

## CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo

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Accepted 28 May 2003

### SUMMARY

The platelet glycoprotein IIb ( $\alpha_{IIb}$ ; CD41) constitutes the alpha subunit of a highly expressed platelet surface integrin protein. We demonstrate that CD41 serves as the earliest marker of primitive erythroid progenitor cells in the embryonic day 7 (E7.0) yolk sac and high-level expression identifies essentially all E8.25 yolk sac definitive hematopoietic progenitors. Some definitive hematopoietic progenitor cells in the fetal liver and bone marrow also express CD41. Hematopoietic stem cell competitive repopulating ability is present in CD41<sup>dim</sup> and CD41<sup>lo/-</sup> cells isolated from bone marrow and

fetal liver cells, however, activity is enriched in the CD41<sup>lo/-</sup> cells. CD41<sup>bright</sup> yolk sac definitive progenitor cells co-express CD61 and bind fibrinogen, demonstrating receptor function. Thus, CD41 expression marks the onset of primitive and definitive hematopoiesis in the murine embryo and persists as a marker of some stem and progenitor cell populations in the fetal liver and adult marrow, suggesting novel roles for this integrin.

Key words: Hematopoiesis, Embryonic yolk sac, CD41, Mouse

### INTRODUCTION

Blood cells first appear in the murine embryo in the extra-embryonic yolk sac on embryonic day 7 (E7.0) with only limiting numbers of primitive erythroid progenitor cells (Ery<sup>P</sup>) detectable (Palis et al., 1999; Wong et al., 1986). Over the next 24 hours, significant expansion in the number of Ery<sup>P</sup> cells occurs in the yolk sac, and mature erythroblasts expressing embryonic hemoglobin ( $\beta H1$ ) appear in the developing blood islands. While primitive erythropoiesis constitutes essentially all primitive hematopoiesis in the early yolk sac (E7.0-8.0), other hematopoietic progenitors are also produced including limiting numbers of macrophage and megakaryocyte progenitors. Definitive hematopoietic progenitor cells (progenitors that display the same culture colony morphology, give rise to the same lineages, and express similar gene products to myeloerythroid progenitors isolated from the fetal liver and bone marrow) including high proliferative potential colony forming cells, can be cultured from the yolk sac at E8.25 (Palis et al., 2001). These progenitor cells and progenitors arising in the para-aortic splanchnopleura (P-Sp) region begin to circulate following the onset of cardiovascular function and migrate to the developing liver by E10. The liver serves as the predominant site of hematopoiesis until just before birth when the spleen and bone marrow compartments become seeded with circulating stem cells. While some of the transcriptional regulation of hematopoietic lineage commitment has been delineated

(Shivdasani and Orkin, 1996), most early markers of the hematopoietic lineage are co-expressed by the intimately associated angioblasts (endothelial precursors) and thus specific identification of the hematopoietic precursors has been elusive (Drake and Fleming, 2000).

The platelet glycoprotein receptor IIb/IIIa ( $\alpha_{IIb}\beta_3$ ; CD41/CD61) is required for normal platelet hemostatic function (Phillips et al., 1988). CD41 is a protein composed of two subunits that interact with CD61 in the presence of calcium to form a functional adhesive protein receptor. Damage to blood vessels results in the release of a variety of intracellular mediators as well as initiation of the hemostatic cascade that in combination activate CD41/CD61 to bind to a variety of proteins including fibrinogen, fibronectin, von Willebrand factor and vitronectin (Shattil et al., 1998; Coller, 1990). Heritable mutations in either CD41 or CD61 subunits results in a bleeding disorder in human patients called Glanzmann's thrombasthenia (Bellucci and Caen, 2002).

CD41/CD61 was once considered to be a platelet specific-receptor complex and has long been used as a phenotypic marker for the megakaryocyte-platelet lineage (Phillips et al., 1988). However, some published information suggests that CD41 expression may not be limited to the platelet lineage (Berridge et al., 1985; Fraser et al., 1986; Tronik-Le Roux et al., 2000; Tropel et al., 1997). CD41 expression was identified on a subpopulation of CD34<sup>+</sup>CD41<sup>+</sup>CD42<sup>-</sup> human cord blood cells that possessed colony forming cells (CFC) and lymphoid

and myeloid repopulating ability (Debili et al., 2001). In contrast, CD34<sup>+</sup>CD41<sup>+</sup>CD42<sup>-</sup> adult mobilized peripheral blood cells were enriched in megakaryocyte and erythroid CFC activity but lacked lymphoid repopulating ability. In murine studies, CD41 co-expression with Kit identified cells in late fetal liver and adult marrow that possessed a variety of CFC activities and gave rise to T lymphocytes in thymic organ cultures *in vitro* (Corbel and Salaun, 2002). Transplantation of adult marrow Kit<sup>+</sup>CD41<sup>+</sup> cells suggested that the sorted cells gave rise to short-term but not long-term hematopoietic repopulation (Corbel and Salaun, 2002). Other studies in fetal and adult mice suggested that CD41 was expressed on most yolk sac progenitor cells at 9.5 days post coitus (dpc) and 10.5 dpc aorta-gonad-mesonephros (AGM) hematopoietic progenitor cells but on few fetal liver or adult marrow progenitors (Mitjavila-Garcia et al., 2002). In addition, CD41 was expressed on most hematopoietic progenitor cells derived *in vitro* from murine embryonic stem (ES) cells (Mitjavila-Garcia et al., 2002). Finally, recent data suggest that CD41 expression may serve as a marker for the onset of definitive hematopoiesis in the murine embryo and in ES cell-derived hematopoietic cells *in vitro* (Mikkola et al., 2002).

In the present work, we have identified CD41 expression on yolk sac cells coincident with the first appearance of primitive erythroid progenitor cells (E7.0) and on all yolk sac definitive hematopoietic progenitor cells (E8.25). Fetal liver and adult marrow hematopoietic progenitor cells were found in both CD41<sup>dim</sup> and CD41<sup>lo/-</sup> populations and both populations demonstrated long-term repopulating ability in multiple lineages upon transplantation, however, repopulating ability was enriched in the CD41<sup>lo/-</sup> cells. These results demonstrate that CD41 is expressed on the first hematopoietic progenitor cells of the primitive and definitive lineages in the murine yolk sac. CD41 expression persists in some hematopoietic stem and progenitor cells in the fetal liver and adult marrow. These data provide new insight into the expression of a molecule previously thought to be lineage restricted and suggest novel roles for the CD41/61 receptor complex.

## MATERIALS AND METHODS

### Maintenance of mouse colonies

C57BL/6J recipient mice were purchased from Jackson Laboratory (Bar Harbor, ME). B.6Gpi-1a/BoyJ donor mice were obtained by breeding B.6Gpi-1a (gift of Dr David Harrison, Jackson Labs, Bar Harbor, ME) with B.6SJL-Pep3B/BoyJ (purchased from Jackson Labs). The Indiana University School of Medicine Institutional Animal Care and Use Committee approved the care and use of all animals in this study.

### Hematopoietic cell isolation

#### Yolk sac

Whole embryo or yolk sac cells were dissected from the decidual tissue of timed-pregnant dams (E6.5-9.5 gestation embryos) and extensively washed in phosphate-buffered saline (PBS; Invitrogen, Grand Island, NY) as described (Yoder and Hiatt, 1997). Presomitic embryos were staged by the presence of tissue landmarks and later stages by somite counting (Downs and Davies, 1993). Dissected yolk sacs were incubated for 60-90 minutes at 37°C in 0.1% collagenase/dispase (Sigma, St. Louis, MO) with 20% fetal bovine serum (FBS, HyClone, Logan, UT) in PBS.

#### Fetal liver

Fetal liver cells were collected from E12.5 embryos. The collected tissue was mechanically dissociated into a single cell suspension using a 5 ml pipette. The cells were washed in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal bovine serum (FBS), 2% penicillin/streptomycin (P/S), 2 mM L-glutamine, and filtered through a 70 µm cell strainer.

#### Bone marrow

Bone marrow was collected from normal C57BL6/J mice and the low-density mononuclear (LDM) cells isolated by density centrifugation as previously described (Yoder et al., 1993).

### Lineage depletion of fetal liver and adult bone marrow cells

Removal of lineage antigen-expressing cells from fetal liver cells was achieved as follows. 0.5-1 µg/ml of rat anti-mouse Gr-1 (granulocytes), B220 (B lymphocytes), TER-119 (erythroid cells), and CD4/CD8 (T lymphocytes) monoclonal antibodies (PharMingen, San Diego, CA) were added to the fetal liver cell suspension for a 20 minute incubation on ice. The stained cell populations were pelleted (300 g for 8 minutes), washed with buffer, repelleted and resuspended in buffer. Goat anti-rat IgG magnetic microbeads (Miltenyi Biotec, Auburn, CA) were added for a 20 minute incubation on ice, and the lineage-depleted cell population was obtained using a magnetic separation device as directed by the manufacturer (Miltenyi Biotec). This procedure was repeated for the adult bone marrow cells, with the exception that purified and fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Mac1 was also used. Lineage-depleted populations of cells were restained with FITC conjugates of the same antibodies and analyzed for purity using a FACS Advantage instrument (Becton Dickinson).

### Fluorescence activated cell sorting

Cells to be stained were incubated with the primary antibodies of interest for 20 minutes on ice, pelleted at 300 g, and resuspended with streptavidin-conjugated allophycocyanin (APC), if necessary. Labeled cells were washed twice, pelleted at 300 g, and resuspended in IMDM with 10% FBS, 1% P/S, and 2 mM L-glutamine. Cell sorting was accomplished using a FACStar instrument (Becton Dickinson). Rat monoclonal antibodies used in this study (all purchased from Pharmingen) included: FITC-conjugated anti-mouse CD41, r-phycoerythrin (PE)-conjugated anti-mouse Kit, biotin-conjugated CD34, APC-conjugated streptavidin, purified and FITC-conjugated CD4, purified and FITC-conjugated CD8, purified and FITC-conjugated B220, purified and FITC-conjugated TER119, purified and FITC-conjugated Mac1, purified, FITC- and PE-conjugated CD45.1, purified, FITC- and PE-conjugated CD45.2, and all appropriate isotype control antibodies.

### HPP-CFC assay

Double-layer agar cultures were prepared as previously described (Yoder et al., 1995). Briefly, the recombinant hematopoietic growth factors human macrophage colony-stimulating factor 1 (M-CSF; 100 U), interleukin 3 (IL-3; 200 U) and IL-1α (500 U), and rat stem cell factor (SCF; 100 ng) were all purchased from Peprotech, Rocky Hill, NJ and added to 10×35 mm gridded tissue culture dishes (Nalge Nunc, Naperville, IL) followed by the addition of 0.5% agar (Bacto-agar; Difco, Detroit, MI). Sorted yolk sac, fetal liver or bone marrow cells (500 cells/dish) were suspended in 0.3% agar and applied as an overlay to the 0.5% agar/growth factor containing dishes. On day 14 of culture, the plates were read and colonies greater than 0.5 mm were scored as HPP-CFC. For all colony assays, cells were plated in triplicate and experiments repeated 2-4 times. In all experiments, statistically significant differences were determined using the Student's *t*-test with a level of significance set at <0.05.

### Primitive erythroid colony assay

Cells were plated in triplicate at  $1 \times 10^5$  cells/ml in 0.9% methylcellulose-based medium (Stem Cell Technologies, Vancouver, CA) that included IMDM, 2 mM glutamine, 1% P/S, 5% protein-free hybridoma medium-II (PFHM-II; Gibco BRL), 50  $\mu\text{g}/\text{ml}$  ascorbic acid (Sigma), 450  $\mu\text{M}$  monothioglycerol (Sigma), 200  $\mu\text{g}/\text{ml}$  iron-saturated holo-transferrin (Sigma), 15% plasma-derived serum (Animal Technology, Antech, TX) and 4 U/ml human erythropoietin (Epo; Amgen, Thousand Oaks, CA). Cultures were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , and colonies were counted on day 7.

### Hematopoietic progenitor cell assay

Sorted cells were plated (500-1000 cells/dish) in triplicate in 0.9% methylcellulose cultures as previously described (Yoder et al., 1995). Briefly, the cells were suspended in 1% methylcellulose, 30% fetal calf serum,  $10^{-5}$  mol/l 2-mercaptoethanol (Sigma), 2 mmol/l glutamine (Invitrogen), 4 U Epo, 200 U IL-3, and 100 ng SCF. The cells were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  in air and groups of more than 50 cells were scored as colonies (CFU). The early appearing CFU-Meg ( $< \text{E}8.25$  plated cells) were identified as clusters of 3 or more large refractile cells on day 3 of culture. These cells were plucked, applied to glass slides, and stained for expression of acetylcholine esterase. These early CFU-Meg were no longer detectable by day 7 of culture (when the definitive progenitors were scored).

### CFU-Meg and BFU-Meg assay

Sorted cells were plated at 500-1,000 cells/ml in 0.3% agar-based McCoy's 5A medium (Invitrogen, Grand Island, NY) which included 10% FBS (HyClone, Logan, UT), 100 U/ml recombinant IL-3, and 50 ng/ml recombinant human Tpo (Peprotech, Rocky Hill, NJ). Cultures were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  in air (Long, 1984). After 7 days (for CFU-Meg) or 14 days (for BFU-Meg) incubation, CFU-Meg and BFU-Meg were scored for their colony morphology and CD41 expression. The early appearing CFU-Meg ( $< \text{E}8.25$  plated cells) did not grow when plated in agar medium.

### Hematopoietic transplantation assays

Sorted B.6Gpi-1a/BoyJ mice yolk sac cells (15,000 cells) were transplanted via the facial vein into six sublethally irradiated (200 centigray) newborn C57Bl6/J recipient mice as previously described (Yoder et al., 1997a). Sorted fetal liver or adult marrow populations from B.6Gpi-1a/BoyJ mice were isolated as above and transplanted intravenously via the tail vein into 6-10 lethally irradiated (11Gy in divided doses 4 hours apart) C57BL/6J recipient animals. The sorted donor cells (500-2000) were mixed with 200,000 low-density bone marrow competitor cells immediately prior to intravenous injection into the recipient mice. At monthly intervals thereafter, peripheral blood was isolated and analyzed for evidence of donor type (CD45.1) blood cells as well as recipient type (CD45.2). After 6 months post-transplant, peripheral blood cells were further analyzed for donor type B lymphocyte, T lymphocyte, and granulocyte cells as evidence of multilineage engraftment.

### Whole-mount immunolabeling for confocal microscopy

C57BL/6J mice were killed by cervical dislocation on days 6.5 to 8.5 of development (day 0.5, morning of vaginal plug). The embryos were dissected and washed three times in PBS, fixed 10 minutes in cold acetone then rinsed three more times in PBS. Embryos were blocked in PBS containing 3% blotting grade non-fat dry milk (Bio-Rad Laboratories, Hercules, CA) and varying amounts of Triton X-100 (0.0125% for day 7 embryos and 0.025% for day 8 embryos) for 1 hour. Directly conjugated primary antibodies were then added to a final concentration of 5  $\mu\text{g}/\text{ml}$  for 12 to 18 hours at  $4^\circ\text{C}$ . TER119, Flk-1 and CD41 purified antibodies were labeled with Rhodamine Red-X protein labeling kit (TER119), Alexa Fluor 488 monoclonal labeling kit (Flk-1) or Alexa Fluor 647 monoclonal labeling kit

(CD41) (Molecular Probes, Eugene, OR). Similarly labeled isotype control antibodies produced no specific staining (not shown). Embryos and yolk sacs were mounted in 70% glycerol/PBS either intact or embryonic dorsal side up following open dissection according to a previously published method (Drake and Fleming, 2000).

### Confocal microscopy and image processing

Embryos were analyzed using a Bio-Rad MRC 1024 laser scanning confocal microscope (Bio-Rad Microscopy Division, Cambridge, MA) equipped with a krypton-argon laser (488, 568, 647 nm) and attached to a Nikon Diaphot inverted microscope (Fryer Co, Huntley, IL). 3D series (Z series) were obtained by imaging serial confocal planes (25-50 planes at 1-2  $\mu\text{m}$  intervals) at  $512 \times 512$  pixel resolution with a Nikon 20 $\times$  oil-immersion objective (2  $\mu\text{m}$  intervals) or a Nikon 60 $\times$  1.2-NA water-immersion objective (1  $\mu\text{m}$  intervals). Z-stacks were converted into 2D projections with MetaMorph (Universal Imaging Corp., Downingtown, PA). Composite two-dimensional (2D) image reconstruction from adjacent confocal fields was performed using Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA). The red, green and blue colors displayed on micrographs indicate that labeling was revealed, respectively, with Rhodamine Red-X (TER119), Alexa Fluor 488 (Flk-1), and Alexa Fluor 647 (CD41) fluorochromes.

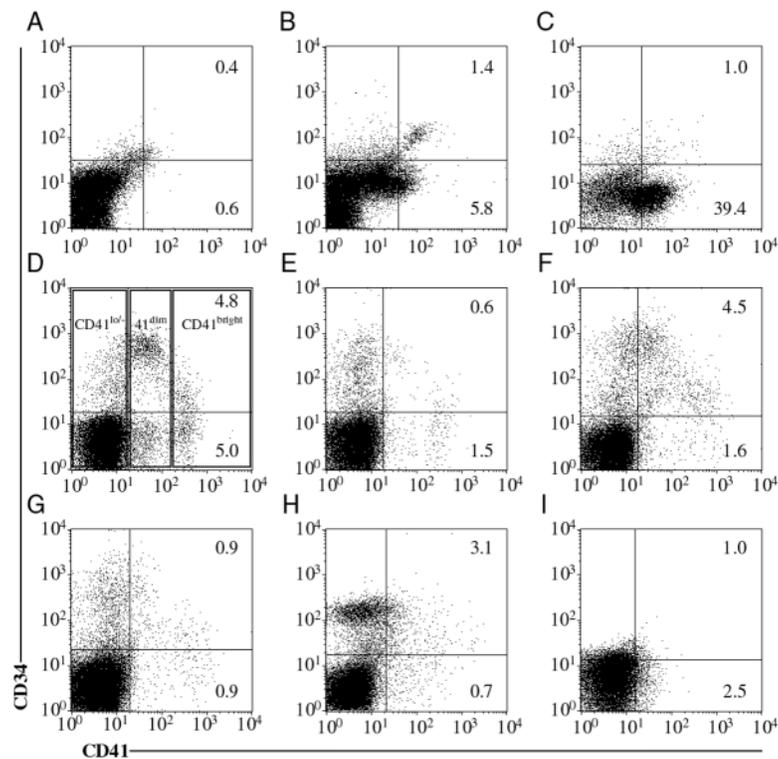
### Fibrinogen staining protocol

Single cell suspensions of YS cells were stained with 1  $\mu\text{l}$  CD61 PE and sorted into CD61 $^+$  and CD61 $^-$  populations using the FACStar instrument. Recovered cells were pretreated with 5  $\mu\text{l}$  of 0.1 M DTT (Invitrogen, Grand Island, NY) for 5 minutes followed by addition of 0.5  $\mu\text{l}$  of fibrinogen conjugated to Oregon Green (stock solution of 1.5 mg/ml in 0.1 M sodium bicarbonate, pH 8.3; Molecular Probes, Eugene, OR). The cells were incubated for 30 minutes at  $37^\circ\text{C}$ . To the fibrinogen and cell suspension, 1  $\mu\text{l}$  CD41 conjugated to Alexa 647 was added for 30 minutes at  $37^\circ\text{C}$ . Following the incubation, cells were pelleted, washed and resuspended in IMDM with 10% FBS, 1% P/S, 2 mM L-glutamine for cell analysis on the FACS Vantage instrument. In some experiments, 1-8  $\mu\text{l}$  of a anti-mouse CD41 blocking mAb (1B5F(ab)'2, 1.6 mg/ml) was added to one aliquot and 1-8  $\mu\text{l}$  of a rat isotype control (99-C7-B3, 1.8 mg/ml) was added (both antibodies were generously provided by Dr Barry Collier, Rockefeller University, New York) to a second aliquot of cells for 30 minutes at  $37^\circ\text{C}$  prior to addition of fibrinogen.

## RESULTS

### CD41 cell surface expression

CD41-expressing cells were first detectable in E7.0 (Fig. 1B) cells isolated from the entire developing embryo. Both the percentage and intensity of staining increased by E9.5 in the yolk sac (Fig. 1D). Several different yolk sac cell populations could be discriminated by the intensity of CD41 expression at E9.5 with CD41 $^{\text{bright}}$ , CD41 $^{\text{dim}}$  and CD41 $^{\text{lo/-}}$  patterns (Fig. 1D). The intensity and percentage of cells expressing CD41 diminished in the fetal liver and bone marrow populations compared with that in yolk sac cell populations (Fig. 1H,I). CD34 is a sialomucin highly expressed on endothelial and some hematopoietic cells (Krause et al., 1996). CD34 and CD41 co-expression was evident in whole embryo (E7.0; Fig. 1B), yolk sac (E8.0-E10.0; Fig. 1C,D,F), embryo proper (E9.5-10.0; Fig. 1E,G), fetal liver (E12.5; Fig. 1H) and adult bone marrow cells (Fig. 1I) with the highest percentage of co-expressing cells present in the yolk sac (Fig. 1D,F). Of interest, the percentage and level of intensity of CD41 expression was



**Fig. 1.** Cell surface expression of CD41 (x axis) and CD34 (y axis) in yolk sac, para-aortic splanchnopleure (P-Sp), aorta-gonad-mesonephros (AGM), fetal liver and adult bone marrow. (A) Isotype antibody staining of E7.0 whole embryo tissue, (B) E7.0 whole embryo tissue, (C) E8.0 yolk sac cells, (D) E9.5 yolk sac cells, (E) E9.5 P-Sp cells, (F) E10.0 yolk sac cells, (G) E10.0 AGM cells, (H) E12.5 fetal liver cells, and (I) adult marrow cells. Numbers listed in upper right corner are the percentage of total cells coexpressing CD41 and CD34 in each tissue.

Representative gates used to identify CD41<sup>lo/lo</sup>, CD41<sup>dim</sup>, and CD41<sup>bright</sup> cells are depicted in panel D. Results are representative data of 4 experiments.

less in cells isolated from the embryo proper (Fig. 1E,G) than in similarly staged and paired yolk sac samples (Fig. 1D,F).

### Colony forming potential of CD41-expressing cells in the early yolk sac

CD41<sup>dim</sup> and CD41<sup>bright</sup> expressing cells were appearing in the yolk sac at times coincident with the appearance of Ery<sup>P</sup> and definitive hematopoietic progenitor cells, respectively, and suggested that these progenitors may be expressing CD41. In fact, CD41<sup>dim</sup> cells arising at E7.0 in the yolk sac possessed all of the Ery<sup>P</sup>-CFC activity at this stage (Table 1). The Ery<sup>P</sup>-CFC activity remained essentially restricted to the CD41<sup>dim</sup> population through E8.5 (Table 1) after which time these progenitors became undetectable (data not shown). All of the CFU-Mac found at E8.0 were also present in cultures initiated with CD41<sup>dim</sup> cells. Even if 20-fold more cells were plated, no CFU-Mac were present in the CD41<sup>lo/lo</sup> population (data not shown). Similarly, all of the CFU-Meg detectable at E7.5 were present in the CD41<sup>dim</sup> fraction. These data indicate that CD41<sup>dim</sup> cells in the early yolk sac contain all of the macrophage, megakaryocytic and primitive erythroid cell activity as these cells first emerge as detectable progenitor cells. Thus, CD41<sup>dim</sup> expression serves as a marker for the onset of megakaryopoiesis, macrophage CFC, and primitive erythroid progenitor cell emergence in the murine yolk sac.

Definitive hematopoietic progenitor cells are first detectable in the yolk sac at E8.25 (Palis et al., 1999). We examined E8.0-8.25 yolk sac cells for expression of CD41 and identified a new population of CD41<sup>bright</sup> cells in addition to CD41<sup>dim</sup> and CD41<sup>lo/lo</sup> cells (Fig. 1C). Plating of these cells in progenitor assays revealed no CFC growth from plated CD41<sup>lo/lo</sup> cells, however, nearly all definitive progenitor cells were present in the CD41<sup>bright</sup> population whereas the CD41<sup>dim</sup> cells retained

the Ery<sup>P</sup>-CFC and low numbers of definitive progenitors (Table 1). As expected,  $\beta$ H1 and  $\beta$  globin major mRNA were expressed in the Ery<sup>P</sup>-CFC-derived erythroblasts present in the cultures of sorted CD41<sup>dim</sup> cells (Fig. 2). Proof that the CD41<sup>bright</sup> population gave rise to definitive erythroid cells was confirmed by the presence of  $\beta$  globin major but not  $\beta$ H1 mRNA in the erythroid cells from isolated BFU-Es (Fig. 2). In summary, these results indicate that essentially all definitive progenitor cell activity is present in the CD41<sup>bright</sup> population of E8.25 yolk sac cells and that nearly all CD41<sup>dim</sup> cells persisting at this stage continue to possess Ery<sup>P</sup>-CFC activity. Thus CD41 expression serves as a marker for the onset of

definitive as well as primitive hematopoiesis in the murine yolk sac.

### Spatial and temporal analysis of the emergence of CD41-expressing cells

We examined E6.5-E8.25 embryos to determine the precise temporal and spatial onset of CD41 expression using confocal

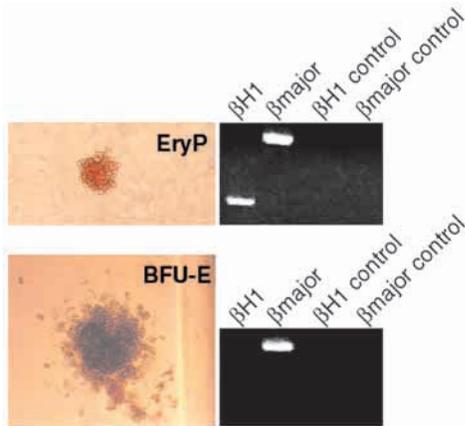
**Table 1. Primitive and definitive progenitors derived from CD41<sup>bright</sup>, CD41<sup>dim</sup> and CD41<sup>lo/lo</sup> cells from murine embryos**

Day	Ery-P	CFU-Mac	CFU-Meg	BFU-E	CFU-mix	CFU-GM
<b>E7.0</b>						
CD41 <sup>bright</sup>	ND	NP	NP	NP	NP	NP
CD41 <sup>dim</sup>	6.2±4.1	NP	NP	NP	NP	NP
CD41 <sup>lo/lo</sup>	0	NP	NP	NP	NP	NP
<b>E7.5</b>						
CD41 <sup>bright</sup>	NP	NP	ND	NP	NP	NP
CD41 <sup>dim</sup>	NP	NP	4.1±2.7	NP	NP	NP
CD41 <sup>lo/lo</sup>	NP	NP	0	NP	NP	NP
<b>E8.0</b>						
CD41 <sup>bright</sup>	NP	ND	NP	NP	NP	NP
CD41 <sup>dim</sup>	NP	8.6±2.3	NP	NP	NP	NP
CD41 <sup>lo/lo</sup>	NP	0	NP	NP	NP	NP
<b>E8.25</b>						
CD41 <sup>bright</sup>	0.5±0.7	0.1±0.2	3.3±0.9	6.0±1.4	14.0±2.8	9.0±2.0
CD41 <sup>dim</sup>	14.8±4.8	12.3±3.4	0.2±0.3	0.3±0.5	1.7±1.6	0.8±1.0
CD41 <sup>lo/lo</sup>	0.8±1.2	0	0	0	0	0

Primitive erythroid (Ery-P), early appearing macrophage (CFU-Mac) and megakaryocyte (CFU-Meg) and definitive progenitor cells (BFU-E, CFU-Mix, CFU-GM) were enumerated from cultures initiated with 1000 sorted cells.

Data presented as mean±s.d. in 2-4 experiments.

NP, not performed and ND, not detectable by flow cytometry.



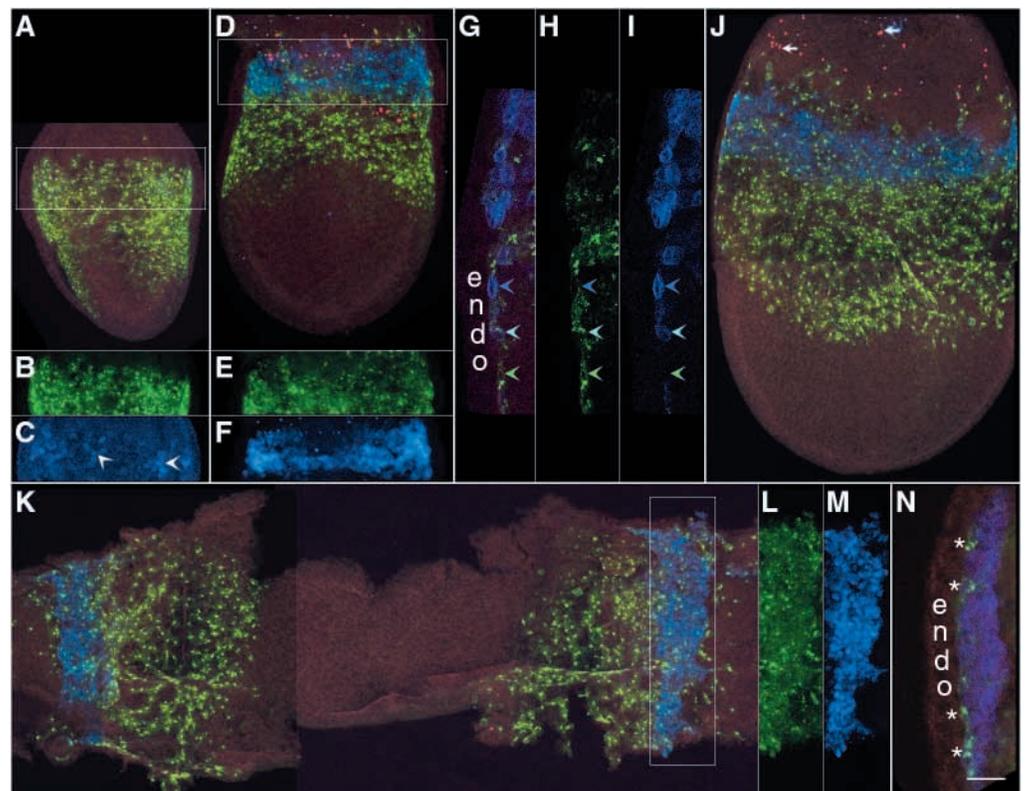
**Fig. 2.** Colony morphology and analysis of hemoglobin mRNA expression. Photomicrograph of an E8.5 yolk sac primitive erythroid cells (EryP) and a definitive erythroid progenitor cell (BFU-E). Magnification  $\times 100$ . Panels to the right of the photomicrographs depict the results of RT-PCR analysis of plucked erythroid colonies and reveal that EryP contain mRNA for  $\beta$ H1 (embryonic hemoglobin) and  $\beta$  globin major (adult hemoglobin) while BFU-E express mRNA for only  $\beta$  globin major. Negative control samples were tested using no reverse transcriptase.

microscopy. Vascular endothelial growth factor receptor 2 (Flk-1) is expressed in mesoderm, angioblasts and some smooth muscle and hematopoietic progenitors in the early yolk sac (Choi et al., 1998; Drake and Fleming, 2000; Yamashita et al., 2000). Mid-streak embryos expressed Flk-1 in differentiating extraembryonic mesoderm cells emigrating

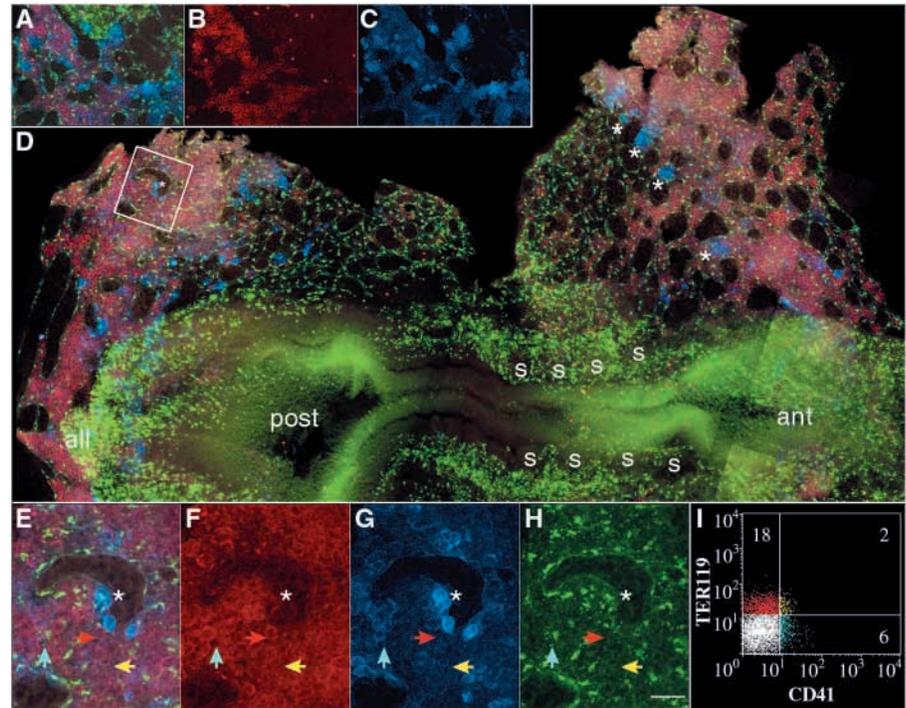
from the primitive streak into the developing yolk sac (Fig. 3A). By late-streak/neural plate stages, Flk-1<sup>+</sup> cells are organizing into capillary-like structures observed between the prospective area of blood island formation and the embryo proper (Flk-1<sup>+</sup>, CD41<sup>-</sup> cells in Fig. 3D-M).

CD41<sup>dim</sup> fluorescence was first detected at the mid-streak stage (E7.0) in a narrow ring of Flk-1<sup>+</sup> cells at the proximal end (farthest from the embryo proper) of the yolk sac (Fig. 3A-C). By the late-streak stage, CD41<sup>+</sup> cells form a uniform band 5-10 cell diameters in width that remains centered around the proximal portion of the yolk sac overlying the upper exocoelomic cavity (Fig. 3D-F). Higher magnification of the yolk sac at this stage permitted visualization of a progression

**Fig. 3.** Expression of Flk-1 and CD41 in the gastrulating murine embryo. (A) E7.0 (mid-streak) embryo reveals Flk-1-expressing putative mesoderm cells migrating from the level of the embryo proper to the more proximal region of the egg cylinder (white box indicates region depicted in B and C). (B) Flk-1 staining only. (C) Emergence of CD41<sup>dim</sup> expression in the E7.0 yolk sac (arrowheads). (D) E7.25 (late-streak stage) embryo expresses both Flk-1 (E) and CD41<sup>dim</sup> (F) cells with the CD41<sup>dim</sup> cells forming a band of cells in the most proximal portion of the yolk sac while Flk-1<sup>+</sup> cells extend toward the distal leading edge of the yolk sac as it approaches the embryo proper. (G) A composite higher magnification image of the proximal yolk sac (in orthogonal view) of an E7.25 embryo. The unlabeled visceral endoderm (endo) overlies Flk-1<sup>+</sup> (H) and CD41<sup>dim</sup> (I) expressing cells. The arrowheads (G-I) reflect cells in transition from Flk-1<sup>+</sup> CD41<sup>lo/-</sup> (green) to Flk-1<sup>+</sup> CD41<sup>dim</sup> (cyan) to Flk-1<sup>dim</sup> CD41<sup>dim</sup> (blue). (J) E7.5 (neural plate stage) embryos reveal a wider distribution of Flk-1-expressing cells with appearance of numerous capillary-like structures. The band of CD41<sup>dim</sup>-expressing cells has expanded but remains centered at the proximal end of the yolk sac. Arrows indicate contaminating maternal red blood cells expressing high levels of the erythroid marker, TER119 (small bright red cells also present in D and Fig. 4B). (K) Reconfiguration of the neural plate stage embryo into a planar fashion by dividing the embryo from the most proximal to distal, results in a clear view of the continuity of the CD41<sup>dim</sup>-expressing cells for the full circumference of the yolk sac. (L) Flk-1 expression alone in the proximal region of the yolk sac. Some of the cells are Flk-1<sup>bright</sup> and many are Flk-1<sup>dim</sup> and these cells correspond to the same cells (M) that are CD41<sup>dim</sup>; the band of CD41-expressing cells is 5-10 cells in width and 1 cell thick. Flk-1 highly expressing cells overlie the band of Flk-1<sup>dim</sup>CD41<sup>dim</sup> cells but an orthogonal view (N) reveals that the Flk-1<sup>bright</sup> cells (asterisks) are interposed between the band of CD41<sup>dim</sup> cells and the outer endoderm layer (endo). Scale bar: 100  $\mu$ m (A-F, J-M) and 33  $\mu$ m (G-I, N).



**Fig. 4.** Emergence of TER119-expressing erythroid cells and CD41<sup>bright</sup> cells in the E8.0-8.25 embryo. (A) A portion of the emerging blood island in an E8.0 embryo (1-somite-pair stage) with the first TER119-expressing cells. (B) Extent of TER119 expression in cells (note the presence of TER119<sup>bright</sup> maternal erythrocytes). (C) Emergence of a new population of CD41<sup>bright</sup> cells among the CD41<sup>dim</sup> cells. (D) E8.25 (4 somite pairs) embryo that has been rendered planar with the anterior (ant) and posterior (post) regions, allantoic stalk (all), and somites (s) identified. Massive expansion in the TER119-expressing cells and extensive vasculogenesis with a network of capillary-like structures is evident as is the emergence of CD41<sup>bright</sup> cell clusters (asterisks). White box indicates region depicted in E-H. (E) High magnification composite image of a blood island containing TER119- (red), Flk-1- (green), and CD41- (blue) expressing cells. Isolated channels are shown in F-H. A red arrow in E-H identifies a cell expressing TER119 (red) but not CD41 (blue). A blue arrow (E-H) indicates a cell expressing only CD41 (blue). A yellow arrow indicates a cell that is expressing TER119 (red) and CD41 (blue). Of interest the CD41<sup>bright</sup> cell in panel G (asterisk) also expresses Flk-1 (E,H). Scale bar: 100  $\mu$ m (A-D) 33  $\mu$ m (E-H). (I) A representative dot plot of sorted E8.25 whole embryos with quadrants color-coded to match arrows in E-H.



of cellular immunophenotypes from Flk-1<sup>+</sup>CD41<sup>lo/-</sup> to Flk-1<sup>+</sup>CD41<sup>dim</sup> to Flk-1<sup>lo/-</sup>CD41<sup>dim</sup> (Fig. 3G-I). The band of CD41<sup>dim</sup>-expressing cells remained constant in size (5-10 cell diameters wide and 1-2 cells thick) and intensity through the neural plate stage of development (Fig. 3J-M). Examination of dissected neural plate stage embryos revealed that the band of CD41<sup>dim</sup> cells was contiguous around the entire circumference of the yolk sac (Fig. 3K-M). Examination of a neural plate stage yolk sac in orthogonal section revealed that the CD41<sup>+</sup> cells were not circumscribed by the Flk-1<sup>+</sup> developing endothelium. These Flk-1<sup>+</sup> nascent endothelial cells (likely angioblasts) are situated between the visceral endoderm and the CD41<sup>+</sup> primitive erythroid elements (Fig. 3N).

### Emergence of the Ery<sup>P</sup> lineage and the definitive progenitors

Expression of the erythroid lineage marker TER119 in a subset of the CD41<sup>dim</sup> cells began soon after the onset of blood island vasculogenesis (Fig. 4A-C). Between E8.0 and E8.25 there was a significant increase in the number of TER119<sup>+</sup> cells and the level of TER119 expression (Fig. 4A,D). Cells with the highest level of TER119 expression had diminished levels of CD41 expression (below dim), thus a reciprocal relationship appeared to exist between the level of TER119 expression and the level of CD41 expression in the CD41<sup>dim</sup> population (Fig. 4E-I). When CD41<sup>dim</sup> and TER119 co-expressing cells (Fig. 4I) were sorted, the frequency of Ery<sup>P</sup> in E8.5 yolk sac CD41<sup>dim</sup>TER119<sup>-</sup> cells was 83/2000 cells plated compared to 41/2000 cells plated for the more mature CD41<sup>lo/-</sup>TER119<sup>+</sup> cells ( $n=2$ ). These observations suggest that maturing Ery<sup>P</sup> and emerging primitive erythroblasts downregulate CD41 expression during differentiation (Fig. 4E-I). However, the

level of TER119 expression in the primitive erythroid lineage never attained that displayed by definitive erythroid cells (e.g. contaminating maternal erythrocytes in Fig. 3J).

The onset of TER119 expression in the CD41<sup>dim</sup> population between E8.0 and early somite stages (E8.25) was accompanied by the emergence of a novel CD41<sup>bright</sup> population of cells (Fig. 4). These CD41<sup>bright</sup> cells were always TER119<sup>-</sup> and seemed to be intimately associated with the endothelium of the developing vasculature (Fig. 4C,G). Some of these CD41<sup>bright</sup> cells coexpressed Flk-1 (Fig. 4E-H asterisk). The CD41<sup>bright</sup> cells were found in clusters of 10-20 cells at the borders between the blood island and the capillary bed leading to the embryo proper (Fig. 4D asterisks). These cells appear to expand in the yolk sac at the time of appearance of the first definitive progenitor cells (E8.25) and since essentially all definitive progenitors are CD41<sup>bright</sup> (Table 1) at least some of the clustered CD41<sup>bright</sup> cells must be definitive progenitors.

### CFC potential of CD41<sup>+</sup> cells in the late yolk sac, P-Sp, fetal liver and adult marrow

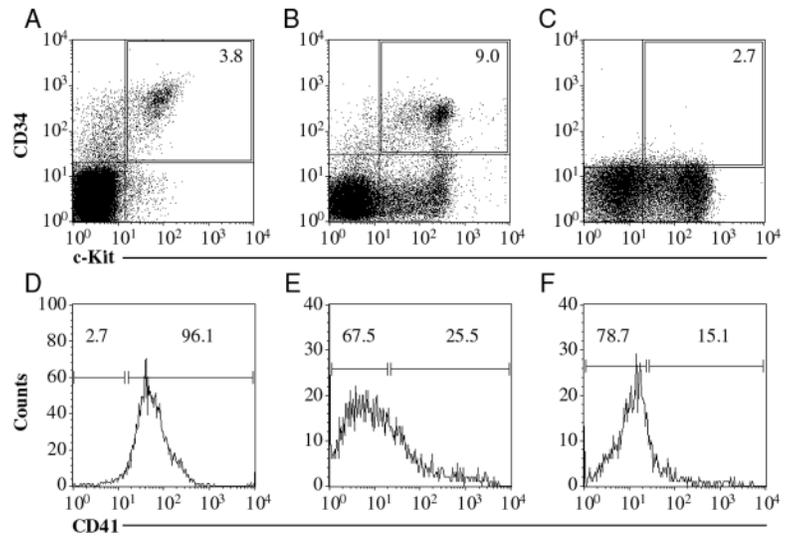
Yolk sac, P-Sp, fetal liver and adult marrow Kit<sup>+</sup>CD34<sup>+</sup> cells are enriched for hematopoietic progenitor cell activity (Ito et al., 2000). We examined Kit<sup>+</sup>CD34<sup>+</sup> cells in the E9.5 yolk sac, E12.5 fetal liver and adult bone marrow for expression of CD41 (Fig. 5). Nearly all Kit<sup>+</sup>CD34<sup>+</sup> cells in the E9.5 yolk sac express CD41 (Fig. 5A). CD41 expression was present on a high percentage of Kit<sup>+</sup>CD34<sup>+</sup> cells in the E12.5 fetal liver (Fig. 5E) but expression was restricted to a small percentage of adult bone marrow cells (Fig. 5F). Since adult bone marrow hematopoietic stem cells are enriched in cells that fail to express CD34, we also examined Kit<sup>+</sup>CD34<sup>-</sup> cells for CD41

expression and less than 1% of these cells expressed CD41 (data not shown). CD41 expression was also examined in E9.5 Kit<sup>+</sup>CD34<sup>+</sup> P-Sp cells and the majority of cells (68±12%) were CD41<sup>+</sup>.

Sorted Kit<sup>+</sup>CD34<sup>+</sup> and Kit<sup>+</sup>CD34<sup>-</sup> cells were subfractionated into CD41<sup>+</sup> and CD41<sup>lo/-</sup> cells and were plated in CFC assays. We observed that nearly all CFC cultured from E9.5 yolk sac cells were present in Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>+</sup> cells (Table 2). E9.5 P-Sp cells contained progenitors in both the CD41<sup>+</sup> and CD41<sup>lo/-</sup> populations (Table 2). E12.5 fetal liver Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>+</sup> cells also contained numerous CFC, however, significantly higher numbers of progenitor cells were present in plated Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>lo/-</sup> cells. A few colonies were also detected in plated Kit<sup>+</sup>CD34<sup>-</sup> CD41<sup>lo/-</sup> fetal liver cells. Adult marrow CFC were present in all subpopulations of cells assayed (Table 2) with the highest frequency of CFC present in plated Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>lo/-</sup> cells.

### Long-term repopulating potential of CD41<sup>+</sup> cells

Essentially all E9.5 yolk sac Kit<sup>+</sup>CD34<sup>+</sup> progenitor cells expressed CD41. Not surprisingly, 2,000 of these cells provided long-term multilineage hematopoietic reconstitution in 4 of 6 sublethally myeloablated newborn mice for at least 4 months post-transplantation (12±8% B lymphocytes, 8±6% T lymphocytes and 21±13% granulocytes; mean±s.d. for *n*=4 recipients). No donor-derived repopulation of hematopoiesis was observed in 22 mice transplanted with Kit<sup>+</sup>CD34<sup>+</sup>CD41<sup>-</sup> cells even if up to 35,000 cells were transplanted (data not shown).



**Fig. 5.** Cell surface expression of CD41 on cells co-expressing CD34 and Kit. CD34 (y axis) and Kit (x axis) staining of (A) E9.5 yolk sac, (B) E12.5 fetal liver and (C) bone marrow cells revealed co-expression of these antigens (number in upper right corner of A-C indicates percentage of total events). (D-F) Analysis of the cells co-expressing CD34 and Kit revealed CD41 expression in (D) most E9.5 yolk sac cells, (E) lower CD41 expression in E12.5 fetal liver cells, and (F) limited CD41 expression on adult marrow cells. These are representative data from 5 experiments.

Since hematopoietic progenitor cells were present in both CD41<sup>+</sup> and CD41<sup>-</sup> subpopulations of fetal liver and adult marrow cells, we wished to determine which subpopulation was enriched in long-term repopulating activity. E12.5 fetal liver and adult marrow cells were first depleted of B and T

**Table 2. Colony forming cell (CFC) potential of sorted cell populations**

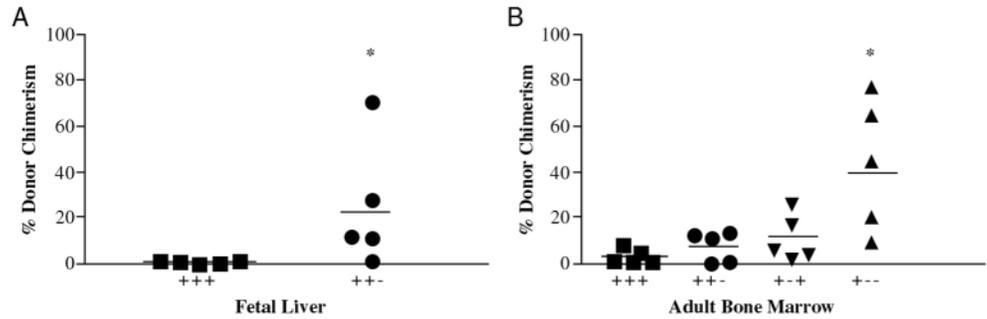
Cell type	Kit <sup>+</sup> CD34 <sup>+</sup> CD41 <sup>+</sup>	Kit <sup>+</sup> CD34 <sup>+</sup> CD41 <sup>-</sup>	Kit <sup>+</sup> CD34 <sup>-</sup> CD41 <sup>+</sup>	Kit <sup>+</sup> CD34 <sup>-</sup> CD41 <sup>-</sup>
<b>E9.5 Yolk sac cells</b>				
HPP-CFC	10±1	1±1	1±1	0
CFU-GM	7±2	0	0	0
BFU-E	3±2	0	0	0
CFU-Mix	10±3	0	1±1	0
CFU-Meg	8±1	0	ND	ND
BFU-Meg	2±1	0	ND	ND
<b>E9.5 P-Sp</b>				
HPP-CFC	3±2	2±2	ND	ND
CFU-GM	3±1	1±1	ND	ND
BFU-E	1±1	2±1	ND	ND
CFU-Mix	2±3	2±1	ND	ND
<b>E12.5 fetal liver</b>				
HPP-CFC	4±2	21±5	0	2±1
CFU-GM	7±3	7±3	0	1±1
BFU-E	1±1	5±1	0	1±1
CFU-Mix	1±1	5±1	0	0
<b>Adult bone marrow</b>				
HPP-CFC	9±2	25±4	9±3	17±3
CFU-GM	5±1	36±2	5±1	21±3
BFU-E	1±1	9±1	1±1	1±1
CFU-Mix	4±2	11±2	3±1	4±1
CFU-meg	4±1	1±1	2±1	0
BFU-meg	2±1	2±1	2±2	0

Data represent the mean±s.d. for triplicate plates in 4 experiments.

Sorted cells from the yolk sac, paraaortic splanchnopleure (P-Sp), fetal liver and adult bone marrow were plated in CFC assays at 500 cells per plate. ND, not determined.

**Fig. 6.** Percentage donor chimerism in the blood of recipient mice transplanted with fetal liver or adult marrow cells. (A) Fetal liver donor cells expressing Kit, CD34 and CD41 (+++) demonstrated minimal but detectable repopulating ability in the competitive repopulating assay, however, cells expressing Kit, CD34, but not CD41 (++) demonstrated high repopulating ability. (B) Adult marrow cells expressing Kit, CD34, and CD41 (+++) or minus CD41

(++) demonstrated minimal but detectable repopulating ability in the competitive repopulation assay. Marrow cells with the phenotype Kit<sup>+</sup>CD34<sup>-</sup>CD41<sup>+</sup> (++) demonstrated slightly greater repopulating ability than the +++ or ++- cells ( $P>0.05$ ). Marrow cells with the phenotype Kit<sup>+</sup>CD34<sup>-</sup>CD41<sup>-</sup> (++) demonstrated significantly greater ( $P<0.05$ ) repopulating ability than all other sorted donor cell populations.

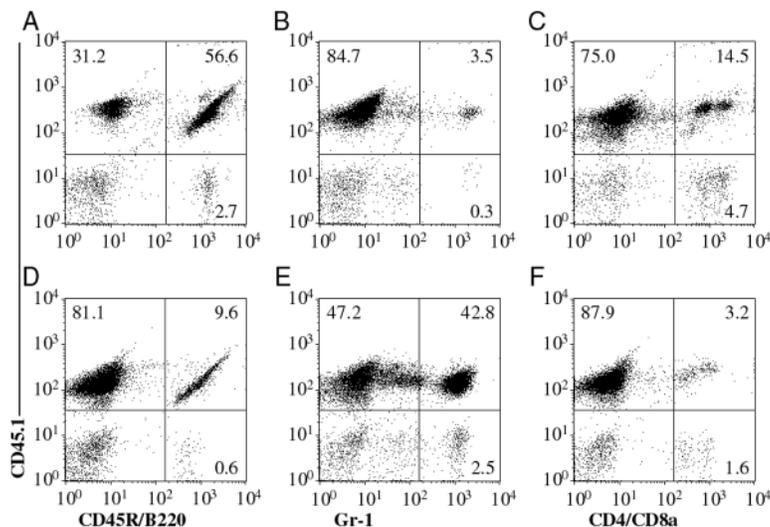


lymphocyte and granulocyte lineage antigen-positive cells (see Materials and Methods). Cells were then fractionated into 4 subpopulations including Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>+</sup>, Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>-</sup>, Kit<sup>+</sup>CD34<sup>-</sup> CD41<sup>+</sup>, and Kit<sup>+</sup>CD34<sup>-</sup> CD41<sup>-</sup> cells. Sorted cells were transplanted into lethally irradiated congenic recipient mice along with fresh low-density marrow competitor cells. Fetal liver Kit<sup>+</sup>CD34<sup>-</sup> CD41<sup>+</sup> and Kit<sup>+</sup>CD34<sup>-</sup> CD41<sup>-</sup> cells failed to demonstrate any (>0.5% donor-derived CD45.1 cells) long-term repopulating activity in the blood of recipient animals at 4 or 6 months post-transplant. Fetal liver Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>+</sup> displayed minimal but persistent and detectable long term repopulating activity ( $1.0\pm 0.3\%$ ,  $n=5$  mice) while Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>-</sup> cells demonstrated significantly greater long-term donor-derived chimerism ( $27.3\pm 24.4\%$ ,  $n=5$ ) in the peripheral blood of the recipient mice at 6 months post-transplant (Fig. 6A). Thus, even though equal numbers of donor cells (500) were originally injected, higher repopulation was observed with Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>-</sup> fetal liver hematopoietic cells. All of the adult marrow sorted cell populations possessed

some degree of long-term repopulating ability (Fig. 6B), though the highest levels of donor blood cell chimerism were present in recipient mice transplanted with 2000 Kit<sup>+</sup>CD34<sup>-</sup>CD41<sup>-</sup> cells ( $42.6\pm 28.3\%$ ,  $n=5$ ). Evidence that both E12.5 fetal liver Kit<sup>+</sup>CD34<sup>+</sup> CD41<sup>-</sup> and adult bone marrow Kit<sup>+</sup>CD34<sup>-</sup>CD41<sup>-</sup> cells contributed for more than 6 months to multiple blood cell lineages including B lymphocytes, T lymphocytes and granulocytes is depicted in Fig. 7 and Table 3. Thus, CD41 is expressed on some long-term reconstituting hematopoietic stem cells, however, repopulating ability is enriched in Kit<sup>+</sup>CD34<sup>+</sup> fetal liver and Kit<sup>+</sup>CD34<sup>-</sup> adult marrow cells that are CD41<sup>-</sup>.

### Co-expression of CD41 and CD61 with fibrinogen binding activity in yolk sac cells

We examined E9.5 yolk sac cells for co-expression of CD41 and CD61 as these proteins interact to bind fibrinogen in mature platelets. CD41 detected on the cell surface of E9.5 yolk sac cells co-localized with CD61, though not all CD61 molecules co-localized with CD41 (Fig. 8). When we isolated CD61-expressing E9.5 yolk sac cells (Fig. 8) and then examined cells for CD41 expression and fibrinogen binding, we observed significant co-localization of the two fluorochromes (Fig. 8). In preliminary studies, addition of monoclonal antibody (1B5F(ab)'2) to the yolk sac cells before exposure to fibrinogen blocked 50% of the fibrinogen binding whereas a non-blocking control antibody had no effect (data not shown). These data indicate that some of the non-megakaryocytic hematopoietic progenitor cells in the E9.5 yolk sac possess the capacity to bind soluble fibrinogen in vitro.



**Fig. 7.** Analysis of donor-derived circulating peripheral blood cells of recipient mice 6 months post-transplantation. Data from single representative recipients of fetal liver Kit<sup>+</sup>CD34<sup>+</sup>CD41<sup>-</sup> cells (A-C) or Kit<sup>+</sup>CD34<sup>-</sup>CD41<sup>-</sup> bone marrow cells (D-F) are depicted. Donor type CD45.1 expression is indicated on the y axis and the B lymphocyte marker B220 (A,D), granulocyte marker Gr-1 (B,E), and T lymphocyte markers CD4 and CD8 (C,F) are depicted on the x axis.

## DISCUSSION

We report the first strategy for the isolation of both primitive and definitive hematopoietic progenitor cells in the murine embryo. CD41 expression marks the first primitive erythroid and essentially all definitive hematopoietic progenitor cells in the yolk sac at the times these progenitors are first detectable with in vitro clonogenic assays. As development proceeds, CD41 expression is lost on some fetal liver and most adult marrow hematopoietic progenitor

**Table 3. Percentage donor type CD45.1 B and T lymphocytes and granulocytes in the peripheral blood of recipient mice 6 months post-transplantation**

	n	Gr-1		B220		CD4/8	
		Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.
ABM +++	6	3.31	1.79	0.74	0.04	0.66	0.66
ABM ++-	5	8.00	4.31	8.77	6.29	24.10	12.51
ABM +-+	4	16.55	7.36	16.70	1.20	14.80	4.04
ABM +--	6	26.50	14.32	22.67	14.48	29.85	11.84

Data represent the mean±s.e.m. of 6-10 mice for each group. ABM + + +, adult bone marrow Kit<sup>+</sup>CD34<sup>+</sup>CD41<sup>+</sup>; ABM + + -, marrow Kit<sup>+</sup>CD34<sup>+</sup>CD41<sup>-</sup>; ABM + - +, marrow Kit<sup>+</sup>CD34<sup>-</sup>CD41<sup>+</sup>; ABM + - -, marrow Kit<sup>+</sup>CD34<sup>-</sup>CD41<sup>-</sup>.

The level of chimerism in recipients of ABM + - - cells was significantly ( $P < 0.05$ ) higher in all lineages compared to all other groups.

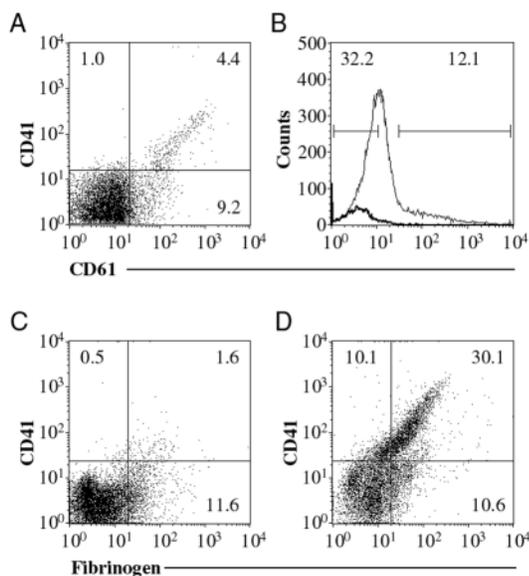
cells. However, CD41 expression is retained on some long-term repopulating hematopoietic stem cells that can reconstitute for at least 6 months following transplantation.

The transient nature, unique morphology and gene expression, and restricted cell types of the primitive erythroid lineage suggest that it is distinct from the definitive erythroid lineage. Support for the separation of primitive and definitive erythropoiesis is also provided by gene-targeting experiments where deletion of certain genes (e.g. *Runx1*, *Gata2*) disrupts definitive erythropoiesis with little change in primitive erythropoiesis (Shivdasani and Orkin, 1996). The early appearing macrophage progenitors identified in the present studies (E8.0) are absent in *Runx1* null embryos and are therefore probably related to the definitive lineage (Lacaud et al., 2002). Whether the early appearing megakaryocyte progenitors identified in the present studies are more related to the definitive or primitive lineage remains to be determined,

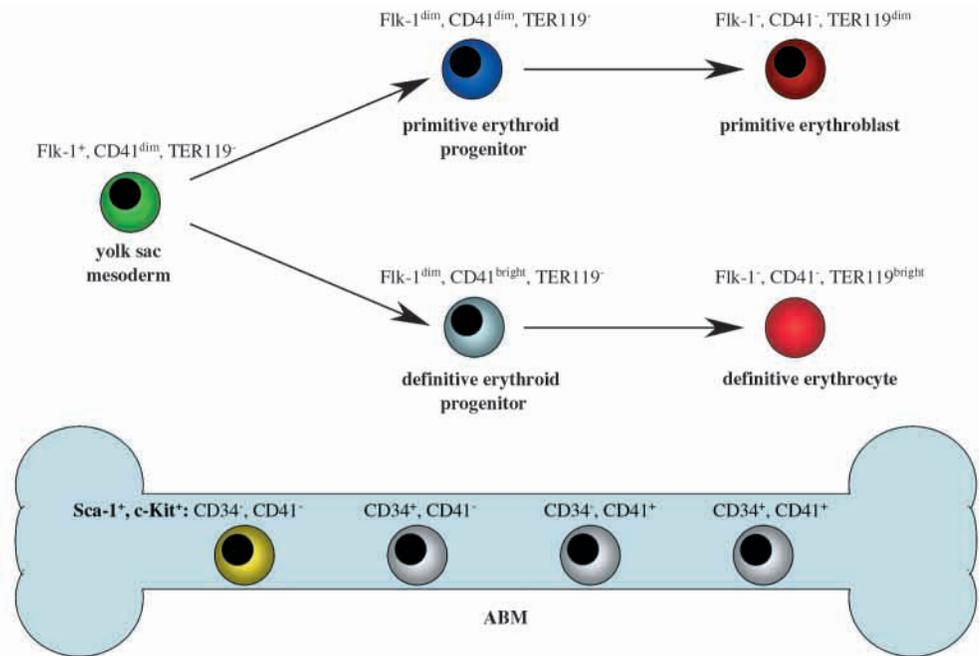
but these progenitors are reported to display features that distinguish them from later appearing definitive megakaryocyte progenitors (Xu et al., 2001). Studies performed with murine embryonic stem (ES) cells suggest that primitive and definitive hematopoietic progenitor cells emerge from a common Flk-1<sup>+</sup> precursor cell called the hemangioblast (Kennedy et al., 1997). Expression of Flk-1 is first evident in the murine embryo in proximal lateral mesoderm during gastrulation and these mesoderm cells give rise to both the hematopoietic and endothelial lineages in the yolk sac (Kataoka et al., 1997; Nishikawa, 1997). While Flk-1 has been used to isolate primitive and definitive progenitor cells from ES cell-derived hematopoietic cultures, Flk-1 has not been used to purify Ery<sup>P</sup> or early appearing CFU-Mac and megakaryocytic progenitors from the early yolk sac. We have identified CD41 expression in the yolk sac in Flk-1<sup>+</sup> cells and have demonstrated that the CD41<sup>dim</sup>-expressing yolk sac cells at E7.0 constitute the entire population of in vitro clonable Ery<sup>P</sup>-CFC. Thus, CD41 expression serves as a marker for both the onset of hematopoiesis in general (early macrophage and megakaryocytic progenitors), and specifically for primitive erythroid progenitor cell emergence.

CD41 expression at a high level in E8.25 yolk sac cells identifies essentially all of the definitive hematopoietic progenitor cells. Flk-1 and vascular endothelial cadherin have previously been utilized to isolate primitive and definitive hematopoietic cells in vitro from ES cell-derived hematopoietic cultures but this combination has not been examined as a tool for isolating primitive and definitive progenitors in the early yolk sac (Kabrun et al., 1997; Nishikawa et al., 1998). The core-binding factor Runx1 is expressed in endoderm, mesoderm and primitive erythroblasts in the yolk sac (North et al., 1999). It remains to be determined whether expression of this transcription factor will identify Ery<sup>P</sup> or CFU-Mac in the early yolk sac. Thus, CD41 is a unique marker for both primitive and definitive hematopoietic progenitor cells in the earliest phases of murine development. These observations both support and extend the recent reports of CD41 expression on hematopoietic progenitor cells from human and murine fetal and newborn subjects and the report that CD41 serves as a marker for the onset of definitive hematopoiesis (Corbel and Salaun, 2002; Debili et al., 2001; Mikkola et al., 2002; Mitjavila-Garcia et al., 2002).

While essentially all the hematopoietic progenitor cells in the yolk sac expressed CD41, CD41 expression diminished in progenitors present in the P-Sp, fetal liver and adult bone marrow compartments. We have summarized the immunophenotype of these progenitors in Fig. 9. E9.0 yolk sac



**Fig. 8.** Cell surface co-localization of CD41, CD61, and fibrinogen on E9.5 yolk sac cells. (A) CD41 (y axis) and CD61 (x axis) expression on E9.5 yolk sac cells indicates a significant percentage of the total cells co-express these markers. (B) E9.5 yolk sac cells expressing CD61 (12.1% of total cells) were isolated from non-expressing cells and further analyzed (C,D) for co-localization of CD41 and fibrinogen. (C) CD61<sup>-</sup> cells failed to demonstrate significant co-localization of CD41 and fibrinogen while CD61<sup>+</sup> yolk sac cells (D) highly co-localized CD41 and fibrinogen (30.1% of the CD61<sup>+</sup> cells). Data are representative of 4 experiments.



**Fig. 9.** Model of CD41 expression on various hematopoietic cell subsets. The results of the present studies lead us to predict a model whereby the primitive and definitive erythroid progenitor cells can be discriminated by the level of CD41 expression. Likewise, CD41 expression varies on long-term repopulating bone marrow stem cell subsets with the cells possessing the greatest repopulating ability expressing neither CD34 or CD41 (yellow cell) on the cell surface.

repopulating stem cells are enriched in cells expressing CD34 and Kit and nearly all of these cells expressed CD41 in the present studies (Yoder et al., 1997b). CD34 and Kit are expressed on hematopoietic stem and progenitor cells in the AGM, fetal liver and adult marrow (Morel et al., 1996; Sanchez et al., 1996). Of interest, fetal liver stem cell activity is enriched in cells expressing CD34 and Kit while adult marrow stem cell repopulating activity is highest in cells expressing Kit but not CD34 (Ema and Nakauchi, 2000; Ito et al., 2000; Zeigler et al., 1994). We observed that transplantation of lethally irradiated adult mice with sorted fetal liver and adult bone marrow cells resulted in very low but persistent long-term chimerism of the peripheral blood of recipient mice with donor cells expressing CD41. However, the highest levels of donor cell chimerism achieved in the competitive repopulation experiments resulted from transplantation of Kit<sup>+</sup>CD34<sup>+</sup> fetal liver and Kit<sup>+</sup>CD34<sup>-</sup> adult marrow cells that did not express detectable cell surface levels of CD41. Further studies to determine whether CD41 is expressed on stem cells derived from the AGM, the site of development of the first stem cells that engraft in adult mice are warranted.

CD41/CD61 interactions on the surface of platelets plays an important role in the adhesion, spreading and aggregation of platelets in areas of vascular injury via interactions with a variety of extracellular matrix and plasma proteins. We have identified fibrinogen binding to CD41- and CD61-expressing hematopoietic progenitor cells in E9.5 yolk sac cells. Plating of cells that bind fibrinogen alone does not isolate or enrich for hematopoietic progenitor cells, however, plating cells that express CD41 or CD61 and fibrinogen enriches for yolk sac progenitors. These results further support the growing appreciation that the CD41/CD61 complex is not restricted to the platelet lineage.

The role CD41 is playing in the primitive and definitive progenitor cells is unknown. One obvious role the CD41/61 receptor complex may play is in the interaction of the progenitor cells with extracellular matrix molecules in the yolk

sac. The specific adhesion interactions between the earliest hematopoietic and endothelial cells that are required for proper formation of yolk sac blood islands remain unknown. Disruption of several integrin and extracellular matrix molecule genes results in embryonic lethality due to aberrant blood island and yolk sac vascular development (with or without defects in hematopoiesis) (Francis et al., 2002). Thus, CD41 may play a role in hematopoietic-endothelial interactions important for early blood island organogenesis. However, mice deficient in CD41 display no obvious defect in yolk sac, fetal liver or adult marrow hematopoiesis other than a platelet adhesion abnormality similar to that seen in human subjects with Glanzmann's thrombasthenia (Tronik-Le Roux et al., 2000). Thus, further studies will be required to define the role of CD41 in hematopoietic stem and progenitor function during murine embryogenesis and throughout ontogeny.

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