positive cells that are distinct from vascular endothelial cells appear. PECAM expression is gradually restricted to endothelial cells and some hematopoietic cells of differentiated blood islands. In embryos, the preimplantation blastocyst contains PECAM-positive cells. PECAM expression is next documented in the postimplantation embryonic yolk sac, where clumps of mesodermal cells express PECAM before the development of mature blood islands. The patterns of PECAM expression suggest that undifferentiated cells, a prevascular cell type, and vascular endothelial cells express this marker during murine development. PECAM expression in blastocysts and by ES cells suggests that PECAM may function outside the vascular/hematopoietic lineage. (*Am J Pathol* 1999, 154:1137-1147)

Platelet endothelial cell adhesion molecule (PECAM) is a member of the immunoglobulin superfamily expressed by endothelial cells and a subset of hematopoietic cells in the adult organism.¹⁻³ PECAM is localized to the cell-cell borders of the vascular endothelial cell where it mediates the extravasation of monocytes and neutrophils during the inflammatory response.^{4,5} PECAM engagement can also result in an up-regulation of integrin function in a variety of hematopoietic cells.⁶⁻¹⁰ During platelet aggregation, PECAM becomes phosphorylated on tyrosine.¹¹ Phosphorylated PECAM can then associate with SHP-2, a positive regulator of signal transduction, suggesting that PECAM may participate in a signaling cascade that results in integrin activation.

PECAM is expressed in the mouse embryo before the need for the inflammatory response, and it is used extensively as a developmental murine vascular marker.^{12,13} Recently, PECAM phosphorylation during embryonic vasculogenesis was documented,¹⁴ suggesting a possible signaling role for PECAM in blood vessel formation. PECAM interactions are also implicated in angiogenesis, the sprouting of new vessels from a pre-existing vascular tree.¹ In postimplantation embryos PECAM is first detected in precursor cells of yolk sac blood islands at day 7.5 and subsequently in the developing embryonic vasculature.^{14,15} The expression of PECAM by yolk sac precursors suggests that PECAM is involved in the earliest steps of vasculogenesis, as the yolk sac is an early site of both vascular and hematopoietic development in mammals.^{16,17}

Murine embryonic stem (ES) cells are derived from the inner cell mass of blastocysts and are totipotent. ES cells differentiated in culture undergo a programmed differentiation that recapitulates normal developmental events in the yolk sac, including vascular and hematopoietic development.¹⁸⁻²³ The endothelial cells and some hematopoietic cells in ES cell differentiation cultures express PECAM.²⁴⁻²⁶ It was recently reported that undifferentiated ES cells express PECAM.²⁷⁻²⁹ Because ES cells resemble the inner cell mass of the blastocyst, this suggested that PECAM may be expressed embryonically earlier than the described postimplantation stages.

To obtain a better understanding of the role of PECAM in early mouse development, we examined PECAM expression in ES cells during a time course of differentiation and in preimplantation and early postimplantation embryos. To our surprise, PECAM was expressed continuously during ES cell differentiation. *In vivo*, PECAM was expressed by blastocysts and in postimplantation yolk sacs, suggesting that PECAM functions outside the vascular/hematopoietic system.

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Materials and Methods

Cells and Embryos

The murine ES cell line, IE6/2, was maintained in an undifferentiated state and differentiated as described,²⁵ except that in some cases ES cells were plated directly into tissue culture dishes. Briefly, ES cells were expanded undifferentiated in medium containing 5637-cell conditioned medium. For differentiation, cells were cultured for 6 to 7 days to allow the gradual depletion of LIF/DIA from the culture medium. These cultures were then dissociated into clumps by treatment with Dispase (Boehringer Mannheim, Indianapolis, IN) on day 0. The cell clumps were either plated directly onto tissue culture dishes or were incubated in suspension in Petri dishes for 3 days before transfer to tissue culture dishes. The culture medium was DMEM-H, 20% fetal calf serum (lot tested for ability to support differentiation), 150 $\mu\text{mol/L}$ α -thioglycerol, and 50 $\mu\text{g/ml}$ gentamicin. ES cells and differentiation cultures were fixed for 7 minutes with either cold 50% methanol/50% acetone (MA) or fresh 4% paraformaldehyde (PFA) and rinsed in phosphate-buffered saline (PBS) before antibody staining.

Cell suspensions for immunostaining were treated with 0.2% collagenase (type II, Sigma, St. Louis, MO). After enzyme treatment, the cells were triturated and then washed with PBS to remove any remaining collagenase. The cell suspension was then either fixed with 4% PFA, then immunostained or first immunostained, then fixed. Both methods yielded identical staining patterns.

For ES rescue experiments, ES cells were treated with Dispase, then plated at a constant density onto multiple duplicate plates and grown in differentiation conditions. Cultures were dissociated with 0.2% collagenase on days 1 to 8 of differentiation, and a duplicate culture was fixed and immunostained for PECAM. After dissociation, cell counts were determined using a hemocytometer and a fixed number of cells was plated and grown in ES cell culture conditions. The cultures were maintained for 4 days, with daily medium replenishment, then fixed and scored for ES cell colonies. The PECAM staining pattern was visually analyzed.

Mouse embryos were obtained from timed matings, and day 0.5 was noon of the day the plug was observed. Blastocysts were isolated by flushing dissected uteri with PBS on day 3.5 followed by overnight fixation with 4% PFA at 4°C. Postimplantation embryos were dissected from the uterus and immediately fixed in cold 4% PFA. After 24 hours, the embryos were dehydrated through a methanol series and stored at -20°C in 100% methanol.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was prepared from ES cells and differentiation cultures by the method of Chomczynski and Sacchi.³⁰ PolyA⁺ RNA was prepared from approximately 65 blastocysts using guanidinium thiocyanate disruption fol-

lowed by oligo(dT)-cellulose chromatography (Quick Prep Micro, Pharmacia, Piscataway, NJ). Either this polyA⁺ RNA or 5 μg of total RNA from cultured cells was reverse transcribed using random primers and Superscript II (Life Technologies, Gaithersburg, MD), as recommended by the manufacturer. The resulting cDNAs were amplified by the polymerase chain reaction (PCR). Cycling conditions were: 94°C, 45 seconds; 55°C, 1 minute, 15 seconds; 72°C, 2 minutes for 35 cycles, followed by a 15-minute incubation at 72°C. The primers used were PECAM 5.1, nucleotides 1704–1723, 5'-CAAGCGGTCGTGAATGACAC-3'; PECAM 3.1, nucleotides 2278–2298, 5'-CACTGCCTTGACTGTCTTAAG-3'; β -actin 1, nucleotides 950–971, 5'-TACTGCTCTGGCTCCTAGCACC-3'; and β -actin 2, nucleotides 1076–1096, 5'-CCGGACTCATCGTACTCCTGC-3'. Products of PCR reactions were analyzed on 7% polyacrylamide gels.

Immunolocalization

Antibody staining was performed as described.^{24,25} The following antibodies were used, with the fixation methods and working dilutions in parentheses: purified rat anti-mouse PECAM antibody Mec 13.3 (PFA or MA, 0.5 $\mu\text{g/ml}$), purified rat anti-mouse CD34 antibody RAM34 (PFA, 1 $\mu\text{g/ml}$) (PharMingen, San Diego, CA), purified rat anti-mouse PECAM antibody EA-3 (MA, 1.5 $\mu\text{g/ml}$), affinity-purified rabbit anti-mouse PECAM antibody (PFA, 1:1000) (both kind gifts of Beat Imhof), and affinity-purified mouse anti-mouse SSEA-1 antibody MC-480 (PFA, 29 ng/ml) (Developmental Studies Hybridoma Bank, Iowa City, IA). The anti-PECAM antibody Mec 13.3 was also biotinylated for use in double staining (PFA, 0.5 $\mu\text{g/ml}$). After blocking with 5% fetal calf serum for 1 hour, cultures were incubated with primary antibodies at 37°C for 1 hour. Blastocysts were incubated in 5 $\mu\text{g/ml}$ Mec 13.3 or 15 $\mu\text{g/ml}$ EA-3 overnight at 4°C.

After washing in 5% fetal calf serum, cultures were incubated with secondary antibodies for 45 to 60 minutes at 37°C. Detection of rat monoclonal antibodies was with B-phycoerythrin-conjugated goat anti-rat IgG (1:1000), B-phycoerythrin-conjugated donkey anti-rat IgG (1:1000), or FITC-conjugated donkey anti-rat IgG (15 $\mu\text{g/ml}$), SSEA-1 was visualized using B-phycoerythrin-conjugated goat anti-mouse IgM (1:500) (Jackson Immunoresearch, West Grove, PA). Biotinylated Mec 13.3 and the PECAM antiserum were visualized using R-PE-labeled streptavidin (0.5 $\mu\text{g/ml}$) and FITC-conjugated goat anti-rabbit secondary antibody (2 $\mu\text{g/ml}$), respectively (Southern Biotechnology Associates, Inc., Birmingham, AL). Blastocysts were incubated with a 1:100 dilution of the B-phycoerythrin-conjugated goat anti-mouse secondary antibody overnight at 4°C. As controls, cultures and embryos were also incubated with secondary antibody alone and with an isotype-matched rat anti-mouse IgE FcR antibody (B3B4, PharMingen) detected with an anti-rat secondary antibody. Purified mouse IgM, κ subtype, was used in conjunction with the anti-mouse IgM secondary antibody as a control for SSEA-1 staining. Staining

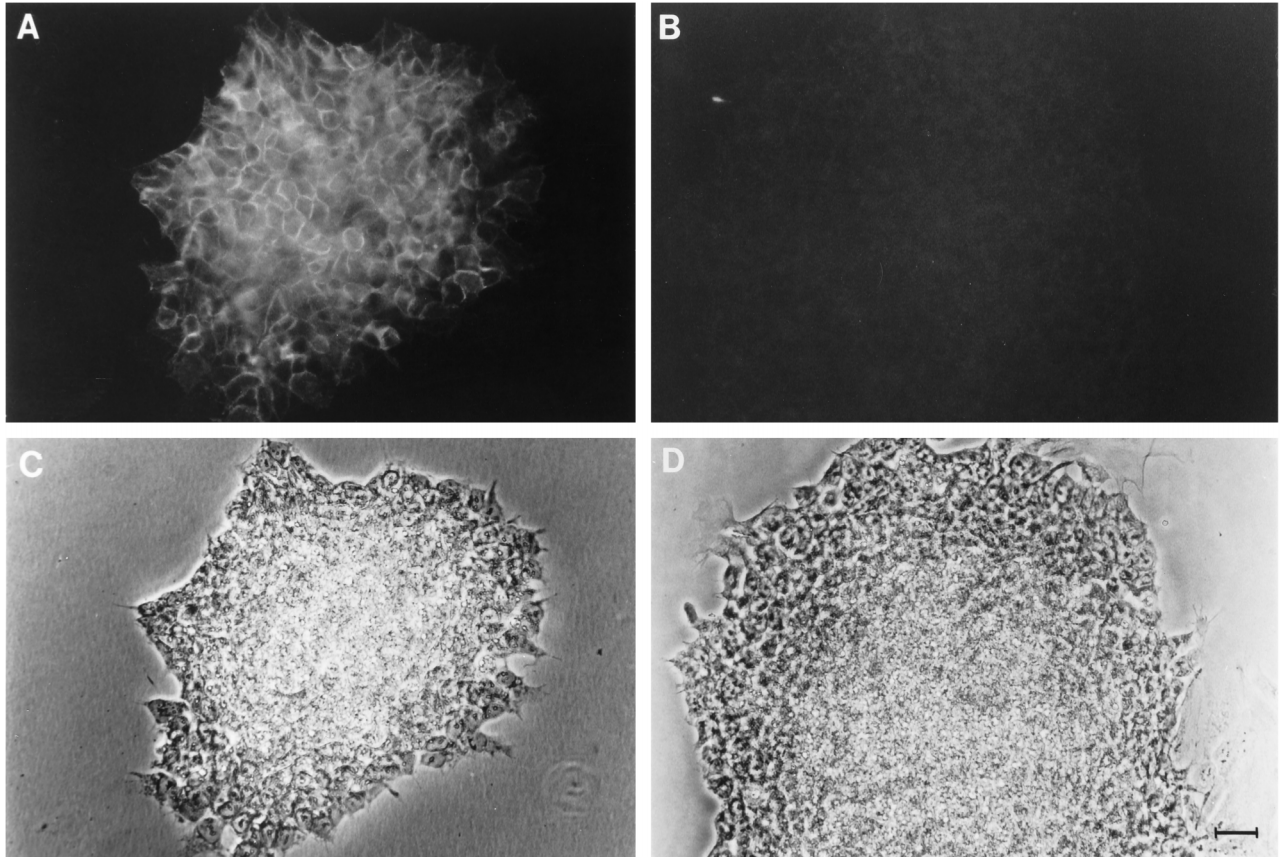


Figure 1. Immunofluorescence of ES cell clumps with an anti-PECAM antibody. ES cells were incubated with the anti-PECAM antibody Mec 13.3 (A and C) or an isotype-matched anti-IgE FcR control antibody (B and D), followed by detection with a B-phycoerythrin-conjugated secondary antibody. Fluorescent (A, B) and phase-contrast (C, D) images are shown. Scale bar, 30 μm .

was visualized with an Olympus IX50 microscope equipped with phase contrast and epifluorescence optics and an Olympus PM-30 camera.

Postimplantation embryos were stained as described by Kispert and Herrmann³¹ with some modifications. Most incubations were performed using embryos placed in nets (Costar 3477) that fit into the wells of 12-well dishes along with 2 ml of each reagent. All incubations were at room temperature on a rocking platform unless otherwise noted. PFA-fixed embryos that were stored in 100% methanol at -20°C were rehydrated in PBS for 30 minutes. Free aldehyde groups were blocked in 1 mol/L glycine, pH 7–8, for 30 minutes, and embryos were then washed in PBS for 30 minutes. Embryos were next incubated in 6% H_2O_2 in PBS for 25 minutes to block endogenous peroxidases, and washed in three changes of PBT (PBS plus 0.1% Tween 20) for 5 minutes each. Embryos were incubated in 10 $\mu\text{g}/\text{ml}$ proteinase K in PBS for 3 to 5 minutes (depending on size), and washed in 2 mg/ml glycine. Embryos were washed in three changes of PBT for 5 minutes each, then in PBTN (PBT plus 5% heat-inactivated fetal calf serum) for 30 minutes. Primary antibody was rat anti-mouse PECAM (Mec 13.3, Pharmingen) diluted to 1:200 in PBTN, and embryos were incubated overnight at 4°C with rocking. The next day, embryos were washed in PBT three times for 5 minutes each, then three times for 30 minutes each, then washed

in PBTN for 30 minutes. Secondary antibody was goat anti-rat horseradish peroxidase (Accurate, IgG (H+L)), diluted to 1:200 in PBTN, and embryos were incubated overnight at 4°C with rocking. The next day embryos were washed in PBT three times for 5 minutes each, four times for 25 minutes each, then transferred to a glass watch dish. Embryos were incubated in a mixture of 0.3 mg/ml 3,3'-diaminobenzidine and 3 mg/ml NiSO_4 in PBT in the dark for 10 minutes, then incubated with the same mixture plus H_2O_2 at 1:1000 dilution. After development (3 minutes for smaller embryos, 5 minutes for larger embryos), the reaction was stopped by washing in PBS 3 times for 5 minutes each. Embryos were photographed using a Nikon SMZ-U stereomicroscope. Embryos were embedded in paraffin, and 8- μm sections were cut and mounted using DPX mounting medium (Fluka). Sections were photographed using a Nikon Optiphot-2 microscope outfitted with DIC optics.

Results

PECAM Expression in ES Cells

To verify that ES cells expressed PECAM,^{27–29} ES cells were fixed either 3 or 6 days after plating and stained with an anti-PECAM antibody. As expected, a majority of the

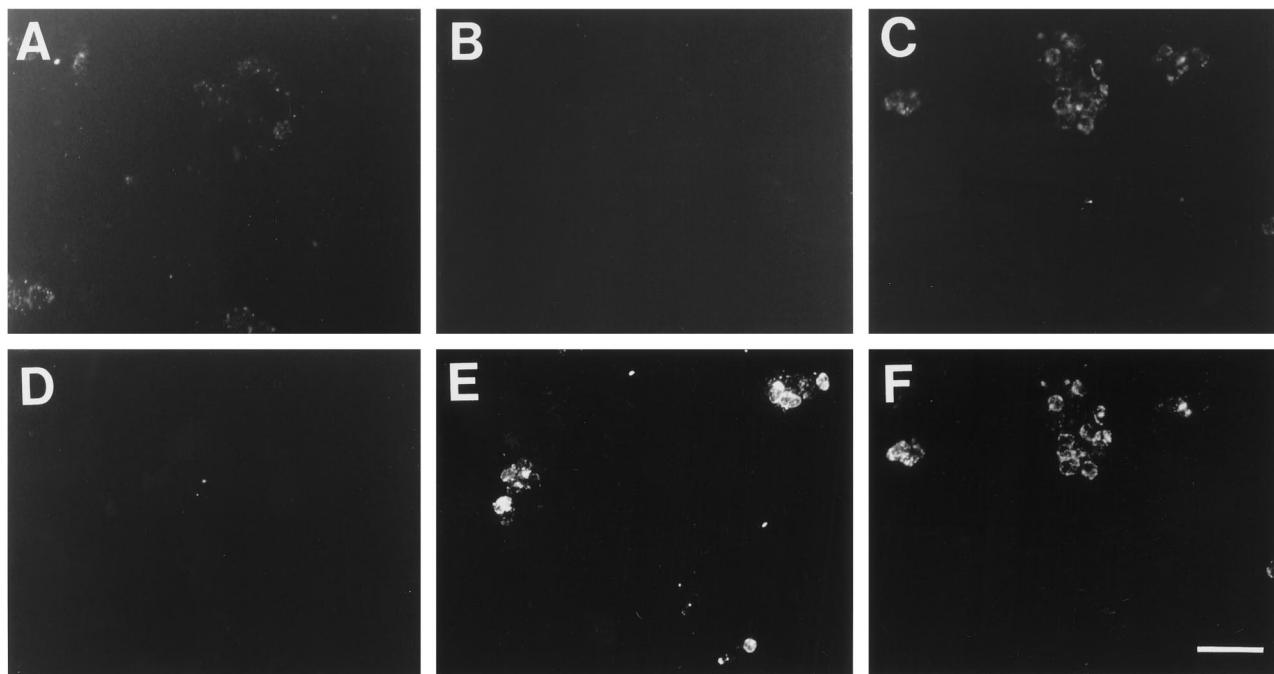


Figure 2. Immunofluorescence of dissociated ES cells. ES cell cultures were treated with collagenase and then incubated with the following antibodies or sets of antibodies: (A, D), anti-PECAM antiserum and mouse IgM control; (B, E), mouse anti-SSEA-1; (C, F), anti-PECAM antiserum and mouse anti-SSEA-1. Secondary antibodies were goat anti-rabbit FITC (green) and donkey anti-mouse B-phycoerythrin (red). A–C: FITC fluorescence; D–F: B-phycoerythrin fluorescence. Scale bar, 60 μ m.

ES cells expressed PECAM, and expression was localized to cell-cell boundaries (Figure 1). To further characterize the stained ES cells, cultures were enzymatically dissociated and double stained for PECAM and SSEA-1 (Figure 2), since SSEA-1 has been used as a marker of undifferentiated and partially differentiated embryonic cells.^{32–34} The majority of the cells stained for both antibodies (Figure 2, C and F), although rare cells stained for either PECAM or SSEA-1. This finding shows that the PECAM⁺ cells in ES cell cultures also express SSEA-1, which suggests that the cells are either undifferentiated ES cells or partially differentiated ES cell derivatives.

PECAM Expression during ES Cell Differentiation

To determine the relationship between the PECAM expression seen on ES cells and in vascular endothelium, RNA analysis was done on a time course of ES cell differentiation. Reverse transcription-PCR of RNA from both ES cells and differentiation cultures from days 1 to 7 showed that a band of the predicted size was amplified with PECAM primers from all of the cultures (Figure 3). This finding suggested that PECAM was expressed throughout the differentiation time course.

Immunofluorescence analysis was used to localize PECAM expression during ES cell differentiation (Figures 4 and 6B). PECAM was expressed throughout the time course. The widespread staining pattern seen in ES cells

was restricted in day 2 cultures, and large areas of unstained cells were interspersed between small clumps of PECAM⁺ cells, in addition to ES-like balls of PECAM⁺ cells (Figure 4A and data not shown). On day 4 of differentiation the pattern was similar to day 2, except that the

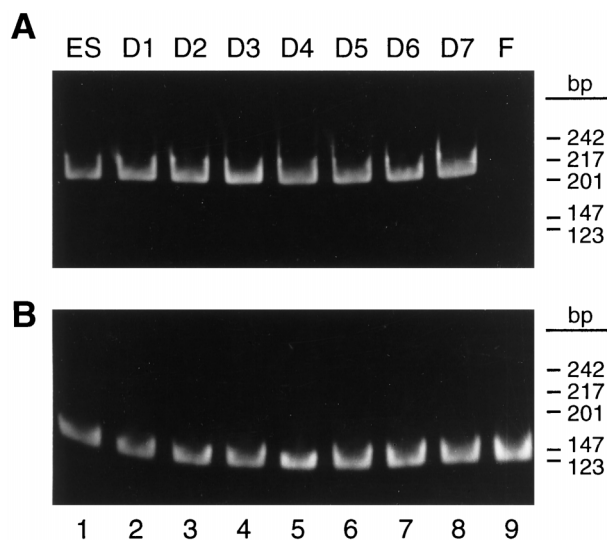


Figure 3. PECAM RNA analysis of ES cell differentiation. Total RNA was reverse transcribed using random primers and then amplified using either PECAM (A) or β -actin (B) primers. Lane 1, ES cells; lanes 2–8, days 1–7 of differentiation; lane 9, 3T3 fibroblasts. The predicted sizes of the PECAM and β -actin products are 215 and 147 bp, respectively. The positions of molecular weight standards are indicated to the right of each gel.

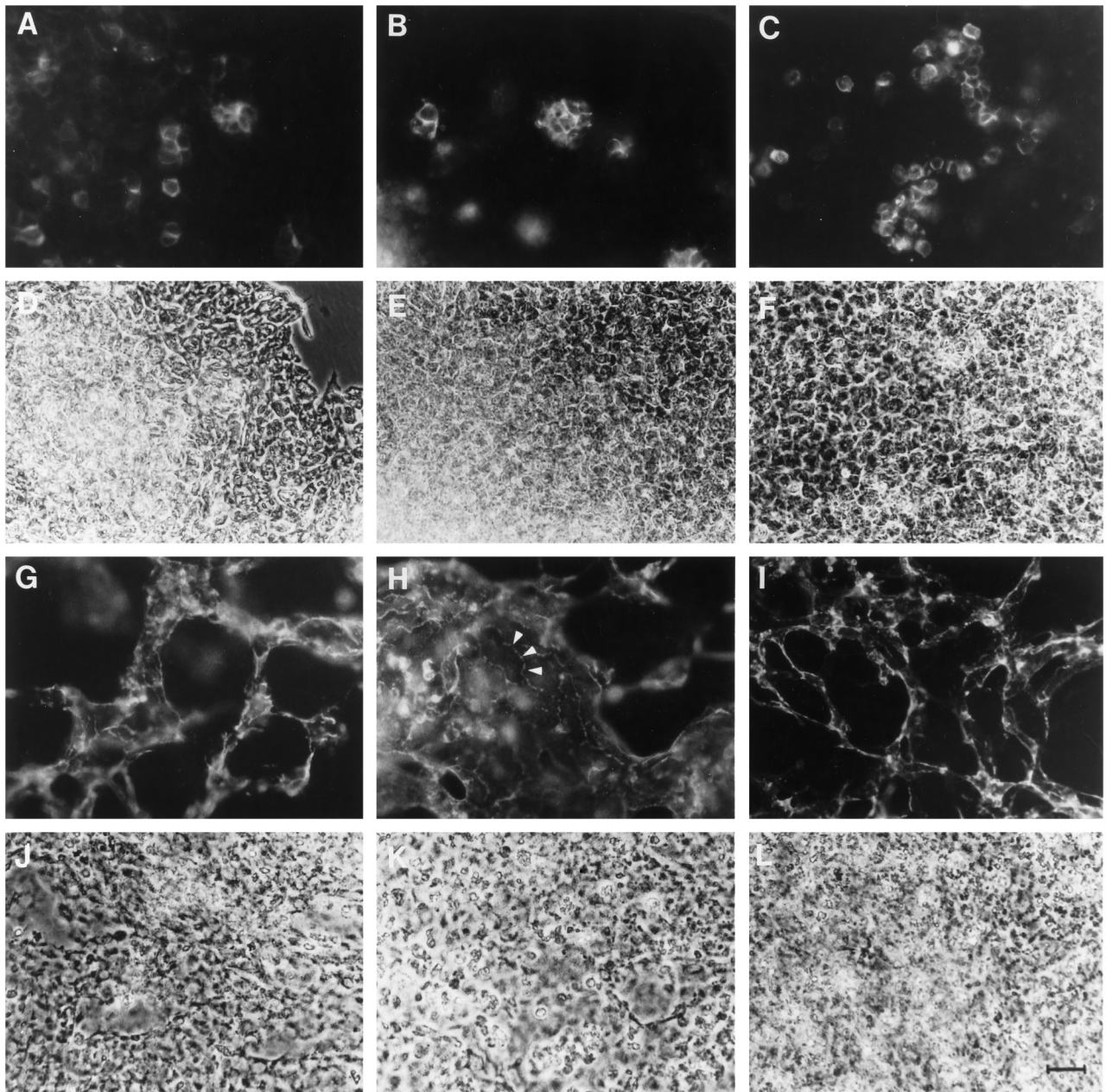


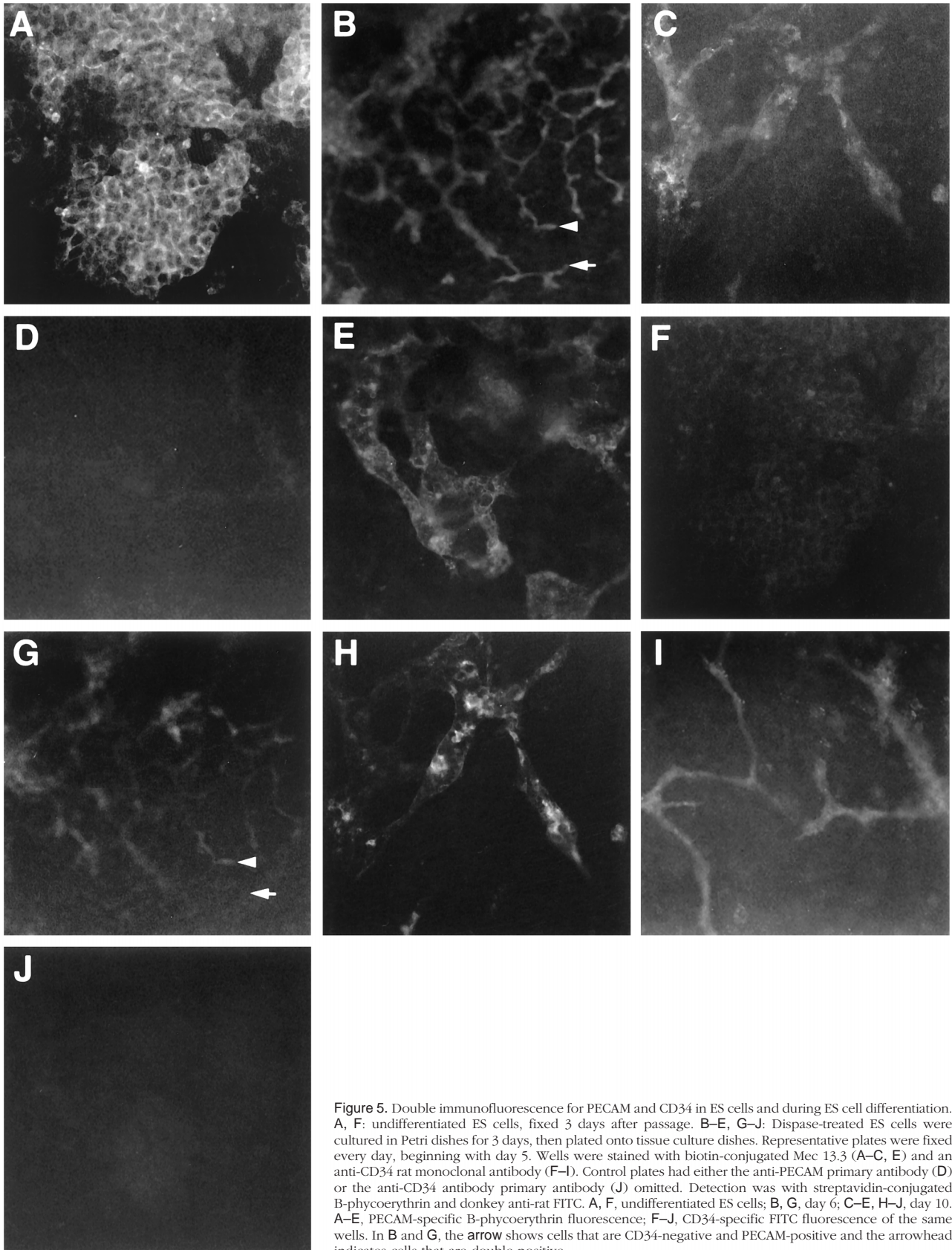
Figure 4. PECAM immunofluorescence of ES cell differentiation. Dispase-treated ES cells were plated onto tissue culture dishes on day 0. Representative plates were fixed every other day. Wells were stained with an anti-PECAM antibody and a B-phycoerythrin-conjugated secondary antibody. A, D: day 2; B, E: day 4; C, F: day 6; G, J: day 8; H, K: day 10; I, L: day 12. A–C, G–I: fluorescence photomicrograph; D–F, J–L: the corresponding phase contrast photomicrograph. Arrowheads in H point to PECAM stain at cell borders. Scale bar, 30 μm .

ES-like PECAM⁺ balls decreased and the clumps of PECAM⁺ cells became more well defined (Figures 4B and 6B). On day 6, cords of PECAM⁺ cells were evident that were morphologically distinct from PECAM⁺ blood vessels that are present at later days (compare Figures 4C and 4G). On day 8 the PECAM⁺ cells were organized into blood vessels and small lacunae began to form (Figures 4G and 6B). By day 10 the blood vessels increased in size and formed blood islands in some areas, although areas of vascular plexus were also evident (Figure 4H). The blood islands and vascular plexus persisted through

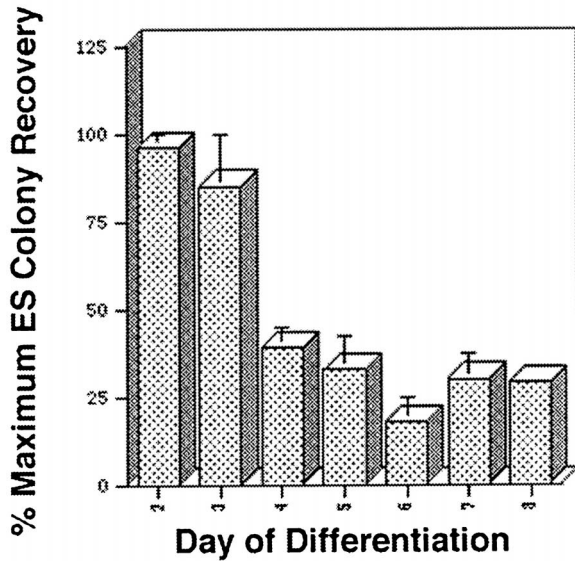
day 12 (Figure 4I), then became less well defined with further time in culture (data not shown). In all cases, the PECAM staining was localized to cell-cell boundaries.

CD34 Is Expressed Subsequent to PECAM during ES Cell Differentiation

Because the PECAM staining seen on days 0–6 of the time course was not associated with patent blood ves-



A. ES Colony Recovery



B. PECAM Staining Pattern

Day	2	3	4	5	6	7	8
Balls	++++	+++	++	++	+/-	-	-
Clumps	+	++	++	++	++	+	+
Vessels	-	-	-	-	+/-	++	+++

Figure 6. A time course of ES colony recovery and PECAM immunostaining. ES cells were treated with Dispase and cultured in differentiation medium. Each day 2 sets of dishes were either fixed and stained for PECAM or treated with collagenase and replated for ES colony formation. **A:** ES colonies were counted 4 days after plating either 200,000 or 500,000 cells treated with collagenase and their number calculated as a percent of maximum colony number. **B:** Parallel cultures were visually examined and the relative amount of PECAM staining in three patterns was assessed: balls, tight clumps of cells with a dense staining pattern, similar to Figure 1A (ES-like); clumps, small to medium aggregations of cells with distinct cell border staining, similar to Figure 4, A-C; vessels, cells arranged with continuous staining at cell-cell borders, similar to Figure 4, G-I. The area covered by each pattern of staining was estimated as follows: ±, trace; +, 2-5%; ++, 5-10%; +++, 10-30%; +++++, >30%.

sels, a second vascular marker was used in conjunction with PECAM to determine the expression profile of non-vessel PECAM⁺ cells. CD34 is a surface marker of vascular endothelium that is expressed during embryonic development.³⁵ Unlike PECAM, CD34 is not expressed on undifferentiated ES cells (Figure 5F). Both PECAM and CD34 are expressed in the vessels of a differentiated culture at day 10. (Figure 5, C and H). In contrast, at day 6 of differentiation, some of the newly formed vessels are stained for both PECAM and CD34 (arrowhead in Figure 5, B and G), while the tips of other vessels are PECAM-positive but CD34-negative (arrow in Figure 5, B and G).

On day 10, a few round cells within the blood islands (Figure 5, C and H) and a smaller number of round cells adjacent to the blood islands (data not shown) are CD34/PECAM double positive. Because CD34 also stains a

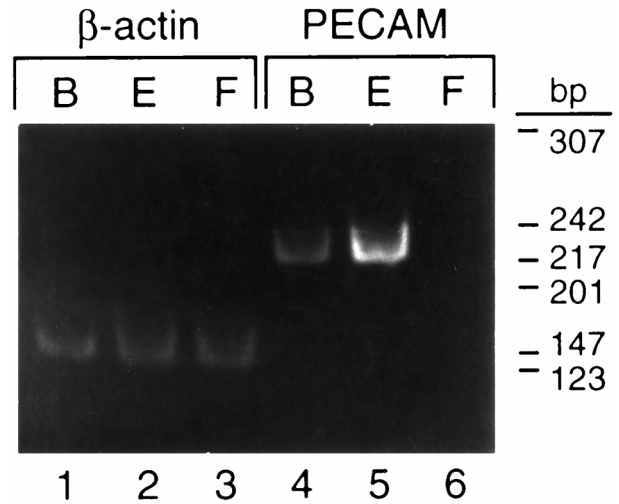


Figure 7. PECAM RNA analysis of mouse blastocysts. Poly A⁺ RNA (blastocysts) or total RNA (cell lines) was reverse transcribed using random primers, then amplified using either β -actin (lanes 1-3) or PECAM (lanes 4-6) primers. Lanes 1, 4: blastocysts; lanes 2, 5: Py-4-1 endothelial cells; lanes 3, 6: 3T3 fibroblasts. The predicted sizes of the PECAM and β -actin products are 215 and 147 bp, respectively. The positions of molecular weight standards are indicated in the right margin.

subset of hematopoietic cells within the developing yolk sac *in vivo*,³⁶ these CD34⁺ cells are most likely hematopoietic cells.

ES Colony Recovery from Differentiating Cultures

Although the PECAM⁺ cells seen in clumps or cords on day 6 of differentiation are distinguished from mature blood island endothelial cells by lack of expression of CD34, they are not distinguished from ES cells by the markers used in our study. To further delineate the relationship between the PECAM⁺ clumps of cells and ES colony-forming potential, a time course of differentiation was analyzed for both PECAM staining pattern and ES colony recovery (Figure 6). As expected, the number of ES colonies recovered from plating differentiation culture cells in ES culture conditions decreased with differentiation time (Figure 6A). A significant drop in the percentage was seen between days 3 and 4, and from day 4 to day 8 the percentage of ES colonies recovered remained fairly constant. In contrast, the PECAM⁺ clumps were first evident on day 2 of duplicate culture plates, and their presence remained fairly constant through day 6 (Figure 6B), although they assumed more "vessel-like" arrangements with time (compare Figures 4A and 4G). Significantly, the appearance of vessels between days 6 and 7 was accompanied by a decrease in the relative area covered by the PECAM⁺ clumps, suggesting a possible precursor relationship between the PECAM⁺ clumps and blood islands. Moreover, the functional assay for ES colony forming potential has a different temporal pattern than the clumps of PECAM⁺ cells, suggesting that the PECAM⁺ cells are not ES cells.

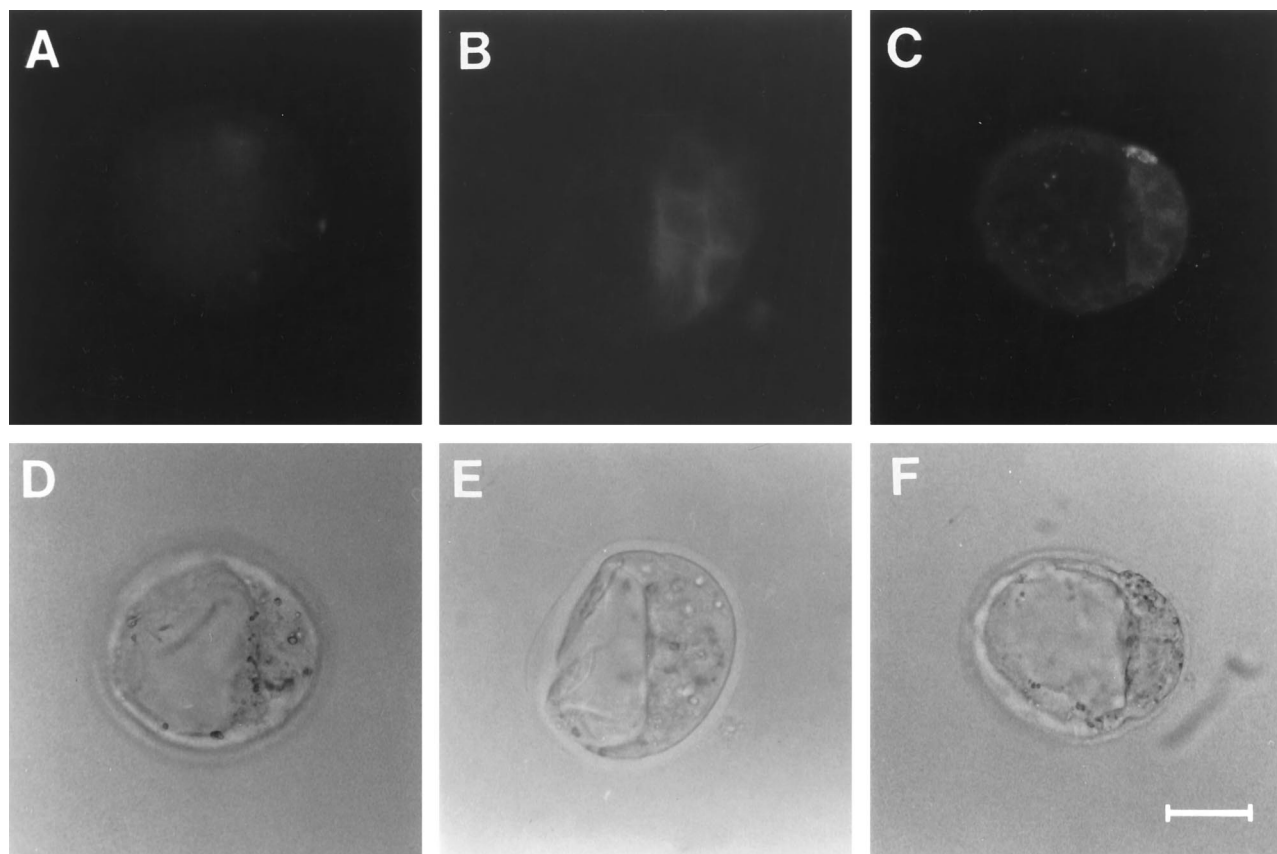


Figure 8. PECAM immunofluorescence of mouse blastocysts. Fixed blastocysts were stained with a control antibody directed against IgE FcR (A, D) or two different anti-PECAM antibodies, Mec13.3 (B, E) or EA-3 (C, F), followed by detection with a goat anti-rat B-phycoerythrin secondary antibody. A–C: fluorescent photomicrographs; D–F: the corresponding phase-contrast micrographs. Scale bar, 25 μ m.

PECAM Expression in Mouse Blastocysts

To determine whether the PECAM expression seen in ES cell cultures recapitulates expression during embryonic development, RNA from blastocysts was analyzed by reverse transcription-PCR for PECAM expression (Figure 7). PolyA⁺ RNA from a pool of blastocysts produced a band of the appropriate size when amplified with PECAM primers. Individual blastocysts were then analyzed by immunofluorescence to localize PECAM expression (Figure 8). Two different PECAM antibodies showed similar patterns, with staining localized to cell-cell junctions. The patterns suggested that the inner cell mass, from which ES cells are derived, may selectively express PECAM protein, but the background of trophoblast cell reactivity made definitive localization difficult.

PECAM Expression in Postimplantation Embryos

In a previous study PECAM expression was detected in angioblasts on day 7.5 in the developing yolk sac.¹⁴ Because PECAM is expressed before overt vascularization during ES cell differentiation, we examined embryos from early postimplantation stages to confirm that PECAM was expressed before the development of mature endothelial cells. Both whole mount *in situ* hybridiza-

tion with a PECAM probe and immunofluorescence of sectioned embryos with an anti-PECAM antibody failed to detect PECAM expression in egg cylinder to early primitive streak stage embryos (data not shown). Both PECAM RNA and PECAM protein were detected in the developing yolk sac of mid-streak stage embryos (Figure 9 and data not shown). Sectioning of the antibody-stained embryos showed that PECAM was expressed in clumps of mesodermal cells before the detection of mature endothelial cells (Figure 9, B–D). The PECAM⁺ cells in the yolk sac *in vivo* resemble in morphology and temporal sequence the PECAM⁺ cell clumps seen during ES cell differentiation, suggesting that an *in vivo* counterpart of the PECAM⁺ clumps of cells does exist.

Discussion

The lack of definition regarding pre-endothelial stages of mouse vascular development, coupled with lack of knowledge of the precise expression pattern of the vascular cell adhesion molecule PECAM, led us to examine PECAM expression during ES cell differentiation and in early mouse embryos. We confirmed that undifferentiated ES cells express PECAM, and we showed for the first time that this early expression mirrors PECAM expression in the mouse blastocyst. PECAM is expressed continuously

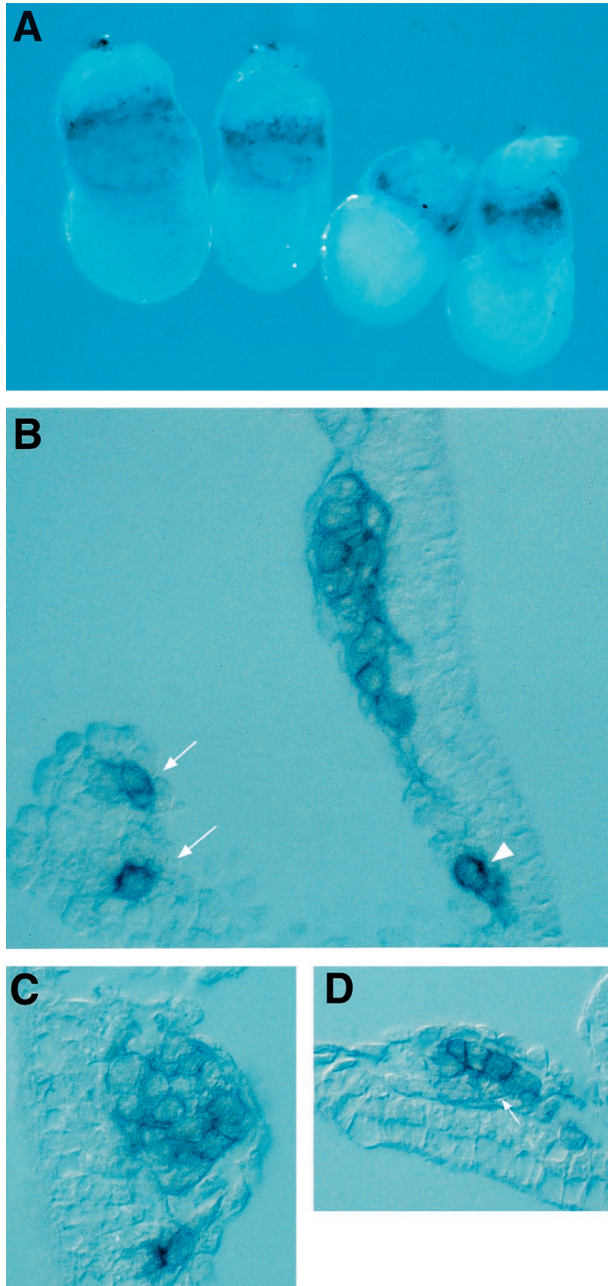


Figure 9. Whole-mount PECAM antibody staining of postimplantation embryos. E 7.5 embryos (mid-streak to early head fold stage) were subjected to whole-mount antibody staining with Mec 13.3 rat anti-mouse PECAM antibody (A). Embryos were then embedded in paraffin and sectioned (B–D). B: cross-section through the yolk sac and allantois of a mid-streak embryo. PECAM staining is seen at the cell borders of a clump of mesodermal cells that, by position, will form a blood island. Additional PECAM reactivity is seen in a small clump closer to the embryonic/extraembryonic border (arrowhead) and in a subset of cells within the allantois (arrows) that are likely to be endothelial precursors. C: another clump of PECAM⁺ yolk sac mesodermal cells from the same embryo. D: a clump of mesodermal yolk sac cells from a slightly older embryo, with a mixture of PECAM⁺ and PECAM⁻ cells (arrow).

during ES cell differentiation and characterizes a population of cells that are PECAM⁺ but not part of patent blood vessels. These cells have a possible counterpart *in vivo* in the yolk sac mesodermal cells that express PECAM before blood island formation. These findings are

consistent with the model that ES cell differentiation recapitulates yolk sac development.^{18,22}

Undifferentiated ES cells express PECAM,^{27–29} and we confirmed this finding using double immunofluorescence analysis with PECAM and SSEA-1, a marker of undifferentiated and partially differentiated embryonic cells.^{32–34} PECAM expression in ES cells is not an artifact of tissue culture, since cells of the mouse blastocyst also express PECAM. This early expression of the PECAM gene during development is also suggested by the report of a PECAM promoter-driven *cre* transgene.³⁷ The ubiquitous excision of the loxP-flanked VCAM-1 target allele indicates that the PECAM gene is expressed early in development, consistent with the blastocyst expression that we have observed. Moreover, PECAM is localized to cell-cell borders in ES cells and blastocysts, suggesting that it may play a role in cell adhesion or transmembrane signaling at this early stage.^{38–42} However, it is unlikely that PECAM is involved in intercellular migration at this early stage of development, since no cellular migration into the blastocoele cavity is known to occur at this time.

To our surprise, PECAM was expressed continuously throughout ES cell differentiation. Although many vascular markers are also expressed in other lineages, continuous expression of a single marker from uncommitted cells through differentiated endothelium has not been observed. This result also differs from the findings of Vittet et al,²⁷ who describe a PECAM-negative stage as ES cells begin to differentiate. However, Ling and Neben²⁹ documented expression of PECAM (named ER-MP12) throughout ES cell differentiation using FACS analysis. In our study using immunofluorescence, PECAM was initially expressed in all or most ES cells, but after several days of differentiation most cells did not express PECAM. The remaining PECAM⁺ cells were found in small clumps that were morphologically distinct from the blood islands that form at later times.

The expression of other vascular markers also distinguishes early PECAM⁺ cells from later PECAM⁺ endothelial cells. The early PECAM⁺ cells do not express CD34, which is expressed by all blood island endothelial cells and a subset of hematopoietic cells (Ref. 36; this report). The early PECAM⁺ cells also do not express ICAM-2, which is expressed by all patent vessels and blood islands that are PECAM⁺ at later stages (M. Harmaty, M. Inamdar, and V. L. Bautch, unpublished observations). Preliminary results suggest that many of the PECAM⁺ cells found at early stages of ES cell differentiation express SSEA-1 (S. Redick and V. L. Bautch, unpublished observations), a marker of undifferentiated and partially differentiated embryonic cells.^{33,43} SSEA-1 is not expressed by PECAM⁺ endothelial cells in blood islands. Thus the expression of PECAM during ES cell differentiation may characterize an endothelial precursor that subsequently matures to a blood island endothelial cell that expresses CD34 and ICAM-2 in addition to PECAM. This model is supported by time course analysis of ES colony recovery and PECAM staining that shows distinct temporal patterns for PECAM⁺ clumps and ES colony potential, suggesting that at least a subset of PECAM⁺ cells present before vessel formation are not ES cells. A pre-

cursor relationship is also suggested by a decrease in PECAM⁺ cell clumps that accompanies vessel formation during ES cell differentiation. Likewise, analysis of mutant ES cells that do not express VEGF shows that relatively large numbers of the early PECAM⁺ cells accumulate, concomitant with severely reduced numbers of blood islands (V. L. Bautch, S. Redick, A. Scalia, P. Carmeliet, and R. Rapoport, manuscript in preparation).

The *in vivo* analysis was consistent with reported analyses^{14,15} that first detected PECAM expressing cells in the developing yolk sac of day 7.0 to 7.5 embryos, at or just before blood island formation. Our detailed analysis confirms that clumps of yolk sac mesodermal cells express PECAM before the development of morphologically distinct endothelial and hematopoietic cells. This finding suggests that yolk sac vascular/hematopoietic precursors express PECAM. Some PECAM⁺ cells are also found in the developing allantois, which is consistent with a recent report that the murine allantois has high vascular potential.⁴⁴ Postimplantation embryonic PECAM expression is first seen slightly later in the lateral mesoderm where the dorsal aorta will form (Refs. 14, 15; V. L. Bautch, unpublished observations). Taken together, these expression patterns suggest that PECAM expression after the blastocyst stage is confined to cells and/or precursors of the vascular/hematopoietic lineages. Moreover, if a common precursor of both lineages called the hemangioblast exists, it is likely to express PECAM.

Because the PECAM⁺ nonvascular cells found just before blood island formation in ES cell differentiation are similar in morphology and temporal appearance to yolk sac blood island precursor cells, it is possible that they are the *in vitro* counterpart of the yolk sac precursor cells. Several recent reports provide evidence that differentiating ES cells at early stages provide an environment conducive to vascular/hematopoietic development. Blast colonies that apparently form from single vascular/hematopoietic precursor cells peak early during *in vitro* differentiation of ES cells.⁴⁵ Up to half of the cells in early embryoid bodies are positive for flk-1, a marker of early vascular and hematopoietic cells.⁴⁶ Differentiation of ES cells mutant for flk-1 revealed an ability to form primitive erythroid cells that was not seen when the same cells were analyzed in chimeras.⁴⁷ One explanation of the data were that ES cell differentiation provided yolk sac-derived signals to mesodermal cells without a concomitant requirement for migration, and these signals promoted hematopoietic differentiation. Thus ES cell differentiation appears to recapitulate the early yolk sac environment and early vascular development. Fortunately, the accessibility of the PECAM⁺ ES-derived cells should allow further characterization of mammalian vascular development with lineage markers as well as functional studies of purified cells.

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