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The Glycoprotein IIb Molecule Is Expressed on Early Murine Hematopoietic Progenitors and Regulates Their Numbers in Sites of Hematopoiesis

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Summary

The α integrin GPIIb is a marker of hematopoietic progenitors. Using a marking strategy based on Cre-loxP technology to trace the fate of GPIIb-expressing cells, we show that GPIIb is expressed during early definitive embryonic hematopoiesis. However, the marked fetal population is distinct from the hematopoietic cells that predominate in the adult, suggesting that at least two waves of progenitors arise concurrently or consecutively in the fetus. Furthermore, using an inactivated allele of *gpIIb*, we provide evidence for a functional role of GPIIb on progenitors. We observe an increase in hematopoietic progenitors in the yolk sac, fetal liver, and bone marrow, an effect which may, in part, be explained by loss of binding to fibronectin.

Introduction

The purification of hematopoietic stem cells and multipotential progenitors is an ongoing challenge (Lemischka, 1991; Spangrude et al., 1991). The need for additional markers is ever important since even though antigen markers such as Sca-1, c-kit, CD34, and AA4.1 are routinely used to sort early hematopoietic cells (Morrison and Weissman, 1994), they are not absolutely specific to these cells (Cumano and Godin, 2001). Conversely, early hematopoietic expression of markers once believed to be restricted to mature cell types is often observed. For instance, the integrin CD11b was originally defined as a macrophage lineage marker but is now known to be expressed on a subset of fetal liver progenitors (Morrison et al., 1995). More recently, the glycoprotein IIb molecule (GPIIb), which is part of the GPIIb/IIIa (αIIbβ3 or CD41/CD61) integrin complex and was viewed as the classical megakaryocyte- and platelet-specific marker (Ginsberg et al., 1995), has also been recognized as a hematopoietic progenitor marker.

Early results using anti-platelet antibodies suggested that GPIIb was present on multipotential myeloid progenitors (Berridge et al., 1985; Fraser et al., 1986) while studies on cycling $CD34^+$ progenitors showed that they expressed *gpIIb* RNA (Debili et al., 1992). These results were supported by the observation of reduced numbers of CFU-GEMM in mice carrying a *gpIIb-tk* transgene following administration of gangcyclovir and consequent cell-restricted metabolic suicide (Tronik-Le Roux et al., 1995; Tropel et al., 1997). More recently, it has been shown that GPIIb is expressed on immature hematopoietic cells in chick and mouse embryos, including sites of stem cell emergence (Corbel and Salaun, 2002; Ody et al., 1999). It has also been detected on human cord blood and bone marrow progenitors (Debili et al., 2001) and on more than 80% of progenitors from ES cell-derived embryoid bodies (Mitjavila-Garcia et al., 2002).

Here, we describe results that shed additional light on the developmental expression of GPIIb on hematopoietic progenitors and reveal a function for this molecule in the regulation of hematopoiesis. Using *gpIIb* promoter-driven Cre recombinase to activate β-galactosidase gene expression through deletion of a LoxP-flanked transcriptional block in the R26R reporter line (Soriano, 1999), we provide evidence for expression of *gpIIb* in early hematopoiesis. Mature myeloid and lymphoid cells in the fetus are marked with the recombined R26R allele, even though Cre recombinase is inactive in mature cells, suggesting that the *gpIIb* gene is expressed in a common hematopoietic progenitor. We also generated a null allele for the *gpIIb* gene and, through analysis of homozygous mice, we demonstrate a functional role for GPIIb in the regulation of progenitor cell number in the yolk sac, fetal liver, and bone marrow. In the absence of GPIIb, we observed a consistent increase in hematopoietic progenitors throughout development. Our results suggest that the loss of GPIIb is associated with decreased function of VLA-4 and VLA-5 in mediating the adhesion of hematopoietic progenitors to bone marrow-derived stroma and to fibronectin.

Results

Generation and Characterization of *gpIIb-Cre* Transgenics

Our first approach in investigating the role of GPIIb in hematopoietic cells was to generate transgenic mouse lines expressing Cre recombinase under the control of the *gpIIb* promoter. We used 2.5 kbp of the *gpIIb* promoter, previously shown to be sufficient for transgenic expression (Tropel et al., 1997), and 2.8 kbp of the *gpIIb* first intron (Figure 1A). Five transgenic lines were generated with copy number varying from 2 to 5. One of the lines did not express Cre while the other four showed similar patterns of expression but at different levels (data not shown). Line NE1/21 was selected for experimental use.

gpIIb-Cre transgene expression in NE1/21 was determined by crossing it to the ROSA-26 (R26R) reporter line and analyzing the extent of recombination in offspring throughout development by whole-mount β-galactosidase staining. Cre-mediated recombination in double transgenic *gpIIb-Cre*::R26R embryos was seen as pale β-galactosidase staining in the yolk sac blood islands at E8.5 with a punctate pattern revealing that only a few cells were positive (Figure 1B). A few circulating β-galactosidase-positive cells were also seen in the developing E8.5 aorta (inset on the top right of Figure 1B). By E9.5 *gpIIb-Cre*::R26R embryos contained β-galactosi-

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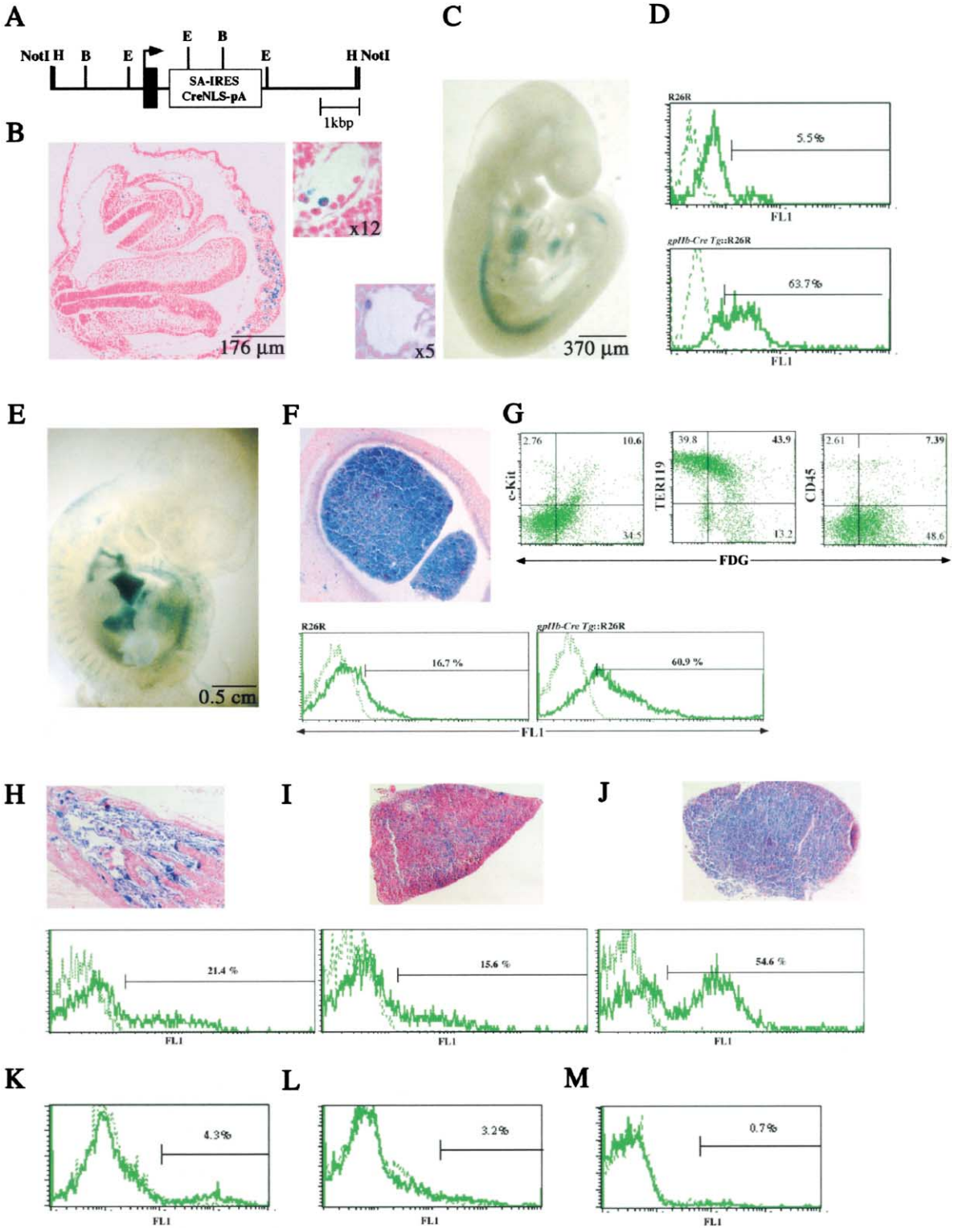

Figure 1. Generation and Characterization of the *gpIIb-Cre Tg* Line

(A) Structure of the *gpIIb-Cre* transgene. The first exon of the murine *gpIIb* gene is depicted as a black box. The lines represent promoter and intron 1 sequences. SA, splice acceptor; IRES, internal ribosome entry site; CreNLS, fusion protein between the Cre recombinase sequences and the SV40 nuclear localization signal; pA, cytomegalovirus (CMV) polyadenylation site. BamHI (B), EcoRI (E), HindIII (H), and NotI restriction endonuclease sites are depicted.

(B) Cross-section of a *gpIIb-Cre Tg*::R26R E8.5 embryo showing staining for β-galactosidase activity in cells of the yolk sac blood islands. The inset (upper right corner) shows a β-galactosidase-positive circulating blood cell in the aorta.

(C) Whole-mount staining of a *gpIIb-Cre Tg*::R26R E9.5 embryo showing β-galactosidase-positive cells in the aorta. The inset (lower left corner) shows a β-galactosidase-positive cell budding off from the endothelium of a blood vessel.

dase-positive cells in the aorta-gonad-mesonephros (AGM) region, a putative site of stem cell emergence (Cumano and Godin, 2001), in particular in the dorsal aorta (Figure 1C). The number of β-galactosidase-positive cells increased dramatically by E10.5 in the circulation and in the fetal liver (Figure 1E). Endothelial cells did not express β-galactosidase except for a few cells with rounded appearance that appeared to be "budding" from the floor of the aorta. Such cells could be detected from E9.5 onward (inset to the left of Figure 1C), had a similar appearance to the intraarterial GPIIb$^+$ clusters reported at E10 (Corbel and Salaun, 2002), and were confirmed to be hematopoietic (data not shown). By E12.5, the majority of the developing fetal liver and circulating blood cells were β-galactosidase positive (Figure 1F). At E18.5, the embryonic thymus, spleen, bone marrow, and circulating blood cells were β-galactosidase positive (Figures 1H–1J).

Further analysis of *gpIIb-Cre*::R26R E9.5 AGM and yolk sac and of E12.5 fetal liver confirmed that a substantial proportion of hematopoietic cells carried the recombined R26R allele. Correlating with the histological stainings, flow cytometric analysis of total hematopoietic cells from E9.5 using fluorescein di-β-D-galactopyranoside (FDG) as the substrate for β-galactosidase revealed that 64% of that population contained the recombined R26R allele (Figure 1D). Of the E9.5 FDG$^+$, 5.4%, 3.1%, and 4.1% were positive for CD41, CD34, and c-Kit, respectively (data not shown). By E12.5 more than 60% of the fetal liver cells contained the recombined R26R allele (Figure 1F), and of these 10.6%, 43.9%, and 7.4% were positive for expression of c-Kit, the erythroid marker Ter119, and the hematopoietic marker CD45, respectively (Figure 1G). Similar analyses of cells from E18.5 fetal bone marrow, spleen, and thymus revealed that 21.4%, 15.6%, and 54.6%, respectively, were positive for expression of β-galactosidase (Figures 1H–1J). In contrast, in a 2-month-old adult the number of β-galactosidase-positive cells had fallen to 4%, 3%, and $<$1% in the bone marrow, spleen, and thymus, respectively (Figures 1K–1M).

Analysis of genomic DNA from E12.5 and E14.5 *gpIIb-Cre*::R26R fetal liver confirmed that the R26R locus had been recombined in over 90% of cells while in other tissues only trace recombination could be detected (Figure 2A and data not shown). However, analysis of DNA from *gpIIb-Cre*::R26R adult bone marrow showed that less than 5% of the cells contained a recombined R26R locus (Figure 2B).

Expression of GPIIb and Cre Recombinase

To determine whether Cre-mediated recombination of the R26R locus by *gpIIb-Cre* reflected endogenous *gpIIb* expression, we compared β-galactosidase expression in *gpIIb-Cre*::R26R embryos to whole-mount antibody detection of GPIIb. At E9.5, the pattern of GPIIb expression matched β-galactosidase expression in *gpIIb-Cre*::R26R embryos, both being expressed in the aorta (Figure 3A). However, by E10.5 the pattern of GPIIb expression only partially overlapped with that of β-galactosidase expression in *gpIIb-Cre*::R26R embryos. GPIIb was detected in the AGM region and in a few cells of the fetal liver (data not shown) whereas stronger β-galactosidase staining was detected in the AGM region and in the majority of E10.5 fetal liver cells in *gpIIb-Cre*::R26R embryos (Figure 1E). These results matched previous studies of GPIIb expression (Corbel and Salaun, 2002) and suggested that GPIIb is present on cells corresponding to the hematopoietic progenitors present in the AGM and fetal liver and that β-galactosidase expression in *gpIIb-Cre*::R26R embryos not only reflects this but also marks the descendants of these progenitors.

We were concerned that Cre might be expressed from the *gpIIb-Cre* in all hematopoietic cells rather than being a reflection of endogenous *gpIIb* expression. We therefore performed whole-mount staining of E10.5 *gpIIb-Cre*::R26R embryos and immunohistochemistry on E12.5 *gpIIb-Cre*::R26R fetal liver sections using an antibody against Cre. Expression could be observed in the AGM region at E10.5 (Figure 3B), coinciding with GPIIb expression and only partially overlapping with β-galactosidase expression in equivalent embryos. At E12.5, Cre expression could only be detected in a few cells of the fetal liver as shown in Figure 3C.

These results demonstrate that GPIIb is expressed at the site of emergence of hematopoietic progenitors in the yolk sac, AGM, and fetal liver, and that this expression is mirrored by Cre expression in *gpIIb-Cre* embryos. By mediating recombination of the R26R allele, this pattern of Cre expression leads to the marking of the descendant definitive fetal liver hematopoietic cells.

Generation of a *gpIIb* Null Allele

As part of our Cre-LoxP cell-marking strategy, we also generated a null allele of the *gpIIb* gene (*gpIIb^{GFPCre}*). Sequences encoding a GFPCre fusion protein were introduced into the first intron of the *gpIIb* locus by gene targeting. An artificial splice acceptor site fused to an internal ribososomal entry site (IRES) was placed upstream of the GFPCre sequences to enable splicing and translation of GFPCre. A *PGK-neo* cassette flanked by Flp-recombinase target sites (FRT) was inserted downstream of the GFPCre cassette in the first intron for the purpose of selection during ES cell targeting (Figure 4A).

(D) Histograms depicting flow cytometric analysis of β-galactosidase activity in R26R (upper panel) and *gpIIb-Cre Tg*::R26R E9.5 (lower panel) hematopoietic cells loaded with FDG (solid lines) compared to cells incubated without substrate (dashed lines).
(E) Whole-mount staining of a *gpIIb-Cre Tg*::R26R E10.5 embryo showing β-galactosidase-positive cells in the fetal liver and in the circulation.
(F) Cross-section through a *gpIIb-Cre Tg*::R26R E12.5 fetal liver showing that the majority of the cells stain positive for β-galactosidase activity. Below are shown corresponding flow cytometric analyses of β-galactosidase activity in R26R (left) and *gpIIb-Cre Tg*::R26R E12.5 (right) as described in (D).
(G) Histograms depicting flow cytometric analyses of *gpIIb-Cre Tg*::R26R E12.5 fetal liver cells loaded with FDG after staining for expression of c-kit, Ter119, or CD45 using specific biotin-conjugated monoclonal antibodies and streptavidin-PE.
(H–J) Cross-sections through E18.5 *gpIIb-Cre Tg*::R26R femur (H), spleen (I), and thymus (J) stained for β-galactosidase activity. The adjacent histograms depict flow cytometric analyses for β-galactosidase activity as described in (D).
(K–M) Flow cytometric analyses for β-galactosidase activity in adult bone marrow (K), spleen (L), and thymus (M) as described in (D).

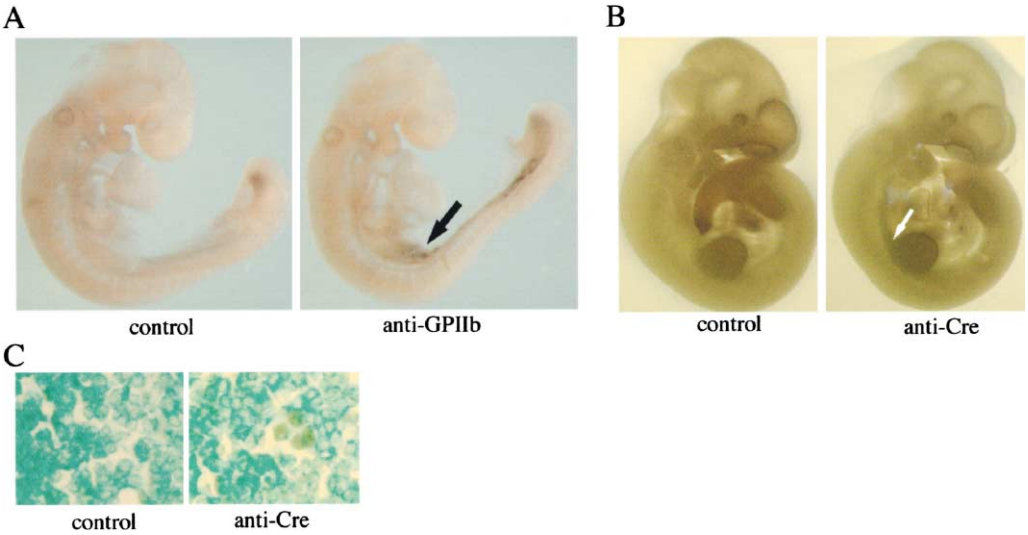

Figure 2. Characterization of GPIIb and Cre Recombinase Expression

(A) Expression of GPIIb in E9.5 embryos. Whole-mount staining of embryos was performed using rabbit polyclonal anti-GPIIb (right panel) or a matched nonspecific control (left). The arrow indicates positive staining in the region corresponding to the location of the AGM. Some nonspecific staining or reagent accumulation can be seen in the tail region.

(B) Expression of Cre recombinase in E10.5 embryos. Whole-mount staining of embryos was performed using rabbit polyclonal anti-Cre (right) or a matched nonspecific control (left). The arrow indicates positive staining in the region corresponding to the location of the AGM. Nonspecific staining was observed in the head, tail, and limb buds.

(C) Immunohistochemical detection of Cre in E12.5 *gpIIb-Cre Tg*::R26R fetal liver. Fetal liver from an embryo which had been stained for β-galactosidase activity (see Figure 1F) was sectioned and labeled using rabbit polyclonal anti-Cre (right) or a matched nonspecific control (left). Specific binding was revealed using a secondary HRP-conjugated anti-rabbit antibody.

gpIIb$^{GFPCre/GFPCre}$ homozygotes are viable and fertile although they have dysfunctional platelets and occasionally anemia because of the resulting bleeding tendancy (N.R. Emambokus et al., submitted). No GPIIb protein could be detected on bone marrow cells from *gpIIb*$^{GFPCre/GFPCre}$ homozygotes either by flow cytometry using a monoclonal antibody specific to CD41 (Figure 4C, upper panels) or by Western blotting analysis using polyclonal anti-GPIIb (data not shown). However, since the number of CD41^{+} cells is normally low (approximately 2.5% of adult bone marrow) and to exclude the possibility that the *gpIIb*^{GFPCre} allele was expressed at lower levels than its wild-type counterpart, we cultured wild-type and *gpIIb*$^{GFPCre/GFPCre}$ bone marrow cells under conditions allowing the selective outgrowth of megakaryocytes. Flow cytometric analysis of cells grown under these conditions showed that up to 49% of the wild-type cells were CD41^{+} while none could be detected in cultures of *gpIIb*$^{GFPCre/GFPCre}$ bone marrow (Figure 4C, lower panels). Analysis of wild-type and *gpIIb*$^{GFPCre/GFPCre}$ hematopoietic tissues during development also revealed that, while wild-type E9.5 yolk sac and E12.5 fetal liver contained 12% and 4% of CD41^{+} cells, respectively, none could be detected in the equivalent tissues from *gpIIb*$^{GFPCre/GFPCre}$ embryos (data not shown).

In contrast to the *gpIIb-Cre* line, expression of Cre from the *gpIIb*^{GFPCre} allele was restricted to the megakaryocyte lineage (unpublished data). Although we could detect Cre by Western blotting of cultured megakaryocytes (data not shown), we could not detect GFP fluorescence, suggesting either that the GFP moiety (unenhanced version) was too weakly fluorescent or that there was relatively low expression of the fusion protein. In crosses with R26R we only obtained recombination in about 10% of megakaryocytes (unpublished data).

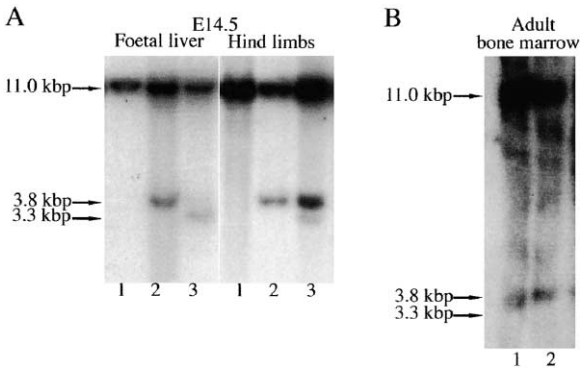

Figure 3. Detection of Deletion in Tissues from *gpIIb-Cre Tg*::R26R Embryos and Adults

Genomic DNA was digested with EcoRV, Southern blotted, and probed with a 500 bp ROSA26-specific probe.

(A) Analysis of DNA from wild-type (lane 1), R26R (lane 2), and *gpIIb-Cre Tg*::R26R (lane 3) E14.5 embryonic fetal liver and hind limbs/tail of the same embryos. The wild-type (ROSA26) and modified (R26R) alleles yield EcoRV fragments of 11 and 3.8 kbp, respectively. Recombination mediated by Cre reduces the modified allele EcoRV fragment to 3.3 kbp. Recombination in the *gpIIb-Cre Tg*::R26R fetal liver was over 90% complete (third lane) compared to <10% in the tissues derived from the lower body.

(B) Analysis of DNA from bone marrow cells derived from R26R (lane 1) and *gpIIb-Cre Tg*::R26R (lane 2) adults. Recombination of the R26R locus can barely be detected in *gpIIb-Cre Tg*::R26R.

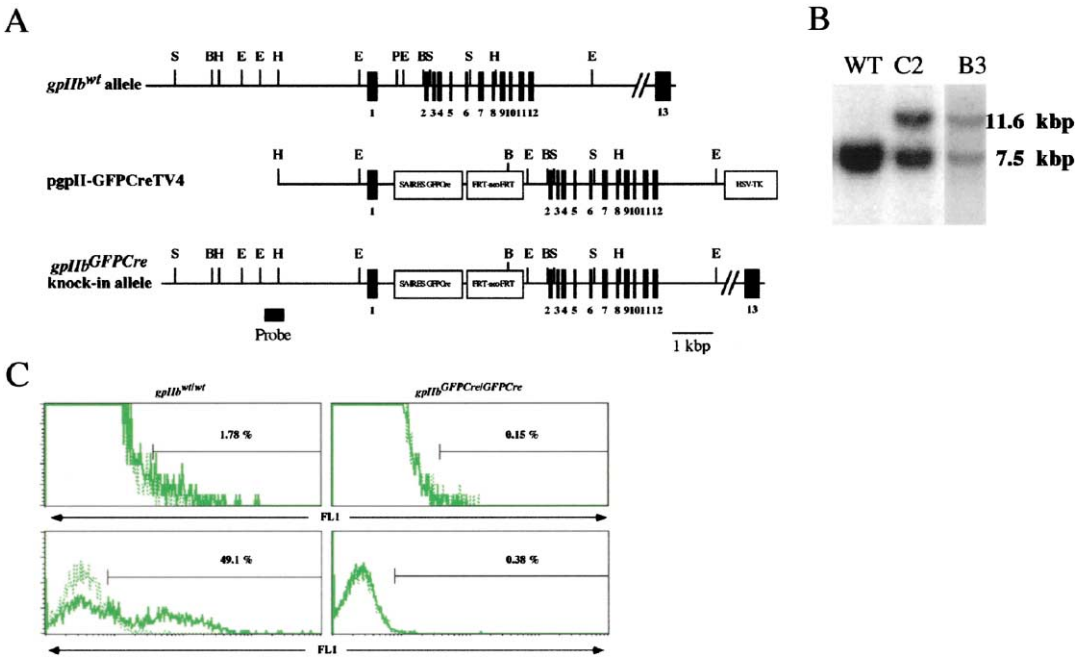

Figure 4. Generation of a Null *gpIIb* (*gpIIb*^{GFPCre}) Allele

(A) Schematic representation of the wild-type *gpIIb* allele showing the promoter and the first 13 exons. Also shown are the gpII-GFPCre targeting vector (pgpII-GFPCre TV4) and the targeted *gpIIb* allele (*gpIIb*^{GFPCre} knockin). The black boxes represent exons. The restriction endonuclease sites ScaI (S), HindIII (H), BamHI (B), PstI (P), and EcoRI (E) are depicted. The pgpII-GFPCre TV4 targeting vector contains a cassette consisting of a splice acceptor (SA), an internal ribosomal entry site (IRES), and GFPCre sequences. It also contains a *neo* resistance cassette (PGK-neo) flanked by FRT sites and an HSV-tk cassette for negative selection. The 700 bp EcoRI-HindIII promoter fragment used for detecting targeted events by Southern blot hybridization is indicated (Probe).

(B) Southern blots of ScaI digests of genomic DNA from wild-type ES cells and the two targeted *gpIIb*^{GFPCre} clones (C2 and B3) which were used to generate the *gpIIb*^{GFPCre} lines. When hybridized with the EcoRI-HindIII probe, the 11.6 kbp targeted ScaI fragment is detected in addition to the 7.5 kbp wild-type ScaI fragment.

(C) Flow cytometric analysis of CD41 expression on bone marrow cells from wild-type (*gpIIb*$^{wt/wt}$, left panels) and *gpIIb* knockout (*gpIIb*$^{GFPCre/GFPCre}$, right panels). Cells were either analyzed directly after collection (upper panels) or after culturing under conditions to favor outgrowth of megakaryocytic cells (lower panels). Cells were labeled with a monoclonal antibody specific for CD41 and the appropriate secondary reagent (solid lines) or with an isotype control (dashed lines).

Increase in Functional Progenitors in *gpIIb*$^{GFPCre/GFPCre}$ Hematopoietic Tissues

To date, no functional relevance has been ascribed to the expression of GPIIb on hematopoietic progenitors. The *gpIIb*$^{GFPCre/GFPCre}$ mice provide a unique means of investigating the consequences of the loss of GPIIb. We assessed hematopoietic progenitors by in vitro colony-forming unit (CFU) assays in methylcellulose under conditions which allow growth of all myeloid progenitors. We grouped CFU-GM, CFU-G, CFU-M, and CFU-Mix as "CFU-Myeloid". To our surprise, we discovered a consistent 1.5- to 2-fold increase in all types of hematopoietic progenitors in the adult bone marrow of *gpIIb*$^{GFP\text{-}Cre/GFPCre}$ animals compared to their matched wild-type siblings (Figure 5A). This difference was observed irrespective of the blood status of the animals (Figure 5B). We did not observe any skewing in the types of colony arising from *gpIIb*$^{GFPCre/GFPCre}$ bone marrow cells, but we did notice a consistent increase in the size of the colonies arising from mutant cells (average diameter of a d14 CFU-Mix was 1.4 mm and 2.1 mm in assays of wild-type and mutant cells, respectively). We also saw a 1.5-fold increase in CFU numbers and colony size when comparing adult spleen cells derived from wild-type and *gpIIb*$^{GFPCre/GFPCre}$ animals (data not shown).

One of our concerns was that the anemia and thrombocytopenia seen in some of the *gpIIb*$^{GFPCre/GFPCre}$ animals might result in an increase in growth factors such as erythropoietin and thrombopoietin which might influence hematopoiesis in general. We addressed this issue by comparing progenitor numbers from wild-type bone marrow ($n = 8$; RBC count $7.52 \pm 0.8\ 10^6/mm^3$; platelet count $740 \pm 186\ 10^3/mm^3$) with samples taken from mutant mice with little or no overt blood cell deficit ($n = 9$; RBC count $6.69 \pm 1.2\ 10^6/mm^3$; platelet count $573 \pm 80\ 10^3/mm^3$) and from those few showing various degrees of anemia and thrombocytopenia ($n = 2$; RBC count $4.80 \pm 2.4\ 10^6/mm^3$; platelet count $103 \pm 86\ 10^3/mm^3$) and saw the same changes between wild-type and knockout as described above (Figure 5B). In addition, we examined the bone marrow from mutant mice which had near normal blood values using Ter119 in combination with CD71 (TfR) to determine the distribution of cells within the erythroid lineage. This in fact showed some evidence for compensation in that the ratio of reticulocytes/late erythroblasts to more immature stages was increased

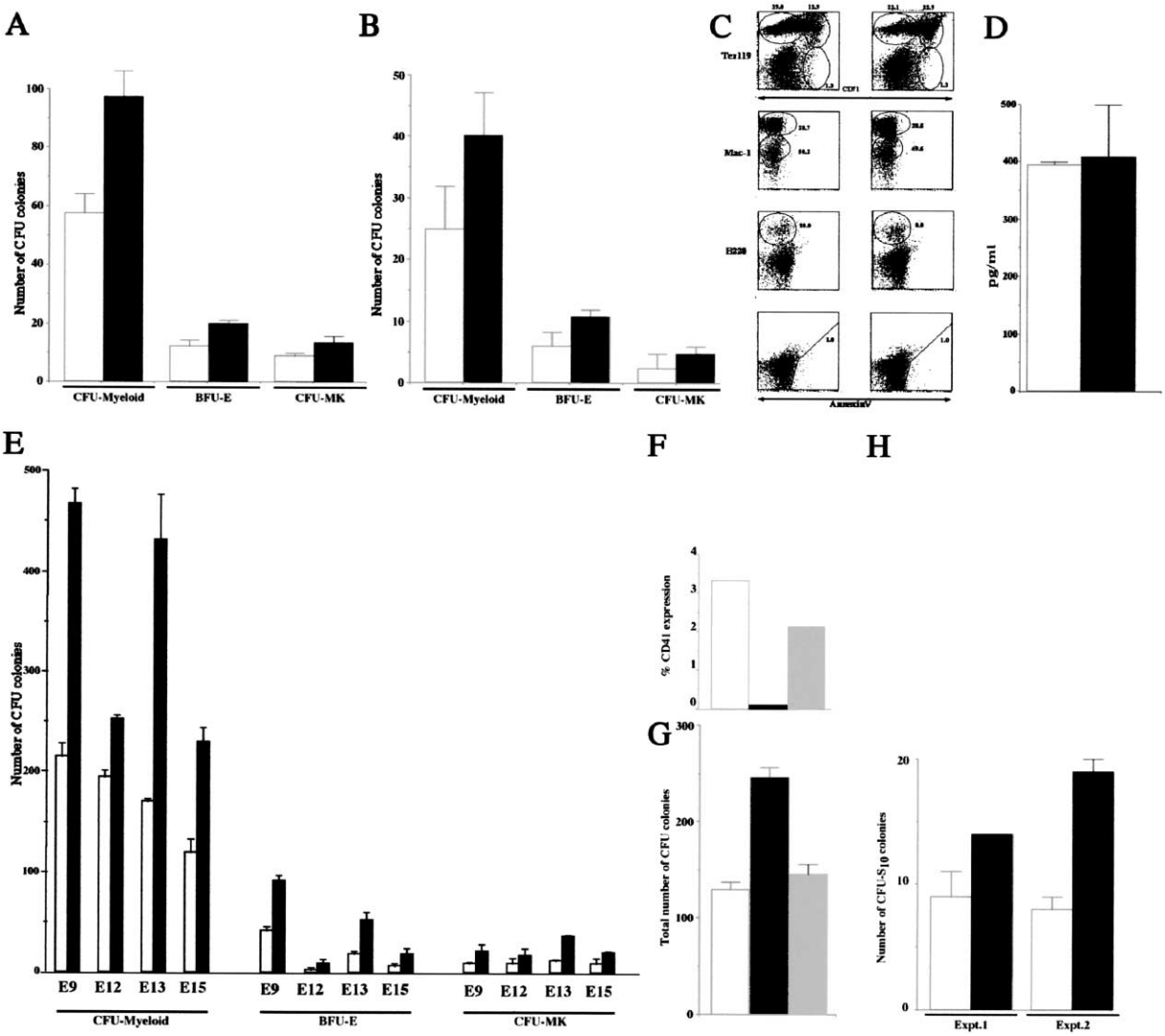

Figure 5. Increase in Functional Progenitors in *gpIIb*$^{GFPCre/GFPCre}$ Animals

(A) Representative assay of colony-forming units (CFU) in *gpIIb*$^{wt/wt}$ (open bars) and *gpIIb*$^{GFCre/GFPCre}$ (black bars) bone marrow. 4×10^4 were cells plated in duplicate in methylcellulose medium supplemented with 25 ng/ml of thrombopoietin (TPO). Colonies were counted at days 5 (BFU-E), 8 (CFU-M, CFU-GM), and 12 (CFU-Mix and CFU-MK). CFU-Myeloid colonies represent all CFU-M, CFU-G, CFU-GM, CFU-Mix. Assays were set up in duplicate from bone marrow derived from healthy wild-type and *gpIIb*$^{GFCre/GFPCre}$ littermate male mice. The error bars represent the standard deviation between duplicates. This experiment was conducted on 23 wild-type and *gpIIb*$^{GFCre/GFPCre}$ littermates in duplicate.

(B) Representative CFU assay on bone marrow from wild-type mice (open bars) and *gpIIb*$^{GFCre/GFPCre}$ animals exhibiting anemia (black bars) were performed as in (A). Three anemic animals were compared in this way to wild-type littermate males.

(C) Flow cytometric analysis of staining with CD71/Ter119, Mac1, or B220 and annexin V binding on bone marrow cells from wild-type (left panels) and *gpIIb* knockout (right panels).

(D) Representative graph of the quantification of TPO levels in the plasma of *gpIIb*$^{wt/wt}$ and *gpIIb*$^{GFCre/GFPCre}$ mice. Three independent *gpIIb*$^{GFPCre/GFPCre}$ animals were compared to sex- and age-matched wild-types. The error bars represent the standard deviation.

(E) Increase in the number of cells with colony-forming ability in *gpIIb*$^{GFPCre/GFPCre}$ yolk sac and fetal liver. 1–4 $\times$ 10^4 E9.5 yolk sac or E12.5, E13.5, and E15.5 fetal liver cells from *gpIIb*$^{wt/wt}$ and *gpIIb*$^{GFCre/GFPCre}$ embryos were assayed for CFU as described in (A). Each bar represents the mean CFU number obtained by comparing two embryos of each genotype from the same litter. One experiment out of three is illustrated.

(F) Restoration of CD41 expression on *gpIIb*$^{GFPCre/GFPCre}$ cells following removal of the neo cassette with FlpE recombinase. The histograms represent the percentage of bone marrow cells staining positively for CD41 expression. *gpIIb*$^{wt/wt}$ (open bars), *gpIIb*$^{GFCre/GFPCre}$ (black bars), and *gpIIb*$^{GFPCre\delta neo/GFPCre\delta neo}$ (gray bars).

(G) Restoration of CFU numbers in *gpIIb*$^{GFPCre\delta neo/GFPCre\delta neo}$ bone marrow. The histogram shows the results of CFU assays of cells as described in (A) performed on bone marrow from *gpIIb*$^{wt/wt}$ (open bar), *gpIIb*$^{GFPCre/GFPCre}$ (black bar), and *gpIIb*$^{GFPCre\delta neo/GFPCre\delta neo}$ (gray bar) animals.

(H) Increase in spleen colony-forming units (CFU-S_{10}) in *gpIIb*$^{GFPCre/GFPCre}$ bone marrow cells. 4×10^4 bone marrow cells from *gpIIb*$^{wt/wt}$ or *gpIIb*$^{GFPCre/GFPCre}$ male littermates were injected intravenously into three lethally irradiated recipients. The spleens were removed and analyzed for macroscopic colonies after 10 days. A single spleen colony was detected in a control group of animals (n = 4) which received no injection of bone marrow cells. Two independent experiments are illustrated. The error bars represent the standard deviation in the group of three injected animals per point.

in knockout mice compared to the wild-type (Figure 5C, upper panel). No other differences could be detected in other cell types (Figure 5C, middle panels) including any change in the number of apoptotic cells as determined by labeling with annexin V-FITC (Figure 5C, lower panel). It should be pointed out that no relative increase in BFU-Es over other CFU colonies was observed in the knockout. Since even a mild degree of thrombocytopenia may have induced changes in the levels of TPO, which is known to be a potent regulator of progenitor development, we measured its level by ELISA in the blood of those animals with a mild platelet phenotype but were unable to detect any differences compared to the wild-type (Figure 5D). Although these results do not completely exclude the possibility of indirect secondary effects in the bone marrow, they show that the increase in progenitors in the *gpIIb* knockout bone marrow does not appear to be caused by obvious hematopoietic compensation.

Next we determined whether an increase in myeloid progenitors could also be seen during embryonic development. CFU assays were performed using cells from E9.5 yolk sac and E12.5, E13.5, and E15.5 fetal liver. Again, we observed a consistent 1.5- to 2-fold increase in all myeloid colonies without any shift in colony type. The differences between wild-type and $gpIIb^{GFPCre/GFPCre}$ were greater at E9.5 and E13.5 (Figure 5E). As in the case of $gpIIb^{GFPCre/GFPCre}$ bone marrow, the colonies arising from $gpIIb^{GFPCre/GFPCre}$ progenitor cells in the yolk sac and fetal liver were larger than in the wild-type assays. Reflecting the results with bone marrow, we were unable to detect any changes in the overall cellular content of E13.5 $gpIIb^{GFPCre/GFPCre}$ fetal liver, but in addition in the latter there were no perturbations to the balance of cells within the erythroid lineage (data not shown)

In order to investigate the effect of restoring GPIIb expression from the $gpIIb^{GFPCre}$ allele, we removed the double FRT-flanked *PGK-neo* resistance cassette by Flp-mediated recombination (Dymecki, 1996). Flow cytometric analysis of CD41 expression on bone marrow cells from $gpIIb^{GFPCre\delta neo/GFPCre\delta neo}$ animals revealed that GPIIb levels were restored to normal (Figure 5F). Correspondingly, the 1.5- to 2-fold increase in the number of progenitors seen in $gpIIb^{GFPCre/GFPCre}$ bone marrow was lost in $gpIIb^{GFPCre\delta neo/GFPCre\delta neo}$ animals (Figure 5G).

Increase in CFU-S_{10} Multipotential Progenitors in $gpIIb^{GFPCre/GFPCre}$ Bone Marrow

To specifically address the question of the effect of GPIIb expression on multipotential progenitors we used the in vivo colony-forming unit-spleen (CFU-S) assay (Till and McCulloch, 1961). We injected 4×10^4 wild-type or $gpIIb^{GFPCre/GFPCre}$ bone marrow cells intravenously into host animals that had been lethally irradiated. Spleens were isolated after 10 days and the number of nodules, representing expansion from a single CFU-S, were counted after fixation. As in the in vitro assays, we observed a 2-fold or greater number of progenitors capable of generating spleen colonies in the bone marrow of $gpIIb^{GFPCre/GFPCre}$ animals compared to wild-type (Figure 5H).

Adhesion to Fibronectin Contributes to the Influence of GPIIb on Progenitor Cell Numbers

Since GPIIb/IIIa has been shown to be a receptor for several extracellular matrix molecules on platelets, we investigated whether GPIIb might have an equivalent role on hematopoietic progenitor cells. The latter develop in close contact with stromal cells and extracellular matrix and express a variety of adhesion receptors which enable them to interact with these components (Prosper and Verfaillie, 2001). Integrin receptors are involved in the homing and retention of pluripotent hematopoietic progenitors in the bone marrow or other specialized hematopoietic microenvironments (Buckley et al., 1998; Verfaillie, 1998), and of the known biologically active ligands which bind GPIIb, likely candidates are fibronectin, fibrinogen, and vitronectin. We compared the CFU potential of wild-type and $gpIIb^{GFPCre/GFPCre}$ bone marrow cells following a step that would allow cells to adhere to immobilized potential ligands. Following a red cell lysis step, 1×10^6 freshly harvested bone marrow cells were plated on either a bone marrow-derived stromal layer or surfaces coated with the purified ligands fibronectin, vitronectin, or fibrinogen. After 6 hr, the adherent and nonadherent fractions were separately collected and plated at equal cell densities into in vitro CFU assays. Under these conditions, the stromal and fibronectin-adherent portion of wild-type bone marrow cells showed a selective enrichment for all myeloid progenitors, which was lost from the $gpIIb^{GFPCre/GFPCre}$ cells (Figure 6A). No difference was seen between wild-type and $gpIIb^{GFPCre/GFPCre}$ cells after allowing adhesion to fibrinogen or vitronectin, therefore showing that specific adhesion to fibronectin was involved.

GPIIb Modulates the Functions of the VLA-4 and VLA-5 Fibronectin Receptors

Since VLA-4 ($\alpha_4\beta_1$) VLA-5 ($\alpha_5\beta_1$) have been shown to be important adhesion receptors for fibronectin (Williams et al., 1991; Kerst et al., 1993; Papayannopoulou and Nakamoto, 1993; Hurley et al., 1995; Craddock et al., 1997; Papayannopoulou et al., 2001), we investigated whether the loss of GPIIb on $gpIIb^{GFPCre/GFPCre}$ cells was related to changes in the expression of these receptors. Flow cytometric analysis of VLA-4 and VLA-5 on bone marrow cells revealed no differences between wild-type and mutant cells (Figure 6B). We performed blocking experiments using specific antibodies against these two integrins to determine whether they are solely responsible for binding to fibronectin or whether GPIIb could be directly involved as well. We incubated bone marrow cells with either anti-VLA-4, anti-VLA-5, or matched isotype control antibodies for 30 min on ice prior to the adhesion step. The results of the CFU assays using these cells showed that in addition to decreased adhesive capacity of mutant cells to fibronectin they had lost most of their binding that could be inhibited by anti-VLA-4 and anti-VLA-5 (Figure 6C), implying a role for GPIIb in modulating the function of VLA-4 and VLA-5.

Discussion

Several questions arise out of the observations that GPIIb is expressed on hematopoietic progenitors. First,

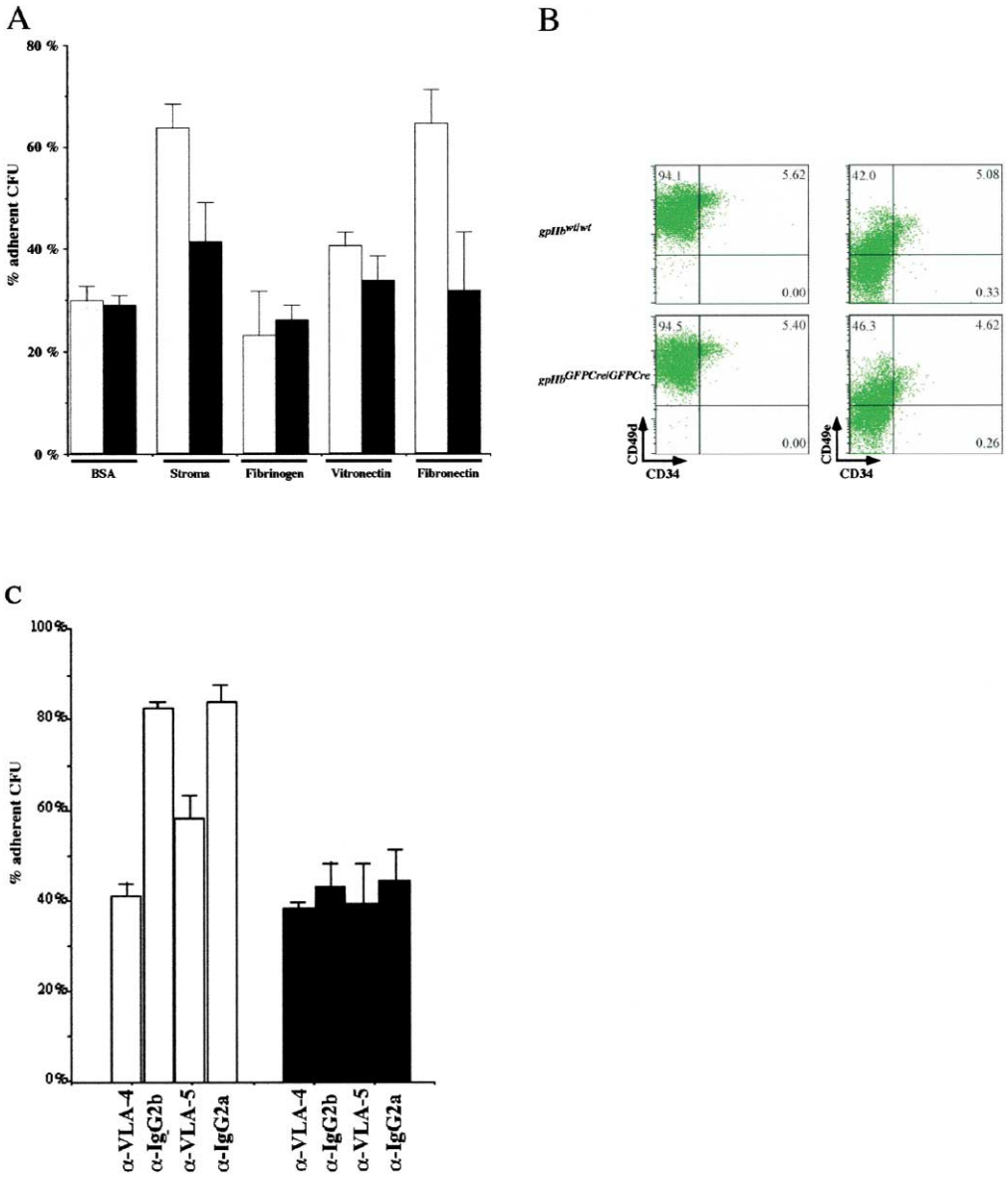

Figure 6. Loss of Fibronectin-Adherent Progenitors in *gpIIb*$^{GFPCre/GFPCre}$ Bone Marrow

(A) Adhesion of bone marrow progenitors to stroma or purified fibronectin is significantly lower for cells derived from *gpIIb*$^{GFPCre/GFPCre}$ compared to wild-type. 1×10^6 unfractionated bone marrow cells were plated on stroma, purified adhesive ligands, or bovine serum albumin (BSA) control for 6 hr in Tyrode's Buffer (pH 7.3). The adherent and nonadherent fractions were collected separately, and 1×10^4 cells were replated in methylcellulose for assay of CFU numbers. The total number of colonies obtained from both fractions was scored after 14 days. The data is represented as the percentage of adherent CFU ([number of CFU in adherent fraction/number of CFU in adherent + nonadherent fractions] $\times$ 100). One out of three experiments is represented. The error bars represent the standard deviation between duplicates.

(B) Two-color flow cytometric analysis of *gpIIb*$^{wt/wt}$ and *gpIIb*$^{GFPCre/GFPCre}$ bone marrow cells using monoclonal antibodies which specifically recognize CD34, α_4 integrin (CD49d), and α_5 integrin (CD49e), showing that there is no significant difference between the wild-type and mutant animals.

(C) Representative anti-α_4 and anti-α_5 integrin blocking experiment. *gpIIb*$^{GFPCre/GFPCre}$ cells appear to have lost the adhesive functions of VLA-4 and VLA-5. 1–2 $\times$ 10^6 unfractionated bone marrow cells were preincubated on ice for 30 min with 10 μg/ml of either anti-mouse CD49d (anti-α_4 PharMingen 01271 D), its isotype control anti-IgG2b (PharMingen), anti-mouse CD49e (anti-α_5 PharMingen 553319), or its isotype control anti-IgG2a (PharMingen). The bone marrow cells were then allowed to adhere to purified fibronectin or BSA control and the CFU potential of the adherent and nonadherent fractions was assayed as described above. One out of three experiments is represented. The error bars represent the standard deviation between duplicates.

how early in the process of hematopoietic commitment is GPIIb expressed? Several markers of hematopoietic progenitors are also detected in endothelial cells, perhaps reflecting the common origin of these two mesodermal lineages; therefore, GPIIb may be expressed at an important developmental junction. Second, do hematopoietic progenitors at all developmental stages express GPIIb equally? There is already some evidence

that this is not the case (Debili et al., 2001); however, it is not clear, for instance, whether GPIIb is expressed on primitive hematopoietic progenitors. Finally, what function, if any, can be ascribed to GPIIb on hematopoietic progenitors, and is this equivalent at all those developmental stages at which it is expressed? We have sought to address these questions using a combination of lineage tracing and analysis of hematopoietic progenitors lacking GPIIb.

Expression of Cre from the *gpIIb-Cre* Transgene Defines a Fetus-Restricted Hematopoietic Progenitor Population

Lineage tracing is a powerful way of following cell fate during development and in the last few years a new approach has been to apply the Cre-LoxP recombination system for this purpose (e.g., Jiang et al., 2000). To apply this strategy to the question of the sites of GPIIb expression, we have utilized a *gpIIb*-driven Cre line in conjunction with the R26R reporter line in which Cre-mediated recombination results in permanent activation of a *lacZ* gene (Soriano, 1999). We believe that the *gpIIb-Cre* transgenic line recapitulates the early expression pattern of the *gpIIb* gene on hematopoietic progenitors because of colocalization of GPIIb protein, Cre protein, and the earliest detectable sites of Cre activity as revealed by the presence of β-galactosidase. Our data from recombination of the R26R allele in *gpIIb-Cre*::R26R mice suggests that the transgene is not active in progenitors of primitive hematopoiesis since we could not detect β-galactosidase in nucleated erythrocytes. However, once circulation is established, Cre is clearly active in hematopoietic progenitors of the yolk sac, AGM, and fetal liver, and the descendants of these cells could be traced to both the myeloid and lymphoid lineages in the fetal liver, spleen, thymus, and bone marrow. Restricted clusters of β-galactosidase-positive cells were also found in close association with the inner layer of the endothelium of blood vessels from E9.5. These were mainly located on the ventral side of the major arteries, which reflected earlier descriptions of GPIIb expression in the aorta in the chick (Ody et al., 1999) and the mouse (Corbel and Salaun, 2002). These intraembryonic clusters are currently believed to represent the main source of definitive hematopoietic stem cells (Manaia et al., 2000) and to be the source of cells populating the fetal liver (Cumano and Godin, 2001; de Bruijn et al., 2000; Garcia-Porrero et al., 1998; Godin et al., 1999). As described previously (Corbel and Salaun, 2002), detection of CD45 expression on the β-galactosidase-positive clusters confirmed their hematopoietic identity although not all β-galactosidase-positive clusters were $CD45^+$ (data not shown). These observations suggest that expression of GPIIb precedes or coincides with that of CD45 in definitive hematopoietic progenitors.

An interesting observation deriving from the lineage tracing experiments is that the majority of adult hematopoietic cells from *gpIIb-Cre*::R26R mice did not contain a recombined R26R allele. Hence, at least 60%–90% of *gpIIb-Cre*::R26R E12.5 or E14.5 fetal liver cells contained the recombined R26R allele, while just prior to birth, recombination was evident in 15% to 55% of hematopoietic cells in the bone marrow, spleen, and thymus. However, only a small proportion of hematopoietic cells (2% to 3%) exhibited the recombined phenotype in adult bone marrow. This suggests that cells marked by Cre expression from the *gpIIb-Cre* transgene do not ultimately give rise to the whole of the adult hematopoietic system and that a separate wave of definitive hematopoiesis is either present concurrently or arises later in the fetus. Our experiments do not allow us to distinguish between these possibilities. Interestingly, distinct classes of GPIIb-expressing progenitors at different stages of development are also proposed by Debili et al. (2001), who showed that sorted neonatal and adult bone marrow $CD41^+$ cells were not necessarily equivalent.

An additional exciting implication is that we may have identified elements which regulate gene transcription of *gp11b* in early hematopoietic progenitors. Although megakaryocyte-specific regulation of the *gpIIb* promoter has been studied quite extensively (Kaluzhny et al., 2001), there is no data presently relating to control elements operating in progenitor cells. Our results suggest that the promoter and intron sequences present in the *gpIIb-Cre* transgene are sufficient for expression in a subset of definitive hematopoietic progenitors but that additional elements may be required for expression in either progenitors that give rise to the majority of adult hematopoiesis or in megakaryocytes.

GPIIb Plays a Role in the Regulation of Hematopoietic Progenitors

The functional significance of GPIIb expression on hematopoietic progenitors has, until now, been unknown. Our findings show that in the absence of GPIIb there is a 1.5- to 2-fold increase in the number of progenitors in hemogenic sites throughout development, suggesting a potential inhibitory role of GPIIb. Although the percentage of GPIIb-expressing cells is higher during development than in the adult bone marrow (12% in E9.5 yolk sac, 4% in E12.5 fetal liver, and 2%–3% in the adult bone marrow), cell sorting data from Corbel and Salaun (2002) show that the actual percentage of $GPIIb^+c\text{-}Kit^+$ cells is similar in E13.5 fetal liver and adult bone marrow (1.2% compared to 0.9%, respectively). Similarity at this level might therefore account for the consistent 1.5- to 2-fold increase that we observed in the *gpIIb* knockout fetus and adult. Our studies do not address whether these observations are a reflection of differences in the developmental functionality of GPIIb-expressing cells, although previous studies by Debili et al. (2001) have shown that $CD34^+CD41^+$ bone marrow cells have a more restricted potential than those derived from cord blood.

Loss of GPIIb Affects the Binding of VLA-4 and VLA-5 to Fibronectin

We suggest that GPIIb plays a role in modulating the adhesion of progenitors to fibronectin on stromal cells and in the extracellular matrix. An extensive network of stromal cells and extracellular matrix ligands present in bone marrow "niches" (Dexter et al., 1990) support stem cell growth, differentiation, and survival (Williams, 1993; Verfaillie et al., 1999). In this context, the interaction of hematopoietic progenitors with fibronectin through integrin receptors has been extensively studied (Hurley

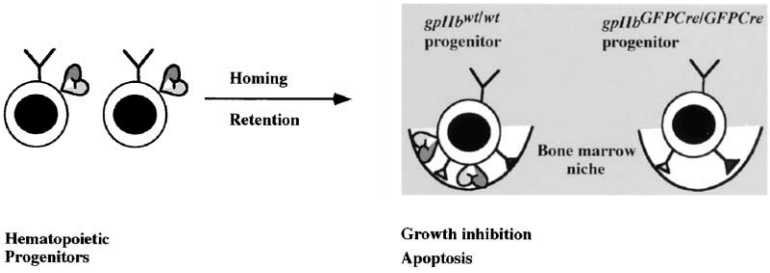

Figure 7. Model for the Role of GPIIb on Hematopoietic Progenitors

Multipotential progenitors home to the bone marrow where GPIIb, through the modulation of VLA-4 and VLA-5, is responsible for their targeting/retention into specialized bone marrow niches where their number is regulated, through inhibition of proliferation or by apoptosis. In the absence of GPIIb, an important component in the regulation of progenitor cell number is lost which results in an increase in multipotential progenitors in the *gpIIb*$^{GFPCre/GFPCre}$ knockout.

et al., 1995; Kerst et al., 1993; Verfaillie et al., 1991; Weinstein et al., 1989; Williams et al., 1991). Several attachment sites to fibronectin have been defined, and it is known that adhesion to fibronectin may directly alter both cell proliferation (in positive and negative ways) (Arroyo et al., 1999; Hurley et al., 1995; Verfaillie and Catanzaro, 1996) and apoptosis (Wang et al., 1998).

Our results show that bone marrow cells lacking GPIIb cannot be selectively enriched for myeloid progenitors by adhesion to a marrow-derived stromal layer or to purified fibronectin. This difference in behavior between wild-type and *gpIIb*$^{GFPCre/GFPCre}$ cells was not seen when the bone marrow cells were plated either on vitronectin or fibrinogen. The loss of GPIIb seems to affect the functionality of the major integrin receptors for fibronectin, VLA-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$), without altering their expression levels. Antibodies against VLA-4 and VLA-5 were able to diminish the ability of wild-type but not mutant progenitor cells to bind to fibronectin. Crossintegrin modulation of binding affinities is a well-described phenomenon (Anzai et al., 1999; Wagers et al., 2002), and our results point to the possibility that GPIIb plays such role on the binding of VLA-4 and VLA-5 to fibronectin. β_1 integrins have been shown to be involved in a cell cycle block at the G1/S phase (Hurley et al., 1995, 1997), and in addition, as we have observed, increased proliferation of hematopoietic progenitors was demonstrated when direct contact between progenitors and stromal cells was prevented (Verfaillie, 1992; Hurley et al., 1995; Verfaillie and Catanzaro, 1996). We therefore speculate that GPIIb, by modulating the VLA-4- and VLA-5-dependent response of hematopoietic progenitors to fibronectin, mediates the anchoring of hematopoietic cells to the bone marrow niche, which, either directly or indirectly, contributes to an inhibition of proliferation of progenitors (Figure 7).

Experimental Procedures

Transgene Construction and Generation of Transgenic Animals

Cre coding sequences were amplified by PCR using pMC-Cre (Gu et al., 1994) as template with the SV40 nuclear localization signal (NLS) fused to it using the primers CreI GTTAATTAAATGCC TAAAAAAAAAAGGAAAGTGTCCAATTTACTGAC and CreII TTAAT TAATGGCTAATCGCCATCTTCC. The NLS-Cre fusion was then cloned into the first intron of the *gpIIb* gene flanked on the 5′ side by a fusion of the mouse engrailed-2 protein splice acceptor (SA) and the ECMV IRES from pGTIRESβgeo (Mountford et al., 1994) and by an SV40 polyadenylation site on the 3′ end to give rise to the *gpIIb-Cre*. Two artificial NotI sites were inserted at either end of the construct, which encompasses 3.0 kbp of the promoter, first exon, and the modified first intron of the *gpIIb* gene to facilitate release of the 8.0 kbp transgene. Purified DNA was injected into the male pronuclei of oocytes. The transmission of the transgene was confirmed by PCR detection of Cre sequences in DNA from tail biopsies (Cre5′ TCGATGCAACGAGTGATGAG and Cre3′ TTCGGCTATACG TAACAGGG). Transgene copy number of *gpIIb-Cre* founders was determined by Southern blotting of EcoRI-digested DNA using a *gpIIb* exon 1-specific probe which identifies a 1.2 kbp fragment.

Targeting Vector Construction

A 129/SvEv mouse phage genomic library (Stratagene Inc.) was screened using a probe encompassing the promoter region of *gpIIb* from −548 to −1 bp (Denarier et al., 1993). A 12 kbp HindIII fragment encompassing 6 kbp of the *gpIIb* promoter, exon 1, and about 5 kbp of intron 1 was ligated into pBluescript IISK$^-$. The targeting construct was made by insertion into intron 1 of a 3.8 kbp cassette consisting of the GFPCre fusion from pBS558 (Gagneten et al., 1997) flanked by a splice acceptor-IRES fusion on the 5′ side from pGTIRESβgeo (Mountford et al., 1994) and by an SV40 polyadenylation site on the 3′ end. A fragment containing the neomycin resistance gene driven by the PGK promoter from the pPNT plasmid (Tybulewicz et al., 1991) was flanked by FRT sites and inserted downstream of the GFPCre cassette in the *gpIIb* intron 1. A *tk* gene driven by the mouse PGK promoter from pPNT (Tybulewicz et al., 1991) was inserted at the 3′ end of the targeting vector, and unique FseI and NotI sites were introduced to facilitate linearization yielding the 20.4 kbp targeting vector pgpII-GFPCreTV4.

ES Cell Targeting and Generation of *gpIIb*^{GFPCre} Mice

The linearized pgpII-GFPCReTV4 vector (50 μg) was electroporated (240 V, 500 μF) into 1 × 10^7 TL1 129SvEv ES cells and maintained as described previously (Winnier et al., 1995). Three hundred sixteen colonies resistant to G418 (280 μg/ml) and gancyclovir (0.2 μM) selection were isolated 7 days after electroporation. Homologous recombination was detected by Southern blot hybridization of ES clone DNA digested with ScaI or BstXI, using a combination of 5′ and 3′ probes which contain sequences external to the targeting vector sequences. An internal *neo* probe, which recognizes only the targeted allele, was used to further confirm the identity of targeted events. Correctly targeted events occurred at a frequency of 1 in 20. Two positive ES cell clones (C2 and B3) were injected into 3.5 day blastocysts harvested from superovulated C57BL/6 donors. Germline transmission was evaluated by backcrossing the resultant chimeras to C57BL/6 mice. The C2 and B3 chimeras gave rise to independent *gpIIb*^{GFPCre} lines, which are indistinguishable. All the results described in this paper are taken from the C2 line.

gpIIb$^{wt/GFPCre}$ heterozygotes, originally on a 129Sv background, were maintained by backcrossing to C57BL/6. Genotyping to discriminate *gpIIb*^{GFPCre} from *gpIIb* used primers that amplify 500 bp of the coding sequence for Cre as described above in conjunction with primers that amplify 435 bp of intron 1 of the *gpIIb* gene (CAG ACTCCTGGCTCATCTAC and TCTGCCTAACTCTGCTTTCC). The *gpIIb*$^{GFPCre/GFPCre}$ embryos were produced by mating *gpIIb*$^{+/GFPCre}$ heterozygotes, the morning of appearance of vaginal plugs being set as day 0.5.

Determination of R26R Recombination by Southern Blotting

DNA was extracted from the fetal livers, hind limbs, and tails of E12.5 and E14.5 *gpIIb-Cre*::*R26R* embryos or bone marrow (with red cell lysis) of adult *gpIIb-Cre*::*R26R*. Twenty micrograms of genomic DNA was digested with EcoRV, run on an agarose gel, and transferred onto a nitrocellulose membrane which was probed with a 500 bp fragment for the ROSA26 locus. In addition to the 11 kbp wild-type ROSA allele, this probe recognizes the mutated 3.8 kbp allele and the recombined 3.3 kbp R26R allele.

Histological Staining and Sectioning

Embryos were dissected out at various stages of gestation and fixed in 0.4%–4% paraformaldehyde for 30 min to 4 hr depending on their size. The embryos were then washed in PBS and dehydrated in a methanol gradient prior to further processing or sectioning. Whole-mount β-galalctosidase staining was performed as described previously (Hogan et al., 1994), and 0.2 μm sections were obtained and lightly counterstained with eosin.

Whole-Mount Antibody Staining

Following dehydration and rehydration, fixed embryos were bleached in 3% hydrogen peroxide for 10 min. After washing in PBS containing 0.2% BSA and 0.1% Triton X-100, the embryos were blocked in 5% goat serum and incubated at room temperature overnight with a 1:300 dilution of a polyclonal Cre antibody (Babco PRB-106C) or a polyclonal CD41 antibody (Santa Cruz, sc-6602). The embryos were then washed and incubated with a secondary goat-anti-rabbit or rabbit anti-goat antibody linked to alkaline phosphatase (DAKO) at a 1:50 dilution for 1 hr at room temperature. After washing, the specimens were developed using NBT/BCIP (Roche).

Immunohistochemistry for Cre Recombinase on Paraffin Sections

Dewaxed sections were bleached in 3% hydrogen peroxide for 30 min, blocked in 1% normal goat serum, and incubated for 30 min at room temperature with a 1:300 dilution of a polyclonal Cre antibody (Babco PRB-106C). After washing, the slides were then incubated with a secondary anti-rabbit biotinylated antibody for 30 min at room temperature and developed using the VIP reagent (Vector Laboratories).

Immunofluorescent Staining and Flow Cytometry

Single-cell suspensions of bone marrow or fetal liver were prepared by standard techniques. Red cells were depleted from the preparations by lysis in ACK buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA). Monoclonal antibodies were used either directly conjugated (Ter119-biotin, c-Kit-PE, CD11b-biotin, CD34-biotin, Sca1-biotin) or indirectly labeled (CD41) using an appropriate isotype-specific conjugated secondary antibody (anti-rat IgG1-FITC or anti-rat IgG1/G2a-biotin). With the exception of anti-rat IgG1-FITC (Sera Labs, United Kingdom), all unconjugated and conjugated antibodies were obtained from PharMingen/Becton Dickinson. Flow cytometric measurement of β-galactosidase activity was performed as described in Sanchez et al. (1999). Stained cells were run immediately on a Becton Dickinson FACSCalibur employing CellQuest software.

Colony-Forming Assay

Cell preparations were obtained from adult femurs or from embryonic fetal livers and yolk sacs of staged embryos. The cells were passed through a 24 gauge needle and then through a cell strainer to obtain single-cell preparations. Adult bone marrow cells were ACK treated. 1–4 $\times$ 10^4 cells were plated in 1% methylcellulose medium containing a cocktail of growth factors (Methocult M3434, Stem Cell Technologies) and supplemented with 25 ng/ml rhTPO (a gift from Genentech). Colony morphology and number was assessed on days 3–5 (BFU-E) and days 10–12 (CFU-MK and myelomonocytic colonies) by scoring under an inverted light microscope.

Spleen Colony-Forming Unit Assay

Six- to eight-week-old C57BL/6 recipient female mice were irradiated with 10 Gy from a cobalt source at a rate of 0.5 Gy/min. 4 $\times$ 10^4 ACK-treated bone marrow cells from independent male donors (either of wild-type or mutant *gpIIb*$^{GFPCre/GFPcre}$) were injected via the lateral tail vein. After 10 days the mice were sacrificed and their spleens were removed and fixed in Tellesniczky's fixative. Colonies were counted after 2 hr of fixation.

Quantification of Thrombopoietin

Age- and sex-matched wild-type and *gpIIb*$^{GFPCre/GFPCre}$ adults were sacrificed, and blood was collected in acid citrate buffer. The samples were spun to remove the cellular content, and the plasma was prepared according to the manufacturer's instructions (Quantikine M Murine TPO Detection Kit; R&D Laboratories) to determine TPO levels by immunoassay. The plate was then read at A_{450} with a correction wavelength of A_{540}.

Adhesion Assay

Adhesive substrata were prepared from bone marrow-derived stromal cells plated at subconfluent density on 6 cm dishes. Alternatively, the plates were coated with 5 μg/ml fibronectin, fibrinogen, or vitronectin (Sigma) in PBS for 2 hr at 37°C. After washing in PBS, the plates containing the purified ligands were then blocked in 3% BSA in PBS for an hour at 37°C. 1–2 $\times$ 10^6 freshly harvested bone marrow cells following red cell lysis were plated on the various substrata on 6 cm plates in Tyrode's Buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM $Na_2HPO_4.12H_2O$, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ [pH 7.3]) and allowed to adhere for 6 hr. The nonadherent fraction was then harvested with two consecutive gentle washes in warm PBS while the adherent fraction was briefly trypsinized. The nonadherent fraction was also briefly trypsinized so that the two fractions had been treated equally. After washing, the cells were resuspended in PBS and counted, and 2–4 $\times$ 10^4 cells from both fractions were plated in methylcellulose. The total number of myeloid colonies (BFU-E, CFU-M, -G, -GM, -MK, and -Mix) was scored 12–14 days after plating.

Antibody-Blocking Assays

1–2 $\times$ 10^6 freshly harvested bone marrow cells following red cell lysis were incubated on ice for 30 min with 10 μg/ml integrin-specific blocking antibody or matched isotype controls: anti-VLA-4 (CD49d, clone R1-2), anti-rat IgG2b, anti-VLA-5 (CD49e, clone 5H10), anti-rat IgG2a (all from PharMingen). The cells were then plated on purified fibronectin in Tyrode's Buffer and allowed to adhere as described above. The nonadherent and adherent fractions were separately harvested and treated in a similar way. The cells were then counted and set up in CFU assays, and colonies were counted after 7 days.

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