

**$\alpha 4\beta 1$  integrin and erythropoietin mediate temporally distinct steps in erythropoiesis: integrins in red cell development**

Shawdee Eshghi, Mariette G. Vogeleyzang, Richard O. Hynes, Linda G. Griffith, and Harvey F. Lodish

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In the months since the publication of our manuscript, an important technical issue has come to our attention. In subsequent work, we learned that the anti-integrin  $\alpha 4$  antibody (Serotec MCA1230) used in the experiments detailed in Figs. 8 C, 9 B, and 10 contained 0.09% sodium azide as a preservative, which can potentially contribute to cell death. The observed inhibition of erythroid cell proliferation by this antibody could have been due at least in part to the azide. We do not feel that this detail minimizes the conclusion of our work that  $\alpha 4\beta 1$  integrin protects terminally differentiating erythroid progenitor cells from apoptosis, but we do wish to bring it to your attention. The role of  $\alpha 4\beta 1$  integrin in erythropoiesis was made clear in the following experiments that did not utilize this antibody preparation:

In Fig. 8 B, the role of  $\alpha 4\beta 1$  integrin in mediating adhesion of erythroid progenitors to fibronectin is shown using mutant fibronectin fragments, without the use of any antibody.

In Fig. 8 C, the addition of the azide-containing  $\alpha 4$  antibody had no effect on the number of cells adhering to the V0 fragment.

In Fig. 9 A, the role of  $\alpha 4\beta 1$  integrin in mediating proliferation of terminally differentiating erythroid progenitors is seen without the use of any antibody.

In Fig. 9, C and D, the specific effect of  $\alpha 4\beta 1$  integrin during the second phase of the culture period is seen without the use of any antibody.

We feel that this additional information could be helpful to those also utilizing in their research function-blocking antibodies to cell surface adhesion receptors.

# $\alpha_4\beta_1$ integrin and erythropoietin mediate temporally distinct steps in erythropoiesis: integrins in red cell development

Shawdee Eshghi,<sup>1,2,6</sup> Mariette G. Vogeleyzang,<sup>3</sup> Richard O. Hynes,<sup>3,4</sup> Linda G. Griffith,<sup>1,2,5</sup> and Harvey F. Lodish<sup>1,2,4,6</sup>

<sup>1</sup>Division of Biological Engineering, <sup>2</sup>Biotechnology Process Engineering Center, <sup>3</sup>Center for Cancer Research, <sup>4</sup>Department of Biology, and <sup>5</sup>Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139

<sup>6</sup>Whitehead Institute for Biomedical Research, Cambridge, MA 02142

Erythropoietin (Epo) is essential for the terminal proliferation and differentiation of erythroid progenitor cells. Fibronectin is an important part of the erythroid niche, but its precise role in erythropoiesis is unknown. By culturing fetal liver erythroid progenitors, we show that fibronectin and Epo regulate erythroid proliferation in temporally distinct steps: an early Epo-dependent phase is followed by a fibronectin-dependent phase. In each phase, Epo and fibronectin promote expansion by preventing apoptosis partly through bcl-xL. We show that

$\alpha_4$ ,  $\alpha_5$ , and  $\beta_1$  are the principal integrins expressed on erythroid progenitors; their down-regulation during erythropoiesis parallels the loss of cell adhesion to fibronectin. Culturing erythroid progenitors on recombinant fibronectin fragments revealed that only substrates that engage  $\alpha_4\beta_1$ -integrin support normal proliferation. Collectively, these data suggest a two-phase model for growth factor and extracellular matrix regulation of erythropoiesis, with an early Epo-dependent, integrin-independent phase followed by an Epo-independent,  $\alpha_4\beta_1$ -integrin-dependent phase.

## Introduction

In mammals, definitive erythropoiesis first occurs in the fetal liver with progenitor cells from the yolk sac (Palis et al., 1999). Within the fetal liver and the adult bone marrow, hematopoietic cells are formed continuously from a small population of pluripotent stem cells that generate progenitors committed to one or a few hematopoietic lineages. In the erythroid lineage, the earliest committed progenitors identified ex vivo are the slowly proliferating burst-forming unit–erythroblasts (BFU-Es; Gregory and Eaves, 1977, 1978). Early BFU-E cells divide and further differentiate through the mature BFU-E stage into rapidly dividing colony-forming unit–erythroblasts (CFU-Es; Gregory and Eaves, 1977, 1978). CFU-E progenitors divide three to five times over 2–3 d as they differentiate and undergo many substantial changes, including a decrease in cell size, chromatin condensation, and hemoglobinization, leading up to the enucleation and expulsion of other organelles (Fawcett, 1997).

Erythropoietin (Epo) has long been understood to be the major factor governing erythropoiesis; its role in regulating the expansion, differentiation, apoptosis, and activation of erythroid-specific genes is well characterized (Richmond et al., 2005).

The first phase of erythroid differentiation is highly Epo dependent, whereas later stages are no longer dependent on Epo (Koury and Bondurant, 1988). Consistent with this, Epo receptors are lost as erythroid progenitors undergo terminal proliferation and differentiation (Zhang et al., 2003). This raises the question of what other signals, if any, these differentiating erythroblasts require to support terminal proliferation, differentiation, and enucleation. The extracellular matrix protein fibronectin has been identified as an important part of the erythroid niche in both the adult bone marrow and fetal liver (Tada et al., 2006), but its precise role in erythropoiesis and potential interaction with Epo-mediated signals is unknown.

Fibronectin is a ubiquitous extracellular matrix molecule that presents developmental cues to many cell types, including hematopoietic cells (Hynes, 1990). Interactions with fibronectin are essential for proper erythropoiesis, as adhesion to fibronectin is required for the enucleation of murine erythroleukemia cells (Patel and Lodish, 1987). Human bone marrow erythroid progenitor cells expand in the presence of fibronectin in a dose-dependent manner and do not form enucleated erythroid colonies in the absence of fibronectin (Weinstein et al., 1989). Collectively, these findings suggest that fibronectin not only provides a supportive niche for erythroid progenitor cells but also plays a role in ensuring proper terminal expansion and differentiation.

Correspondence to Harvey F. Lodish: lodish@wi.mit.edu

Abbreviations used in this paper: BFU-E, burst-forming unit–erythroid; CFU-E, colony-forming unit–erythroid; Epo, erythropoietin; FAK, focal adhesion kinase.

Fibronectin is a large multidomain glycoprotein that contains binding sites for heparin, collagen, fibrin, and gelatin in addition to a number of cell surface receptors. Adhesion of hematopoietic cells to fibronectin is mediated by at least two integrin pairs.  $\alpha_5\beta_1$  integrin (VLA-5) mediates adhesion to the canonical RGDS sequence in the 10th type III repeat. There are two cell-binding sequences in the type III connecting segment that mediate adhesion to  $\alpha_4\beta_1$  integrin (VLA-4). The LDV sequence forms a high affinity binding site, whereas the REDV sequence forms a binding site with much lower affinity (Humphries et al., 1986; Komoriya et al., 1991).

$\alpha_4$  integrins appear to be essential for the efficient differentiation and expansion of erythroid progenitors *in vivo* and *in vitro*. Deletion of  $\alpha_4$  integrin has no effect on the number of fetal liver erythroid progenitors but results in decreased numbers of differentiated erythroid cells. In *in vitro* erythropoiesis assays, fetal liver  $\alpha_4$ -null erythroid cells formed only small pale colonies, whereas wild-type cells transmigrated beneath the stroma, expanded, and formed large red colonies (Arroyo et al., 1999). Early studies have shown that fetal liver erythroid cells express both  $\alpha_4$  and  $\alpha_5$  integrins and that these integrins mediate attachment of the CFU-E to fibronectin and stromal cells (Vuillet-Gaugler et al., 1990; Rosemblatt et al., 1991; Verfaillie et al., 1994). However, the biological significance of this adhesion is not yet known.

In this study, we sought to characterize the precise role that fibronectin plays in erythropoiesis by using an *in vitro* model of fetal erythropoiesis (Zhang et al., 2003). In this system, populations of erythroid progenitors at varying phases of differentiation can be purified from the fetal liver based on expression of the cell surface markers CD71 and TER119; these

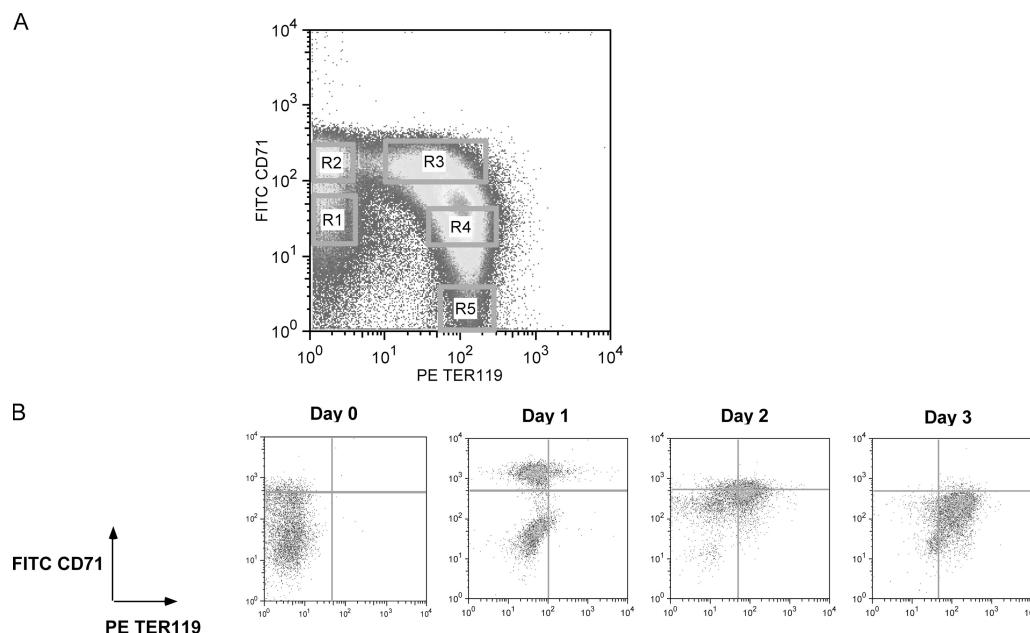
same markers can then be used to track the differentiation of progenitor cells cultured *in vitro*. We present data to support a novel model for erythropoiesis wherein Epo and fibronectin each play a distinct, essential role. We show that erythroid expansion proceeds in two phases, with an early Epo-dependent phase followed by a fibronectin-dependent phase. We also determine that  $\alpha_4\beta_1$  integrin is the primary molecular mediator of the observed fibronectin response and that signals emanating from  $\alpha_4\beta_1$  engagement by fibronectin act to protect cells from apoptosis in a manner similar to the binding of Epo to its receptor.

## Results

### Erythroid expansion proceeds in temporally distinct Epo and fibronectin-mediated phases

We used murine fetal liver cells to study erythroid differentiation. As previously described, fetal liver erythroid cells can be separated into five distinct populations of progressively differentiated cells based on the expression of CD71, the transferrin receptor, and TER119, an erythroid-specific glycoprotein (Fig. 1 A; Zhang et al., 2003). Early erythroid progenitors (the R1 population) express moderate levels of CD71 and are TER119<sup>-</sup>. Later progenitor cells (R2) express higher levels of CD71 but are still TER119<sup>-</sup>. As cells continue to divide and differentiate, TER119 expression is induced, and CD71 expression is down-regulated, as indicated by the R3, R4, and R5 populations. R3–5 populations contain no CFU-E activity.

The expression of CD71 and TER119 can also be used to track erythroid differentiation *in vitro*. In this system, purified embryonic day (E) 14.5 TER119<sup>-</sup> progenitor cells are cultured

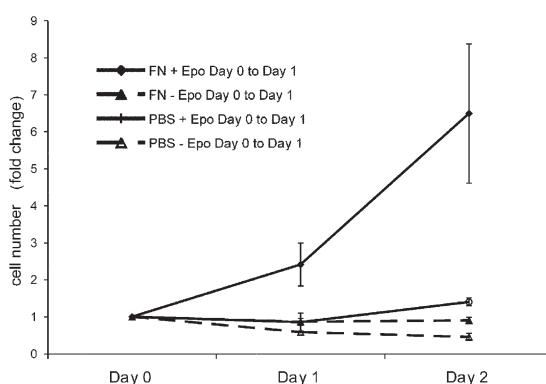


**Figure 1. CD71 and TER119 can be used as markers of erythroid differentiation in vivo and in vitro.** (A) Five populations of sequentially differentiated erythroid cells can be isolated from day 14.5 fetal liver based on the expression of CD71 and TER119 as previously described (Zhang et al., 2003). (B) CD71 and TER119 can be used as markers of erythroid differentiation *in vitro*. Day 14.5 TER119<sup>-</sup> fetal liver cells were cultured on fibronectin-coated plates in Epo-containing media for 1 d and in Epo-free media for an additional 2 d. On each day, cells were dissociated and stained with antibodies to CD71 and TER119 for FACS analysis.

on fibronectin-coated plates in two phases: first in the presence of Epo for 16–18 h and then without Epo for an additional 24 h. On each day, cells are dissociated with PBS containing EDTA, counted, and stained with CD71 and TER119 antibodies for FACS analysis. In the first phase, CD71 is up-regulated, and there is a modest expansion in cell number. By day 3, TER119 is up-regulated, and CD71 is down-regulated (Fig. 1 B).

We used this *in vitro* model to study the role of fibronectin in erythropoiesis. We observed a dramatic increase in cell number between days 1 and 2 for TER119<sup>−</sup> progenitor cells cultured on fibronectin but not on uncoated substrates despite the presence of Epo between days 0 and 1 in both cases (Fig. 2). As previously shown, the withdrawal of Epo during the first day of culture leads to an expansion defect even in the presence of fibronectin (Fig. 2, dashed lines).

This result led us to hypothesize that Epo and fibronectin regulate erythroid expansion in temporally distinct regimes. To test this hypothesis, we varied the presentation of extracellular matrix and growth factor cues during the course of the 2-d culture period. When TER119<sup>−</sup> erythroid progenitors were cultured on a control substrate for 1 d and were transferred to a fibronectin substrate for the second day, the level of expansion was indistinguishable from that achieved in the presence of fibronectin for the entire 2-d period, suggesting that fibronectin is only required during the second day of erythroid culture (Fig. 3 A). In another test, we isolated TER119<sup>+</sup> cells from the fetal liver. As described previously, TER119 is a marker of differentiated erythroid cells (Kina et al., 2000), so we hypothesized that the expansion of these cells is fibronectin but not Epo dependent. Indeed, when we cultured differentiated TER119<sup>+</sup> erythroid cells, we found that they expanded on fibronectin and not on the control substrate and that the addition of Epo had no effect (Fig. 3 B). It is important to note that these freshly isolated TER119<sup>+</sup> cells have not been exposed to Epo *ex vivo*, and, thus, the response can be attributed solely to the presence of fibronectin. Collectively, these results suggest a two-phase model for erythroid expansion in which the presence of Epo on the first day of culture followed



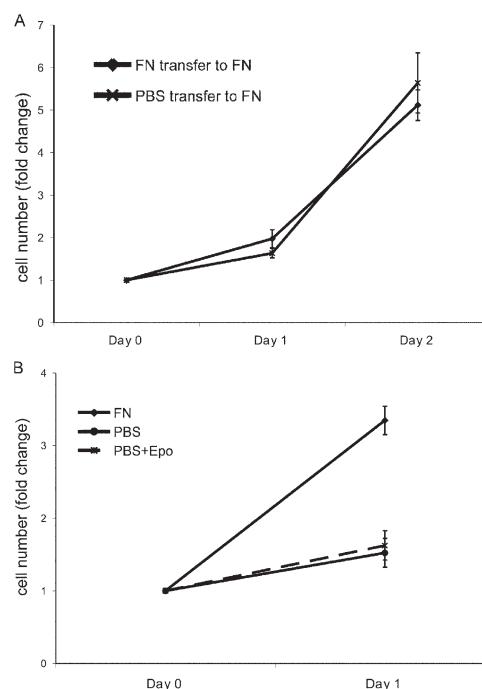
**Figure 2. Fibronectin supports the expansion of erythroid progenitors.** TER119<sup>−</sup> fetal liver erythroid progenitor cells were cultured on fibronectin or control (uncoated) substrates with Epo-containing media for 1 d and then in Epo-free media for a second day. At days 1 and 2, the number of live cells was counted. Progenitor cells cultured on fibronectin undergo dramatic expansion in cell number, but cells cultured on a control substrate do not. Each point is the mean of three to five independent experiments. Error bars represent the SD.

by the presence of fibronectin on the second day of culture is each essential for proper erythroid expansion.

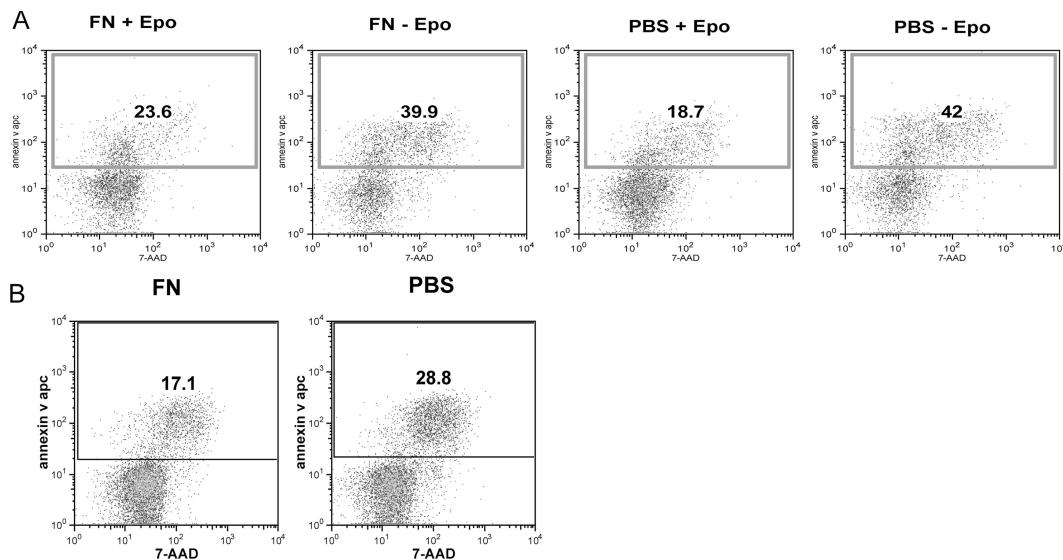
### Fibronectin protects from apoptosis in the second phase of erythroid expansion

It is well known that Epo protects early erythroid progenitors from apoptosis, (Socolovsky et al., 1999) so we tested the hypothesis that fibronectin acts in a similar manner. First, we examined the roles of Epo and fibronectin in preventing the apoptosis of early erythroid progenitors in the first phase of erythropoiesis. We found that nearly 40% of TER119<sup>−</sup> cells cultured overnight on fibronectin in the absence of Epo were annexin V positive (Fig. 4 A). When Epo was present in the culture medium, the percentage of annexin V-positive cells dropped to 23.6%, confirming that Epo plays an antiapoptotic as well as proliferative role during the first phase of erythropoiesis. However, the absence of fibronectin either in the presence or absence of Epo did not have an effect on the level of apoptosis (Fig. 4 A, 23.6% FN + Epo vs. 18.7% PBS + Epo and 39.9% FN-Epo vs. 42.0% PBS-Epo).

To test the role of Epo and fibronectin in preventing apoptosis during the second phase of erythropoiesis, TER119<sup>−</sup> progenitors were cultured overnight on uncoated substrates with Epo as in Fig. 4 A and were transferred to fresh fibronectin or control substrates in the absence of Epo. 4 h after Epo removal, 28.8% of the cells from control wells were annexin V positive



**Figure 3. Fibronectin is only required on the second day of culture.** (A) TER119<sup>−</sup> erythroid progenitors were cultured on either fibronectin or uncoated surfaces for 1 d in Epo-containing media and were dissociated and transferred to fresh fibronectin surfaces in Epo-free media. A dramatic expansion in cell number was observed regardless of which substrate was present on the first day of culture. (B) TER119<sup>+</sup> cells were cultured on fibronectin or uncoated surfaces in the absence of Epo. Only those cells cultured on fibronectin proliferate; the addition of Epo to the culture media (dashed line) had no effect on cell number. Each point is the mean of three to five independent experiments. Error bars represent the SD.



**Figure 4. Epo and fibronectin protect against apoptosis.** (A) TER119<sup>-</sup> progenitor cells were cultured overnight on fibronectin or uncoated substrates in the presence or absence of Epo. Annexin V binding (shown on the y axis) was assayed via flow cytometry, and the percentage of positive cells is indicated in each plot. The absence of Epo during the first day of culture leads to an increase in the percentage of apoptotic cells, but the absence of fibronectin does not have an effect. (B) Fibronectin protects from apoptosis in the second stage of proliferation. TER119<sup>-</sup> progenitor cells were cultured overnight on uncoated surfaces in the presence of Epo and were dissociated and transferred to fresh fibronectin or control surfaces in Epo-free media. Annexin V binding was assayed at 4 h. The absence of fibronectin in the second phase of culture led to an increased percentage of annexin V-positive cells. These data are representative of three to five independent experiments.

versus 17.1% of those on fibronectin (Fig. 4 B). Thus, the presence of fibronectin during the second phase partially protects erythroid cells from apoptotic death.

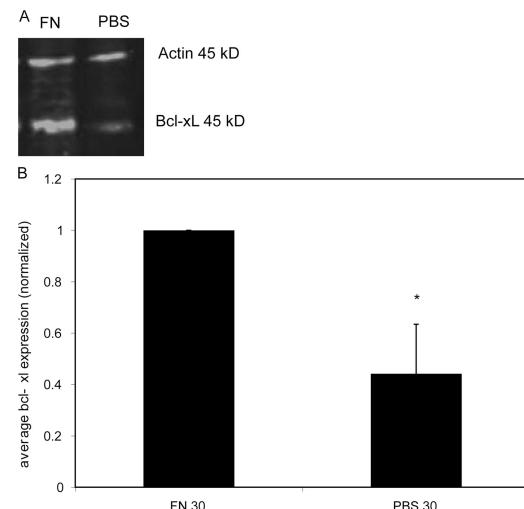
One of the mechanisms of apoptosis protection mediated by Epo is through up-regulation of the antiapoptotic protein bcl-xL (Socolovsky et al., 1999), so we tested whether fibronectin protects against apoptosis in a similar manner in the second phase of erythropoiesis. TER119<sup>-</sup> progenitors were cultured overnight on uncoated substrates in the presence of Epo and were serum starved for 1 h before being transferred to fresh fibronectin or control substrates in the absence of Epo. Cells were lysed at 30 min, and bcl-xL protein expression was assessed via Western blotting. As shown in Fig. 5 (A and B), bcl-xL expression is distinctly higher in cells cultured on fibronectin than in those cultured in control wells. Similar results were obtained via quantitative PCR as well as flow cytometry (unpublished data). These data support the idea that fibronectin induces signals that act to prevent apoptosis in the second phase of erythropoiesis, much like the binding of Epo to its receptor does in the first phase.

#### Erythroid cells express fibronectin receptors $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins

We were interested in determining the molecular mediators of the observed fibronectin response, so we focused on integrins as fibronectin receptors. As previously shown, E14.5 fetal liver cells can be separated into five distinct phases of erythroid development on the basis of the relative expression of CD71 and TER119 (Fig. 1 A; Zhang et al., 2003). This system allowed us to examine the expression of candidate fibronectin receptors at distinct phases in erythropoiesis that correlate with our two-phase model.

We used flow cytometry to determine fibronectin receptor expression on the surface of fetal liver erythroid progenitors.

By costaining E14.5 fetal liver cells with antibodies to CD71 and TER119 as well as a panel of integrin subunits, we determined that  $\alpha_4$ ,  $\alpha_5$ , and  $\beta_1$  are the most highly expressed integrins on these cells. The expression of  $\alpha_2$ ,  $\alpha_v$ ,  $\beta_2$ , and  $\beta_3$  integrins

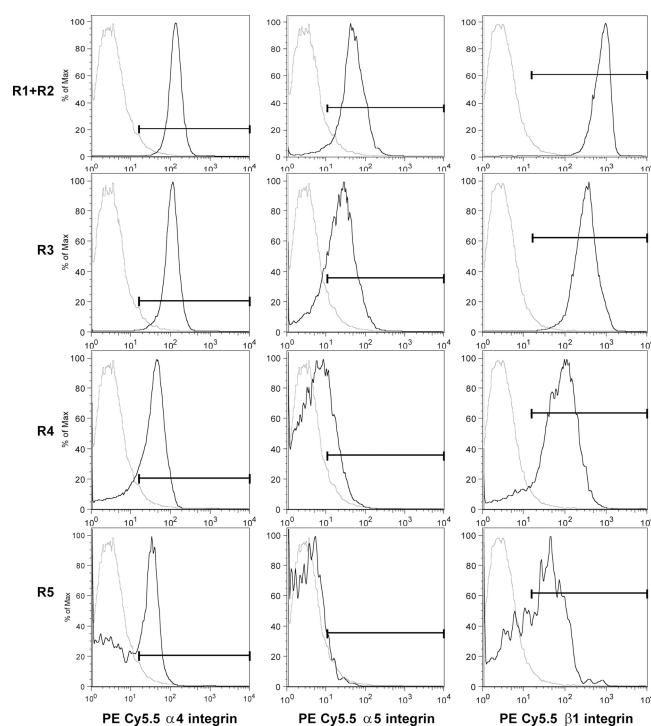


**Figure 5. Fibronectin up-regulates bcl-xL in the second stage.** TER119<sup>-</sup> progenitor cells were cultured overnight on uncoated surfaces in the presence of Epo and were dissociated and serum starved for 1 h before being transferred to fresh fibronectin or control surfaces in Epo-free media. bcl-xL expression was assayed at 30 min via Western blotting. (A) Representative Western blot indicating the up-regulation of bcl-xL on fibronectin and the equivalent expression of actin as a loading control. The anti-actin and anti-bcl-xL antibodies were raised in different hosts, thereby allowing the visualization of both proteins on the same membrane. (B) Quantification of five identical experiments. Integrated fluorescence intensity on the control substrate was normalized to that on fibronectin, indicating a statistically significant decrease in the absence of fibronectin. \*, P ≤ 0.01. Error bars represent the SD.

was not distinguishable from the background fluorescence on erythroid progenitor R1 and R2 cells, a pooled population of progenitors we refer to as R1 + R2 cells (unpublished data). The expression of  $\alpha_4$ ,  $\alpha_5$ , and  $\beta_1$  integrins is down-regulated as cells differentiate (Fig. 6). R1 + R2 cells express the highest levels of each integrin subunit, and the expression levels in the R3, R4, and R5 populations progressively decrease. Although R4 and R5 cells have completely lost the expression of  $\alpha_5$  integrin, the expression of  $\alpha_4$  and  $\beta_1$  integrins is clearly distinct from the background in these populations.

#### Adhesion to fibronectin is correlated with integrin receptor expression

To test the functional role of the loss of integrin expression on fetal liver erythroid cells, we adapted a quantitative cell-extracellular matrix adhesion assay for use with the fetal liver erythroid system. A centrifugation assay is ideal for this application because it is highly quantitative and repeatable while requiring no special equipment (Hertl et al., 1984). Using this assay, we demonstrated a progressive loss in adhesion to fibronectin during erythroid development (Fig. 7 A). Sorted R1–5 cells were allowed to attach to plates coated with 10 or 3  $\mu\text{g}/\text{ml}$  human plasma fibronectin and were centrifuged at an acceleration of  $\sim 1,000 \text{ g}$ . Whereas 75% of R1 + R2 cells adhere to 10  $\mu\text{g}/\text{ml}$



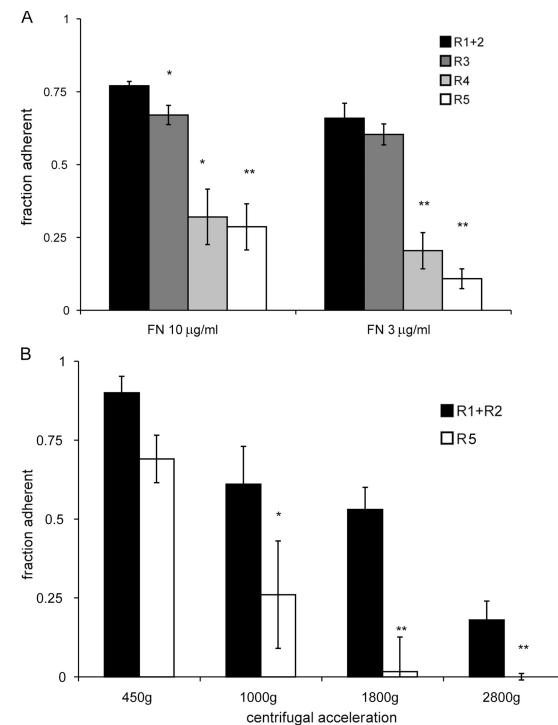
**Figure 6. Erythroid progenitors express fibronectin receptors.**  $\alpha_4$ ,  $\alpha_5$ , and  $\beta_1$  integrins are highly expressed on early erythroid progenitors, and expression is down-regulated as cells differentiate. Fetal livers were stained with antibodies against CD71, TER119, and each of  $\alpha_4$ ,  $\alpha_5$ , and  $\beta_1$  integrins. Cells were gated into regions as in Fig. 1 A, and integrin expression within each population was determined. The light trace is the background staining of the secondary antibody alone. These data are representative of at least three experiments. The horizontal bars represent the fraction of the population with integrin expression above the level of the background control.

fibronectin under these conditions, the fraction of adherent cells decreases progressively in the R3–5 populations. Adhesion of all cell populations was greater on 10  $\mu\text{g}/\text{ml}$  fibronectin than on 3  $\mu\text{g}/\text{ml}$ . The progressive decrease in adhesion on fibronectin parallels the loss of  $\alpha_4$ -,  $\alpha_5$ -, and  $\beta_1$ -integrin expression shown in Fig. 6.

The optimum centrifugal force for cell detachment was determined by applying a range of centrifugal accelerations to R1 + R2 and R5 cells attached to plates coated with 10  $\mu\text{g}/\text{ml}$  fibronectin (Fig. 7 B). A low centrifugal force was not enough to distinguish between R1 + R2 and R5 cell populations, whereas higher forces removed all of the R5 cells. A centrifugal acceleration of 1,000  $\text{g}$  provided enough force to distinguish between the adhesion of R1 + R2 and R5 cells without removing all of the R5 cells; thus, 1,000  $\text{g}$  was used for all further studies.

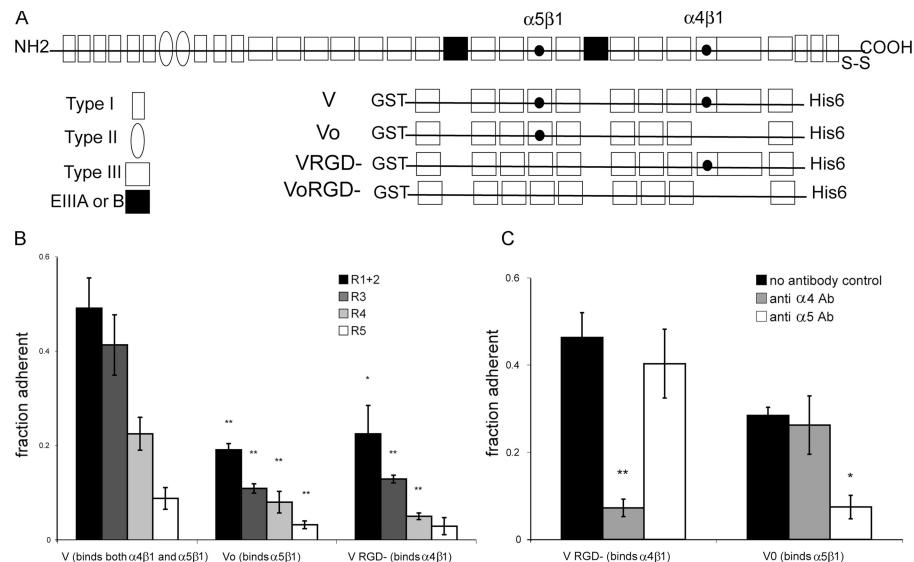
#### Role of integrins in adhesion of erythroid progenitors

To test the role of specific integrin subunits in mediating adhesion to fibronectin, we used recombinant fibronectin fusion proteins. These recombinant proteins contain either the  $\alpha_4\beta_1$ -binding site (VRGD<sup>−</sup>), the  $\alpha_5\beta_1$ -binding site (Vo), or both binding sites (V; Fig. 8 A). Cell adhesion assays were performed as in Fig. 7.



**Figure 7. Adhesion to fibronectin is correlated with integrin receptor expression.** (A) Adhesion of R1 + R2, R3, R4, and R5 cells to either 10 (left) or 3  $\mu\text{g}/\text{ml}$  (right) human plasma fibronectin at an acceleration of  $\sim 1,000 \text{ g}$ . Cells from each population were sorted and allowed to adhere to fibronectin-coated plates for 30 min before the adhesion assay. Adhesion to fibronectin decreases as cells differentiate. (B) Adhesion of R1 + R2 and R5 cells to 10  $\mu\text{g}/\text{ml}$  fibronectin at a range of centrifugal accelerations. (A and B) In all cases, each bar represents the mean fraction adherent from three wells, and error bars represent the SD. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$  when compared with R1 + R2 cells.

**Figure 8. Role of integrins in the adhesion of erythroid progenitors.** (A) Schematic diagram of recombinant fibronectin fusion proteins. The V fragment contains both the  $\alpha_4\beta_1$ - and  $\alpha_5\beta_1$ -binding sites, Vo contains only the  $\alpha_5\beta_1$ -binding site, VRGD<sup>-</sup> contains only the  $\alpha_4\beta_1$ -binding site, and VoRGD<sup>-</sup> contains neither integrin-binding site. (B) Characterization of the adhesion of sorted R1 + R2, R3, R4, and R5 cells to 10  $\mu$ g/ml V, Vo, or VRGD substrates at an acceleration of  $\sim$ 1,000 g. (C)  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$  integrins mediate adhesion to different fibronectin domains. Adhesion of sorted R1 + R2 cells to 10  $\mu$ g/ml recombinant fibronectin fragments Vo or VRGD<sup>-</sup> with and without pretreatment with function-blocking antibodies to  $\alpha_4$  and  $\alpha_5$  integrins. Each bar represents the mean fraction of cells adherent in each of three replicate wells. In all cases, error bars are the SD. \*, P  $\leq$  0.05; \*\*, P  $\leq$  0.01.



As shown in Fig. 8 B, adhesion to all fragments mirrors that on fibronectin in that the fraction of adherent cells on V, Vo, and VRGD<sup>-</sup> decreases progressively in R1 + R2, R3, R4, and R5 populations. For all populations of cells, adhesion was greatest to the V fragment, which contains both  $\alpha_4\beta_1$ - and  $\alpha_5\beta_1$ -integrin-binding sites.

We then repeated the adhesion assay with the addition of function-blocking antibodies. Sorted R1 + R2 cells were incubated with function-blocking antibodies to  $\alpha_4$  or  $\alpha_5$  integrin before being added to precoated plates. When  $\alpha_4$  integrins were blocked on R1 + R2 cells, adhesion to the  $\alpha_4\beta_1$ -binding fragment VRGD<sup>-</sup> was almost completely abrogated, whereas adhesion to the  $\alpha_5\beta_1$ -binding fragment Vo was unaffected (Fig. 8 C). Similarly, blocking  $\alpha_5$  integrins on R1 + R2 cells had no effect on cell adhesion to  $\alpha_4\beta_1$ -binding fragments but abrogated adhesion to  $\alpha_5\beta_1$ -binding fragments. These results indicate that  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$  integrins on fetal liver erythroid progenitor cells mediate adhesion to distinct sequences on fibronectin.

Changes in the levels of integrin expression and corresponding adhesion to fibronectin is physiologically relevant, as erythroid progenitors and erythroblasts must be retained in the bone marrow or fetal liver, whereas the more differentiated reticulocytes must be released into the circulation. Because fibronectin is a major extracellular matrix protein in both the fetal liver and bone marrow, we conclude that adhesion of erythroid progenitors by both  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$  integrins is crucial in retaining immature erythroid cells in the marrow, and loss of these integrins is likely crucial in triggering their release.

#### $\alpha_4$ but not $\alpha_5$ integrin mediates the fibronectin response

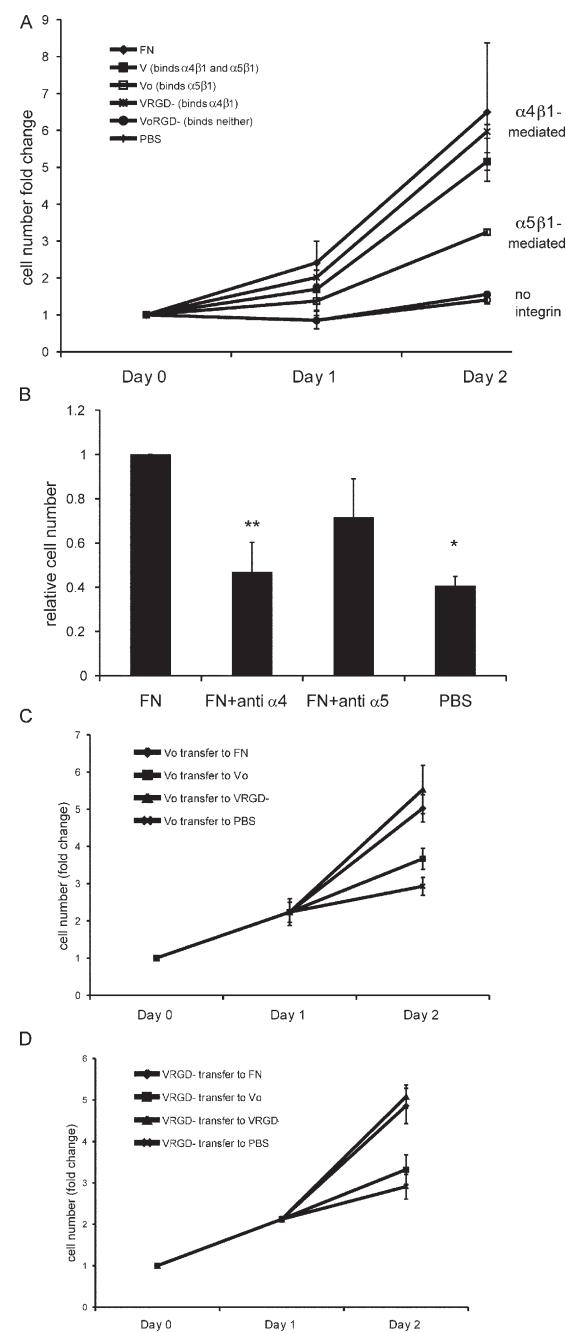
We were interested to determine whether both  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$  integrins were responsible for the observed proliferative and antiapoptotic effects of fibronectin in the second phase of erythropoiesis. To this end, we cultured TER119<sup>-</sup> erythroid progenitors on the various recombinant fibronectin fragments in Epo-containing media for 1 d and then in Epo-free media for a second day. Importantly, only those cultured on  $\alpha_4\beta_1$ -binding

substrates V and VRGD<sup>-</sup> underwent a dramatic expansion between days 1 and 2 similar to cells cultured on intact fibronectin (Fig. 9 A). In contrast, cells cultured on the  $\alpha_5\beta_1$ -binding fragment Vo exhibited a defect in cell expansion, indicating that  $\alpha_4\beta_1$ -mediated adhesion to fibronectin is necessary for maximum numbers of terminal erythroid divisions.

We obtained the same result when we blocked integrin engagement with antibodies. TER119<sup>+</sup> cells were isolated from the fetal liver as in Fig. 3 B and were incubated with function-blocking antibodies against  $\alpha_4$  or  $\alpha_5$  integrins for 15 min on ice. Cells were then added to fibronectin-coated or control plates in Epo-free media. As shown in Fig. 9 B, blocking  $\alpha_4$  integrin blocked expansion significantly on fibronectin (P = 0.0023). Blocking  $\alpha_5$  integrin had a lesser effect.

Our two-phase model of erythroid expansion predicts that  $\alpha_4\beta_1$ -integrin engagement is only necessary during the second, Epo-independent phase. We tested this prediction by varying the extracellular matrix cues presented to the cells during the first and second phases of culture, as in Fig. 3 B. As seen in Fig. 9 C, cells cultured on the  $\alpha_5\beta_1$ -binding substrate Vo for the first day exhibit normal expansion when transferred to  $\alpha_4\beta_1$ -binding substrates (fibronectin and VRGD<sup>-</sup>) for the second day. Conversely, there is a marked defect in expansion if cells are cultured in the absence of  $\alpha_4\beta_1$ -mediated adhesion on the second day even if  $\alpha_4\beta_1$  integrin is engaged during the first day of culture (Fig. 9 D). Collectively, these results indicate that the proliferative effect of fibronectin on the second day of erythropoiesis is  $\alpha_4\beta_1$  integrin dependent.

We went on to characterize the role of  $\alpha_4\beta_1$  integrin in preventing the apoptosis of differentiating erythroid cells. TER119<sup>-</sup> progenitor cells were cultured overnight on PBS in the presence of Epo and were transferred to fresh fibronectin or PBS wells in the presence or absence of function-blocking antibodies to  $\alpha_4$  and  $\alpha_5$  integrins. Blocking  $\alpha_4$  integrin increased the percentage of annexin V-positive cells 4 h after Epo removal, whereas blocking  $\alpha_5$  integrin had little effect on the percentage of apoptotic cells (Fig. 10). Representative flow cytometry data are shown in Fig. 10 A, and the normalized means of three independent



**Figure 9.  $\alpha_4$  integrin supports terminal proliferation.** (A)  $\alpha_4\beta_1$ -Mediated adhesion is required for the proliferation of differentiating fetal liver erythroid cells. TER119<sup>+</sup> fetal liver erythroid progenitor cells were cultured on V, Vo, VRGD<sup>-</sup>, or VoRGD<sup>-</sup> substrates with Epo-containing media for 1 d and then in Epo-free media for a second day. At days 1 and 2, the number of live cells was counted. Only  $\alpha_4\beta_1$ -binding substrates V and VRGD support an expansion in cell number similar to that seen on intact fibronectin.  $\alpha_5\beta_1$ -Binding substrate Vo supports a moderate level of expansion, and VoRGD, which does not engage either integrin, does not support any cell expansion. (B) Blocking  $\alpha_4$  integrin with an antibody blocks proliferation. TER119<sup>+</sup> cells were isolated and incubated with function-blocking antibodies to either  $\alpha_4$  or  $\alpha_5$  integrin and were cultured on fibronectin-coated or control substrates for 1 d. The mean normalized increase in cell number from three experiments is shown. \*, P ≤ 0.05; \*\*, P ≤ 0.01. (C and D) Expansion of erythroid progenitors during the second day occurs only in conditions in which  $\alpha_4\beta_1$  is engaged. TER119<sup>+</sup> cells were cultured on Vo (C) or VRGD<sup>-</sup> (D) substrates in Epo-containing media for the first day and were dissociated and transferred to wells coated with each of the various integrin-binding substrates in Epo-free media for a second day. Error bars represent the SD.

experiments are shown in Fig. 10 B. Collectively, these results establish that  $\alpha_4\beta_1$  integrin protects differentiating erythroid cells from apoptosis much like the action of Epo on early erythroid progenitors.

## Discussion

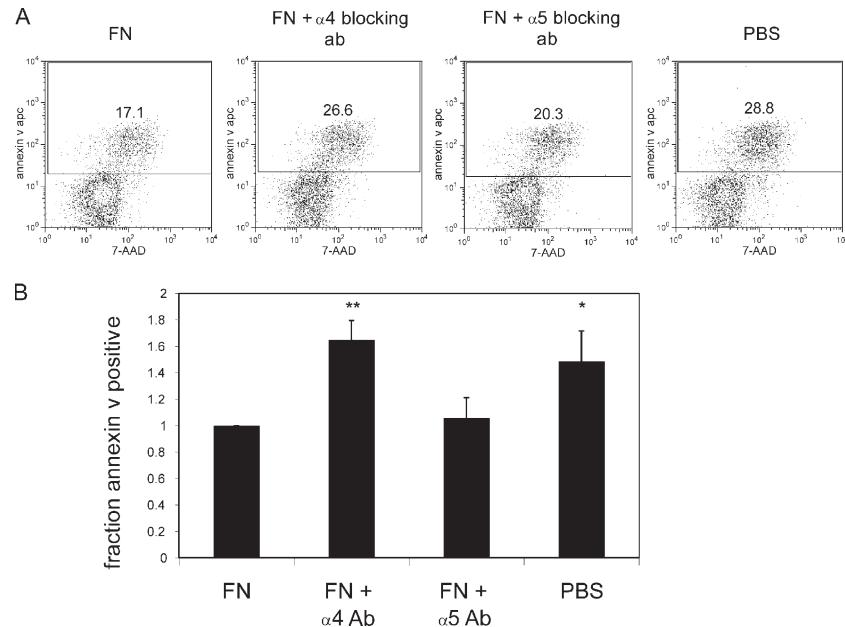
Over the course of erythroid differentiation, progenitor cells undergo three to five cell divisions, during which they undergo profound morphological and biochemical changes. These include up-regulation of the cell surface transferrin receptor CD71, which is required for import of the iron essential for hemoglobin function, followed by the induction of many erythroid-important enzymes and other proteins, including  $\alpha$  and  $\beta$  globin; red cell-specific membrane proteins such as glycophorin, TER119, and the anion exchange protein AE1 (also called Band III); and enzymes in the heme biosynthetic pathway. Subsequently, during the final two or three cell divisions, there is a marked decrease in cellular volume, increase in chromatin condensation, and inactivation of DNA transcription before the final steps of enucleation and expulsion of other organelles (Fawcett, 1997). These changes are used to define the sequential stages of erythroid differentiation: proerythroblasts, basophilic, and polychromatophilic erythroblasts, orthochromatic erythroblasts, and reticulocytes. We have used the sequential developmental up-regulation of CD71, the induction of TER119, and the down-regulation of CD71 as a tool for monitoring these stages of erythroid differentiation by FACS analysis both during culture *in vitro* and *in vivo* (Socolovsky et al., 2001; Zhang et al., 2003).

Epo and its specific receptor are crucial for promoting the survival, proliferation, and differentiation of mammalian erythroid progenitors (Richmond et al., 2005). The first stage of erythroid differentiation, from CFU-E to late basophilic erythroblast, is highly Epo dependent, whereas differentiation beyond this stage is no longer dependent on Epo (Koury and Bondurant, 1988). Consistent with this, Epo receptors are lost as erythroid progenitors undergo terminal proliferation and differentiation (Zhang et al., 2003). In our culture system for primary fetal liver erythroid progenitors, Epo is required only during the first day, when the progenitors undergo the approximately two initial divisions, and is not essential for the later stages of terminal cell proliferation and differentiation. This raises the question of what extracellular signals, if any, these differentiating erythroblasts require to support terminal proliferation, differentiation, and enucleation.

The main point of this study is that engagement of  $\alpha_4\beta_1$  integrin by fibronectin provides signals necessary for the terminal expansion of these differentiating erythroblasts. Our experiments indicate that erythroid expansion proceeds in two temporally distinct phases, the first governed by the binding of Epo to its receptor and the second by engagement of  $\alpha_4\beta_1$  integrin by fibronectin. In each phase, Epo and fibronectin act to promote cell expansion by inducing antiapoptotic signals through bcl-xL, but it is likely that both induce and repress the expression of many other genes as well.

Although fibronectin is known to be the major extracellular matrix protein in the erythroid niche, its role in erythropoietic

**Figure 10.  $\alpha_4$  integrin provides protection from apoptosis.** (A)  $\alpha_4$  integrin engagement provides protection from apoptosis in the second phase. TER119<sup>+</sup> fetal liver cells were purified and cultured overnight on uncoated substrates in Epo-containing media. Cells were then dissociated, incubated with or without antibodies to  $\alpha_4$  or  $\alpha_5$  integrins, and cultured in Epo-free media on fibronectin or control substrates. 4 h after Epo removal, the cells were dissociated and stained with annexin V and 7-AAD to assay for apoptosis. Annexin V binding is shown on the y axis. Data are representative of three independent experiments. The percentage of positive cells is indicated in each plot. (B) Summary of apoptosis data. Error bars represent the SD. \*, P ≤ 0.05; \*\*, P ≤ 0.01.



differentiation and survival was unknown. We used our established *in vitro* model of fetal erythropoiesis to systematically vary the extracellular matrix and growth factor cues presented to erythroid progenitors. Our experiments indicate that withdrawal of Epo during the first day of the culture period leads to defects in cell expansion resulting from increased apoptosis (Figs. 2 and 4 A). In contrast, the presence or absence of Epo on the second day of culture had no effect on the extent of expansion of these more differentiated erythroid cells (Fig. 4 B). Fibronectin plays a reciprocal role in that it has no effect on the survival or expansion of early erythroid CFU-E progenitors, but its absence on the second day of culture leads to increased apoptosis and a reduction in cell division (Figs. 2, 3 B, and 4 B). These results establish that erythropoiesis is governed by an early Epo-dependent phase followed by a later fibronectin-dependent phase.

To determine the molecular mediators of the observed fibronectin response, we measured integrin expression levels in erythroid progenitor cells; on these cells,  $\alpha_4$ ,  $\alpha_5$ , and  $\beta_1$  were the mostly highly expressed integrins. Furthermore, we showed a progressive down-regulation of  $\alpha_4$ ,  $\alpha_5$ , and  $\beta_1$  integrins over the 3-d course of erythroid differentiation (Fig. 6). A quantitative adhesion assay indicated that the loss of integrin expression is correlated with the loss of adhesion to fibronectin (Fig. 7 A). Changes in the levels of integrin expression and corresponding adhesion to fibronectin are physiologically relevant, as erythroid progenitors and erythroblasts must be retained in the bone marrow or fetal liver, whereas the more differentiated reticulocytes must be released into the circulation. In this respect,  $\alpha_4$  and  $\alpha_5$  integrins play a similar role in that they both function as adhesion receptors (Fig. 8 B). Because fibronectin is a major extracellular matrix protein in the bone marrow, we conclude that adhesion of erythroid progenitors by both  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$  integrins is crucial in retaining immature erythroid cells in the marrow, and the loss of these integrins is likely crucial in triggering their release.

By culturing erythroid progenitor cells on recombinant fibronectin fragments, we demonstrated that only  $\alpha_4\beta_1$ -integrin

engagement supports expansion on fibronectin-coated surfaces (Fig. 9 A), indicating that  $\alpha_4$  integrins play an additional role in transducing signals from the extracellular matrix. A previous study has identified  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$  as the predominant integrins on erythroid progenitors (Rosemblatt et al., 1991), but in the present study, for the first time, we distinguish between the roles of these two fibronectin receptors on erythroid cells.

As predicted by our two-phase model,  $\alpha_4\beta_1$ -integrin engagement is necessary only during the second phase of erythropoiesis, when erythroblasts undergo the approximately two final cell divisions and enucleate. As shown in Fig. 9 (B and C), robust cell expansion is observed when  $\alpha_4\beta_1$ -integrin engagement is provided in the second, Epo-independent phase of the culture period regardless of whether fibronectin or any of its fragments was present during the first, Epo-dependent phase. Thus, our work suggests that  $\alpha_4\beta_1$  integrin plays two roles in erythroid development. In the first phase of development,  $\alpha_4\beta_1$  seems to function solely as an adhesion receptor for fibronectin, whereas in the second phase, it functions to activate pathways that are necessary for erythroid expansion.

Interestingly, we found that integrin engagement is not essential for the differentiation of erythroid progenitors, as measured by the expression levels of CD71 and TER119 (unpublished data), supporting the view that erythroid differentiation and expansion are decoupled and regulated by separate pathways (Arroyo et al., 1999). Withdrawal of Epo during the first phase of erythropoiesis and the lack of engagement of  $\alpha_4\beta_1$  integrin during the second phase both lead to increased apoptosis. Dead cells cannot proliferate or differentiate, and, thus, it is difficult to determine whether signals downstream of the Epo receptor or  $\alpha_4\beta_1$  integrin directly activate signal transduction pathways leading to cell proliferation or differentiation. Alternatively, by preventing apoptosis, these signals could allow previously inscribed signaling pathways to support both the cell division and induction of erythroid-specific genes.

In other systems, cytokine and integrin-mediated signals interact to direct cell behavior. For example, the differentiation of mammary epithelia requires signals initiated by the binding of prolactin to its receptor as well as the simultaneous engagement of  $\beta_1$  integrins by the basement membrane protein laminin (Streuli et al., 1995). Similar coordination is evident in neurogenesis, in which simultaneous signals from FGF and  $\beta_1$  integrins are necessary for neural stem cell maintenance and proliferation (Campos, 2005). One important distinction of these developmental pathways with erythroid development is that growth factor and integrin-mediated signals regulate erythroid cells at very different stages of differentiation, whereas in these other systems, they occur simultaneously.

Integrin-mediated adhesion to the extracellular matrix initiates a diverse set of intracellular signaling pathways that are specific to each integrin dimer and cell type. Little is known about the precise downstream pathways activated after integrin receptor–ligand binding in erythroid cells. In fibroblasts, integrin engagement often results in the formation of focal adhesions, which form linkages to the cytoskeleton and bring together many different scaffolding proteins and enzymes to activate downstream signaling pathways such as MAPK and Ras (Wozniak et al., 2004). Although focal adhesion kinase (FAK) has emerged as the key signaling component of focal adhesions, our preliminary data and that of others suggest that the FAK family member PYK2 rather than FAK itself is activated in erythroid cells in response to integrin activation (Avraham et al., 2000). Identifying the downstream signaling pathways and transcription factors that are activated by integrin-mediated adhesion in these cells is one important area for future work. In addition, examining any interaction between Epo- and integrin-mediated signals would provide additional insight into the regulation of erythroid development. For example, it is likely that Epo-mediated signals during the first day of proliferation and differentiation of erythroid progenitors generate cells that are primed to receive and transduce integrin-mediated signals during the final days of erythropoiesis. In particular, signals activated downstream of the Epo receptor may affect integrin activation. Determining how  $\alpha_4\beta_1$  integrin affects apoptosis and cellular expansion and how these signals are integrated with Epo-mediated signals is the focus of our future work.

## Materials and methods

### Animals

C57BL/6 mice were purchased from The Jackson Laboratory and maintained at the Whitehead Institute animal facility.

### Fetal liver erythroid progenitor cells

C57BL/6 E14.5 fetal livers were dissected into Hank's Balanced Salt Solution containing 2% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10 mM Hepes, pH 7.4 (called HBSS+) at a concentration of two livers per milliliter. Liver tissue was disaggregated by vigorous pipetting followed by passage through a 70- $\mu$ m cell strainer. Cells were blocked with a 1:50 dilution of ChromPure Rat IgG (The Jackson Laboratory) and labeled with a 1:200 dilution of phycoerythrin TER119 and FITC CD71 antibodies (BD Biosciences) for 15 min on ice. Cells were washed and resuspended in HBSS+ and labeled with 1  $\mu$ g/ml propidium iodide for cell sorting on a MoFlow3 cell sorter (Becton Dickinson).

### In vitro erythroid culture

Fetal liver differentiation assays were performed as previously described (Zhang et al., 2003). In brief, day 14.5 fetal livers were suspended in PBS + 2% FBS at a concentration of two fetal livers/milliliter. Cells were incubated with 1:50 rat IgG for 15 min on ice followed by 1:100 biotinylated anti-Ter119 antibody (BD Biosciences). Cells were washed, resuspended at the same concentration, and further labeled with 1:10 tetrameric streptavidin complex (StemCell Technologies) for 15 min followed by 60  $\mu$ l of magnetic colloids per milliliter of cells (StemCell Technologies) for 15 min. Ter119 $^+$  cells were purified using a StemSep magnetic column (StemCell Technologies) according to the manufacturer's instructions. Cells were then seeded into 24-well plates coated overnight with 0.5 ml of 20  $\mu$ g/ml human plasma fibronectin, V, Vo, or VRGD $^-$  recombinant fusion proteins and washed twice with PBS. Cells were seeded at a density of 100,000 cells/ml. For the first 16–18 h, they were cultured in day 1 media, which consists of Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 15% FBS (StemCell Technologies), 1% BSA (StemCell Technologies), 10  $\mu$ g/ml recombinant human insulin (Sigma-Aldrich), 200  $\mu$ g/ml recombinant human holotransferrin (Sigma-Aldrich), 10 $^{-4}$  M  $\beta$ -mercaptoethanol, 1% penicillin/streptomycin, 2 mM glutamine, and 2 U/ml Epo (Amgen). After 16–18 h, the medium was changed to day 2 media, which consists of Iscove's Modified Dulbecco's Medium supplemented with 20% FBS (Invitrogen), 10 $^{-4}$  M  $\beta$ -mercaptoethanol, 1% penicillin/streptomycin, and 2 mM glutamine. At each time point, cells were dissociated with PBS containing 5 mM EDTA and 10% FBS for 5 min at 37°C. Cells were counted using a hemocytometer (BrightLine; Hauser Scientific) and were stained with 1:200 FITC CD71 and phycoerythrin TER119 for 15 min on ice. Cells were washed and analyzed using a flow cytometer (FACSCalibur; Becton Dickinson). FACS data were analyzed using FlowJo 6.0 software (Tree Star, Inc.).

In experiments in which the substrate was varied from days 1 to 2, cells were cultured in day 1 media for the first day, dissociated with PBS containing 5 mM EDTA and 2% FBS for 5 min at 37°C, counted using the BrightLine hemocytometer, and added to fresh plates coated with 20  $\mu$ g/ml human plasma fibronectin, V, Vo, or VRGD $^-$  recombinant fusion proteins in day 2 media. After 24-h culture, cells were dissociated and counted.

In experiments using TER119 $^+$  cells, E14.5 fetal livers were blocked and labeled with biotinylated TER119 antibody. Cells were then washed, resuspended in HBSS+, and incubated with streptavidin-coupled microbeads (Miltenyi Biotec) according to the manufacturer's instructions. TER119 $^+$  cells were isolated on an autoMACS column (Miltenyi Biotec) using the PosseL\_s program.

### Recombinant fibronectin proteins

Recombinant fibronectin fusion proteins were expressed in *Escherichia coli* grown to late log phase and induced with 1 mM IPTG for 4 h. Cells were harvested by centrifugation and stored at –80°C in PBS containing a cocktail of protease inhibitors (Sigma-Aldrich). Bacterial pellets were thawed, and the buffer was adjusted to 50 mM sodium phosphate, pH 8.0, 10 mM imidazole, and 300 mM NaCl. Pellets were incubated with 2 mg/ml lysozyme and DNase for 30 min at 4°C, lysed using a French press, brought to 1% Triton X-100, and centrifuged at 20,000 g for 20 min. Supernatants were incubated with TALON resin (CLONTECH Laboratories, Inc.) and washed in 50 vol of wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 10 mM imidazole), and proteins were eluted with 2 vol of elution buffer (wash buffer adjusted to 250 mM imidazole). Proteins were dialysed against CAPS buffer (20 mM CAPS, pH 11.0, and 150 mM NaCl). The final protein concentrations were determined by UV absorption, and protein sizes were confirmed on SDS-PAGE.

### Apoptosis assay

To measure apoptosis, TER119 $^+$  cells were isolated and cultured on uncoated surfaces in day 1 media for 16–18 h. At this time, cells were dissociated, washed, and incubated with 10  $\mu$ g/ml of function-blocking antibodies to either  $\alpha_4$  (clone PS/2; Serotec) or  $\alpha_5$  (5H10-27; BD Biosciences) integrin for 15 min on ice. The cells were then transferred to fresh fibronectin or control substrates in Epo-free day 2 media. 4 h after Epo removal, cells were dissociated and stained with annexin V-allophycocyanin (BD Biosciences) and 7-AAD (BD Biosciences) according to the manufacturer's instructions. bcl-xL expression was assayed by fixing and permeabilizing the cells and staining with 1:500 antibody to bcl-xL (clone 54H6; Cell Signaling Technology) for 15 min at room temperature followed by 1:1,000 AlexaFluor647 secondary antibody for 15 min at room temperature (Invitrogen). Expression data were then collected on a flow cytometer (FACSCalibur; BD Biosciences) and analyzed using FlowJo software. Cells were gated based on forward scatter and side scatter properties before then analyzing annexin v and 7-AAD levels.

**Integrin staining**

C57/Bl6 E14.5 fetal livers were dissected and stained as above with the addition of a 1:100 dilution of the following biotinylated antiintegrin antibodies, which were all obtained from BD Biosciences:  $\alpha_4$  (9C10),  $\alpha_5$  (5H10-27), and  $\beta_1$  (Ha2/5). Cells were washed, resuspended, and stained with 1:1,000 streptavidin phycoerythrin Cy5.5 (Caltag Laboratories) for 15 min on ice. Cells were washed, resuspended in HBSS+, and labeled with 1  $\mu$ g/ml propidium iodide for FACS analysis. Data were collected on a cell sorter (MoFlow3; Becton Dickinson) and analyzed with FlowJo 6.0 software (Tree Star, Inc.).

**Adhesion assays**

96-well half-area plates (Costar) were precoated with various concentrations of human plasma fibronectin (Sigma-Aldrich) diluted in PBS overnight at 4°C and washed twice with PBS before cell seeding. 5,000 freshly sorted fetal liver R1–5 erythroid progenitors were resuspended in 100  $\mu$ l Alpha Modified Essential Medium (Invitrogen) and added to each well. Cells were incubated at 37°C for 30 min. To perform the adhesion assay, wells were filled to the top with medium and covered with clear sealing tape (Costar), ensuring no entrapped air bubbles. Plates were inverted and spun at the various speeds for 5 min. Each well was photographed, and the number of cells was enumerated before and after spinning. Fraction adherent was calculated as the number of cells present in each well after spinning divided by the number of cells present before spinning. Cells were enumerated using ImageJ image analysis software (National Institutes of Health).

The force experienced by the cells is given by the following equation:

$$F_{cell} = RCF \times V \times (\rho_{cell} - \rho_{medium})$$

$$RCF \propto \omega^2 \times r_c$$

where  $V$  is the volume of the cell,  $\rho_{cell}$  is the density of the cell,  $\rho_{medium}$  is the density of the medium,  $RCF$  is the relative centrifugal force,  $\omega$  is the centrifugal velocity, and  $r_c$  is the radius of the centrifugation. In our centrifuge,  $r_c$  was 10 cm.

For integrin inhibition studies, cells were incubated with 10  $\mu$ g/ml of the following function-blocking antibodies before cell seeding:  $\alpha_4$  clone PS/2 (Serotec),  $\alpha_5$  clone 5H10-27 (BD Biosciences), and  $\beta_1$  clone Ha2/5 (BD Biosciences). Cells were stained for 30 min at 37°C.

**Statistical analysis**

Significance levels were determined using the *t* test algorithm in Excel (Microsoft). For the data in Figs. 5, 8 (B and C), 9 B, and 10 B, a homoscedastic two-tailed distribution was used. In Fig. 7 (A and B), a heteroscedastic two-tailed distribution was used.

**bcl-xL expression**

TER119<sup>+</sup> cells were isolated and cultured in day 1 media on uncoated surfaces overnight. Cells were then dissociated and serum starved in Iscove's Modified Dulbecco's Medium + 1% BSA for 1 h before being transferred to fresh fibronectin or uncoated wells. At the indicated time points, cells were lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, and 0.5% NP-40) containing protease inhibitors (Complete mini [Roche], 4 mM NaF, and 4 mM Na<sub>3</sub>VO<sub>4</sub>) for 20 min at 4°C. Lysates were ultracentrifuged at 13,000  $\times g$  for 10 min at 4°C and analyzed via SDS-PAGE. Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences) for 1 h and incubated with 1:1,000 bcl-xL antibody clone 54H6 (Cell Signaling Technology) and 1:1,000 actin antibody I-19 (Santa Cruz Biotechnology, Inc.) overnight. Proteins were visualized using the Odyssey infrared imaging system (LI-COR Biosciences).

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