The Role of Integrins in Hematopoiesis

by

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Abstract

Hematopoietic stem cells (HSCs) hold great promise for the treatment of disease. The rare frequency at which HSCs occur in the bone marrow under homeostatic conditions is a limiting factor in both their study and clinical use. *ex vivo* expansion of these cells is therefore a necessary step to maximizing their potential. In this thesis I explore the concept that signals from the extracellular matrix can direct differentiation, survival and self-renewal decisions in hematopoietic cells, and thus can provide a foundation for the design of *ex vivo* expansion strategies. This work is focused on the role integrins, the major class of cell-extracellular matrix adhesion molecules, play in mediating these signals to hematopoietic cells at two developmental stages. In the erythroid lineage, I show that expansion of committed erythroid progenitors is regulated by growth factor and integrin-mediated signals in temporally distinct regimes. I establish a biologically relevant role for $\alpha_4\beta_1$ but not $\alpha_5\beta_1$ integrins in erythropoiesis and provide evidence that erythroid differentiation and expansion are regulated by separate processes. In the study of uncommitted HSCs, I identify several integrin subunits that are differentially expressed on highly purified HSC populations that correlate with long term repopulating ability. One of these subunits, α_2 integrin, specifically mediates adhesion of HSCs to bone marrow extracellular matrix proteins, thereby providing a potential mechanism for stem cell self-renewal. This work establishes that integrin-mediated interactions between hematopoietic cells and the extracellular matrix are dynamic and provide important developmental cues.

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Mariette Vogelezang of the Hynes lab not only provided key reagents but also contributed critical reading of manuscripts as well as general knowledge of fibronectin and integrins. Glenn Paradis, the director of the Flow Cytometry Core Facility, was immensely important in teaching me about flow cytometry and in maintaining the facilities on campus. As a thesis committee member, Richard Hynes always made key insightful comments at every step of the project. Doug Lauffenburger, as chair of the thesis committee, made himself available to me to discuss aspects of quantitative cell biology. In addition, Doug's efforts to foster a strong sense of community within the Department of Biological Engineering were much appreciated.

I am indebted to my co-advisors Linda Griffith and Harvey Lodish. They gave me enormous independence in designing and carrying out this project and were able to aid and support me when needed. They work together quite well and are a model for a professional collaboration; I feel lucky to have been a part of their team.

Finally I would like to thank my many friends and family for all of their support during my time at MIT. In particular, my parents, Abdi and Goli, and my brother, Bardia, have been the best role models, friends and inspiration I could have ever imagined. My parents have made every sacrifice in their lives so that I could have every opportunity to succeed and I only hope they know how grateful I am.

Contents

Chapter 1

Introduction

Cell behavior is influenced by a combination of cues from soluble growth factors, the extracellular matrix and neighboring cells. Recent advances in the study of hematopoiesis have focused the field on the importance of interactions with the niche in directing cellular differentiation and survival [41]. In this thesis, I explored the idea that the extracellular matrix is an active component of the hematopoietic niche in that cellular adhesion to matrix proteins influences cell differentiation and survival decisions. In particular, I focused on the role that integrins, a broad class of cell surface adhesive proteins, play in hematopoiesis. Chapter 1 provides a concise review of hematopoiesis and integrin structure and function. The major focus of my thesis work was on growth factor and integrin-mediated regulation of erythropoiesis, which is presented in Chapters 2 and 3. The focus of Chapter 4 shifts towards integrins on self-renewing hematopoietic stem cells (HSCs). Chapter 5 discusses the relevance of this work within the field and suggests avenues for further investigation.

1.1 Motivations

Since the landmark discovery of bone marrow cells with the capacity for both self-renewal and differentiation [6], there has been much interest in precisely defining the characteristics of these so-called hematopoietic stem cells. The ability of these cells to develop into any type of blood cell while at the same time maintaining self-renewal

capacity in at least some of the progeny holds great promise for the treatment of hematopoietic disease. Hematopoietic stem cells are routinely used in bone marrow transplants for the treatment of cancers and inherited immune disorders. In the future, HSCs may be used in combination with gene therapy to correct monogenetic hematological disorders or even to endow HSCs with enhanced properties [48].

The rare frequency at which HSCs occur in the bone marrow under homeostatic conditions, 1 in 100,000 in the adult human [95], is a limiting factor in both their study and clinical use. *ex vivo* expansion of these cells is therefore a necessary step to maximizing their potential. To this end, much effort in the last decade has been dedicated to developing a cytokine cocktail to stimulate stem cell self-renewal *in vitro*, with relatively little attention to cues that may come from the extracellular matrix. With a more detailed, mechanistic understanding of how hematopoietic cells interact with their environments we can better realize the therapeutic potential of these cells.

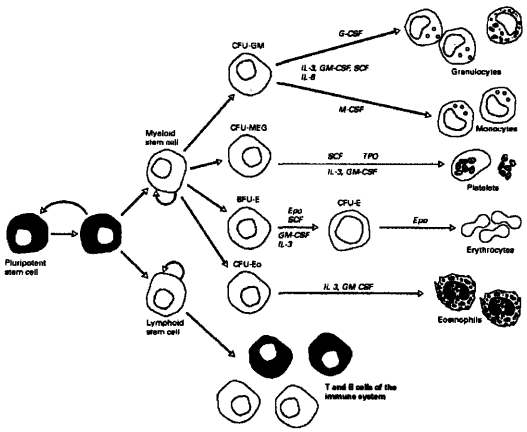

Figure 1-1: **Schematic of the adult hematopoietic hierarchy.** Reprinted from Lodish et al, with permission.

1.2 Hematopoietic development

Blood is among the most highly self-renewing tissues in the body, producing on order 10^{11} new cells each day in an adult. This self-renewing capacity depends upon a

constant source of hematopoietic stem cells, which arise from the mesoderm in the developing embryo shortly after gastrulation. In mice, these first hematopoietic cells are found in the extra-embryonic yolk sac at around embryonic day 7.5 and most likely contribute to embryonic red cell production, or primitive hematopoiesis. Some of these cells migrate to the aorta-gonad-mesonephros region, where the first definitive hematopoietic stem cells are found around E8.5. Circulating HSCs from the yolk sac and perhaps other sites begin to seed the fetal liver at E9.5-10.5, from which point on the fetal liver functions as the primary hematopoietic organ until just after birth [96].

Developing hematopoietic cells are thus exposed to a variety of microenvironments in the fetus. This compartmentalization of fetal hematopoiesis serves the developing embryo's dual needs for mature as well as primitive cells. Some anatomical sites provide inductive signals for production of functional red blood cells for immediate use, while others support the proliferation of undifferentiated HSCs to serve the embryo in the future.

In the adult, steady state hematopoiesis occurs in the bone marrow. The hard exterior of calcified bone protects these critical cells from injury while the highly vascularized interior allows access to circulating growth factors as well a mechanism for transport of differentiated cells to the tissues. Under extreme stress conditions, extramedullary hematopoiesis can occur, most often in the spleen. In addition, at any given time, a small number of HSCs can be found in the circulation [88].

1.3 Hematopoietic niches

1.3.1 Endosteal niche

The bone marrow is the primary source of HSCs in the adult. In recent years, much progress has been made in defining the bone marrow HSC niche and importantly, the mechanism through which it supports stem cell self -renewal and differentiation. Current thinking allows for at least two distinct niches within the bone marrow, an

endosteal niche and a vascular niche; further defining their relative roles and potential interaction remains a major challenge.

The first evidence that hematopoietic cells may develop within specific microenvironments in the bone marrow came in 1975 when Lord and Testa found an unequal distribution of HSCs in the bone marrow [44]. Bone marrow samples taken from the endosteal surface of mouse femurs had greater spleen repopulating ability than did those from the center of the marrow. Soon after, Schofield first proposed the concept of a hematopoietic stem cell niche, wherein cell-cell intereactions allow stem cell self-renewal but prohibit differentiation [67].

More recently, two independent approaches have provided evidence for an interaction between HSCs and the osteoblasts that line the endosteal surface. Genetically increasing the number of osteoblasts by expressing a constitutively active form of parathyroid hormone (PTH) or the PTH/PTH-related protein receptor, both positive regulators of osteogenesis, resulted in a concommitant increase in the number of HSCs [9]. A second group arrived at the same result by knocking down expression of bone morphogenetic protein receptor 1A, a negative regulator of osteogenesis which is normally expressed on endosteal osteoblasts [93]. In addition, both studies provide evidence for a direct, physical interaction between HSCs and osteoblasts.

1.3.2 Vascular niche

Despite the landmark work in identifying ostoblastic niches for HSCs, the fact that hematopoiesis can take place in extramedullary sites, in the absence of osteoblasts, implies that other supportive HSC niches also exist. The derivation of HSCs and endothelial cells from a common progenitor, the hemangioblast, suggests the existence of an endothelial HSC niche.

Recent advances in HSC purification have enabled *in situ* imaging of HSCs and revealed possible endothelial niches [34]. This purification strategy takes advantage of a family of receptors that are differentially expressed among functionally distinct progenitor cells. Using this code, competitive reconstitution analysis indicated that 1 in 2.1 $CD150^{+}CD48^{-}Sca\text{-}1^{+}$lineage $c\text{-}kit^{+}$ cells were true long-term repopulating

HSCs, the best purification to date. This same population of cells was found associated with the sinusoidal endothelium in spleens of animals treated with G-CSF to mobilize stem cells. Similarly, in the bone marrow, 60% of these cells that were imaged were in contact with sinusoidal endothelium. Consistent with this, endothelial cells isolated from heart and liver were able to maintain HSC activity *in vitro* [40]. Together, these data provide strong evidence for an endothelial niche for HSCs in the bone marrow and other hematopoietic tissues.

One prominent model linking the endosteal and vascular niches proposes that the endosteal niche provides a microenvironment suited to quiescence and HSC maintenance, while the vascular niche promotes proliferation and differentiation [38]. These niches are discussed in more detail in Chapter 4 and Chapter 5.

1.4 *ex vivo* HSC expansion

The area of stem cell biology with the most fervent activity has been identification of growth factors to support HSC expansion in culture. Most successfully, this has involved identification of factors secreted by primary stromal cells that support HSC expansion in co-culture experiments [91]. This approach led to the isolation of angiopoietin-like proteins, which support up to a 30-fold net expansion of long term HSCs. Progress in this realm can be combined with developments in culture systems, bioreactors and biomaterials to expand HSCs. Notably, low oxygen conditions in culture seem to promote HSC activity [14], which is attributed to their hypoxic environment *in vivo* [52]. In addition, advances in biomaterials have made it possible to culture stem cells on substrates that present ligands to specifically activate signaling pathways [75].

Another approach to expanding stem cells is to create HSCs from pluripotent embryonic stem cells. Because ES cells can be maintained in culture without differentiation, they represent an attractive source for HSCs. The most impressive results towards this end thus far have been obtained by overexpressing key hematopoietic transcription factors in mouse ES cells [84]. Expression of *Cdx4* and *HoxB4* together

in ES cells led to commitment to hematopoietic mesoderm, and, importantly, development of both lymphoid and myeloid cells in a transplant model. However, much is still unknown about the transcriptional programs that control hematopoietic differentiation and self-renewal and how such genetic modification could impact the ultimate cell behavior. Influencing cell behavior by controlling the microenvironment therefore offers a safer approach to generation of large numbers of HSCs.

1.5 Overview of integrins

Integrins are the major class of cell-matrix adhesion molecules. Integrins are $\alpha\beta$ heterodimeric molecules formed by combinations of 8 β subunits and 18 α subunits. Most integrins recognize several different ECM proteins, and individual matrix proteins can bind to several integrins [27]. Through their characteristic bidirectional signaling mechanism, integrins allow for exquisite coordination of the external and internal environments of the cell [20]. Furthermore, the interactions of integrins with soluble growth factors allow for another level of control [17]. Not surprisingly, integrins are involved with a wide variety of cellular processes, including control of cell growth, shape, survival and cell cycle progression. Integrins are highly developmentally regulated, again allowing for a dynamic relationship between the cell and its environment.

Integrins play a major role in hematopoiesis, the β_1 subunit in particular. $\beta_1$null embryos have significant defects in migration of hematopoietic cells to the fetal liver and other sites of hematopoiesis [8] and adult $\beta_1$null hematopoietic progenitors generated using a conditional knockout system were not able to repopulate primary sites of hematopoeisis [58]. The β_1 subunit can dimerize with 10 different α subunits (α_1 through α_9 and α_v) leading to much variety in the ligands with which it can interact. β_1 integrins are thought to be expressed on all hematopoietic cells, so changes in expression of the α subunit could be a mechanism by which cells at different stages modulate their interaction with different matrix molecules.

1.6 Discussion

The overall motivation for this thesis is the idea that adhesive interactions on hematopoietic cells are dynamic, responding to the unique needs of cells at distinct developmental stages. In approaching this project, I first focused on the erythroid lineage. In Chapter 2 I present data to support a model where erythropoiesis is regulated by growth factor- and integrin-mediated signals in temporally distinct regimes. In Chapter 3, the downstream signaling pathways behind the observed cellular behavior are explored. In Chapter 4, the focus shifts toward expression and function of integrins on hematopoietic stem cells. In Chapter 5, I discuss the relevance of this work within in the field of stem cell biology and present thoughts on future directions.

Chapter 2

Growth factor and extracellular matrix-mediated regulation of erythropoiesis

2.1 Introduction

Erythropoietin (Epo) has long been understood to be the major factor governing erythropoiesis; its role in regulating expansion, differentiation, apoptosis and activation of erythroid-specific genes is well characterized [62]. The first phase of erythroid differentiation is highly Epo-dependent, whereas later stages are no longer dependent on Epo [39]. Consistent with this, Epo receptors are downregulated as erythroid progenitors undergo terminal expansion and differentiation [94]. This raises the question of what other signals, if any, these differentiating erythroblasts require to support terminal expansion, 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 [74], but its precise role in erythropoisis and potential interaction with Epo-mediated signals was unknown.

I sought to characterize the precise role that fibronectin plays in erythropoiesis by utilizing an *in vitro* model of fetal erythropoiesis [94]. In this system, populations

of erythroid progenitors at varying phases of differentiation are purified from fetal liver based on expression of the cell surface markers CD71 and TER119; these same markers can then be used to track differentiation of progenitors cells cultured *in vitro*. I present data to support a novel model for erythropoiesis wherein Epo and fibronectin each play a distinct, essential role. My work shows that erythroid expansion proceeds in two phases, with an early Epo-dependent phase followed by fibronectin-dependent phase.

2.2 *in vitro* model of fetal erythropoiesis

In mammals, definitive erythropoiesis first occurs in the fetal liver with progenitor cells from the yolk sac [51]. 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-erythroids (BFU-E) [22, 23]. Early BFU-E cells divide and further differentiate through the mature BFU-E stage into rapidly dividing colony-forming unit-erythroids (CFU-E) [22, 23]. CFU-E progenitors divide three to five times over two to three days as they differentiate and undergo many significant changes including decrease in cell size, chromatin condensation and hemoglobinization leading up to enucleation and expulsion of other organelles [96]. These changes are used to define the sequential stages of erythroid differentiation: proerythroblasts, basophilic and polychromatophilic erythroblasts, orthochromatic erythroblasts and reticulocytes. Earlier work in the lab used the sequential developmental upregulation of CD71, induction of TER119, and then downregulation of CD71 as a tool for monitoring these stages of erythroid differentiation by flow cytometry both during culture *in vitro* and *in vivo* [71, 94]. This work formed the basis for the *in vitro* model I used to study erythropoiesis.

Fetal liver erythroid cells can be separated into five distinct populations of progressively differentiated cells based on expression of CD71, the transferrin receptor,

and TER119, an erythroid-specific glycoprotein (Figure 2-1). Early erythroid progenitors, the R1 population, express moderate levels of CD71 and are TER119$^-$. Later progenitor cells, R2, express higher levels of CD71 but are still TER119$^-$. As cells continue to divide and differentiate, TER119 expression is induced and then CD71 expression is downregulated, as indicated by the R3, R4 and R5 populations. R3-R5 populations contain no CFU-E activity [94].

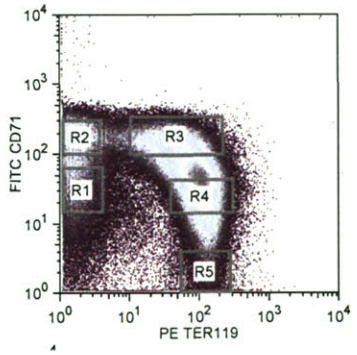

Figure 2-1: **Five populations of sequentially differentiated erythroid cells can be isolated from Day 14.5 fetal liver based on expression of CD71 and TER119.** R1 and R2 populations contain all of the CFU-E activity.

Expression of CD71 and TER119 can also be used to track erythroid differentiation *in vitro*. In this culture system, purified Day 14.5 TER119$^-$ progenitor cells are cultured on fibronectin-coated plates in two phases: first in the presence of erythropoietin for 16-18 hours and then without Epo for an additional 24 hours. On each day, cells are dissociated with PBS containing EDTA and stained with CD71 and TER119 antibodies for analysis by flow cytometry. In the first phase, CD71 is upregulated and there is modest expansion in cell number. By Day 3, TER119 is upregulated and CD71 is downregulated, recapitulating the staining pattern seen in freshly isolated fetal liver cells (Figure 2-2).

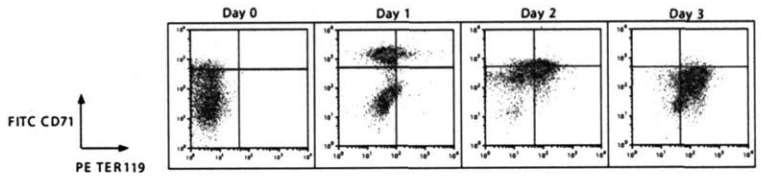

Figure 2-2: **CD71 and TER119 can be used as markers of erythroid differentiation *in vitro*.** TER119$^-$ cells were cultured on fibronectin-coated plates in Epo-containing media for one day and in Epo-free media for two additional days. Over three days, the staining pattern recapitulates that seen in freshly isolated fetal liver.

2.3 Role of fibronectin in erythropoiesis

2.3.1 Background

Fibronectin is a ubiquitous extracellular matrix molecule that presents developmental cues to many cell types, including hematopoietic cells [28]. Interactions with fibronectin are essential for proper erythropoiesis, as adhesion to fibronectin is required for enucleation of murine erythroleukemia cells to form reticulocytes [53]. 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 [86]. Taken together, 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.

2.3.2 Two-stage model for erythroid expansion

I used the *in vitro* model described above to study the role of fibronectin in erythropoiesis. I observed a dramatic increase in cell number between Day 1 and Day 2 for TER119$^-$ progenitor cells cultured on fibronectin, but not on an uncoated substrate, despite the presence of Epo between Day 0 and Day 1 in both cases (Figure 2-3, solid lines). As previously shown, withdrawal of Epo during the first day of culture led to an expansion defect, even in the presence of fibronectin (Figure 2-3, dashed lines).

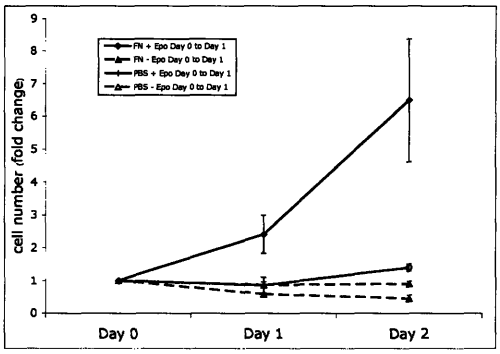

Figure 2-3: **Fibronectin supports expansion of erythroid progenitors.** TER119$^-$ cells were cultured on fibronectin-coated or control wells in Epo-containing media for one day and in Epo-free media for a second day (solid lines) or without Epo at all (dashed lines). In the presence of Epo, progenitor cells cultured on fibronectin undergo dramatic expansion in cell number but cells cultured on a control substrate do not. Withdrawal of Epo during the first day of culture results in an expansion defect regardless of the substrate.

This result led to the hypothesis that Epo and fibronectin regulate erythroid expansion in temporally distinct regimes. To test this hypothesis, I varied the presentation of extracellular matrix and growth factor cues during the course of the two-day culture period. When TER119$^-$ erythroid progenitors were cultured on a control substrate for one day and then 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 two-day period, suggesting that fibronectin is only required during the second day of erythroid culture (Figure 2-4).

In another test, I isolated TER119$^+$ cells from the fetal liver. As described previously, TER119 is a marker of differentiated erythroid cells [35], so I predicted that expansion of these cells is fibronectin-dependent but not Epo-dependent. Indeed, when I cultured differentiated TER119$^+$ erythroid cells I found that they expanded on fibronectin and not on the control substrate, and that the addition of Epo had no effect (Figure 2-5). It is important to note that these freshly isolated TER119$^+$ cells have not been exposed to Epo *ex vivo* and thus the response can be attributed solely

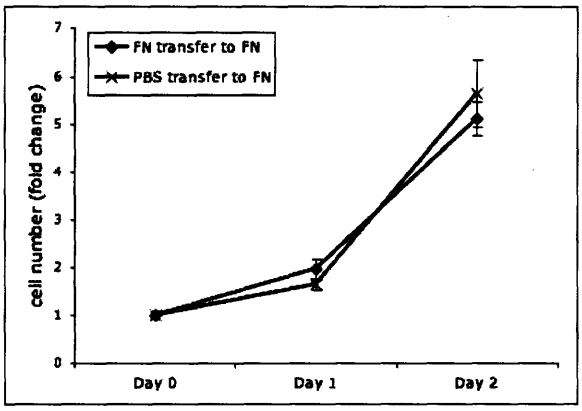

Figure 2-4: **Fibronectin is only required on the second day of culture.** TER119$^-$ cells were cultured on fibronectin-coated or control wells in Epo-containing media for one day and then dissociated and transferred to fresh fibronectin surfaces in Epo-free media for a second day. A dramatic expansion in cell number was observed regardless of which substrate was present on the first day of culture.

to the presence of fibronectin.

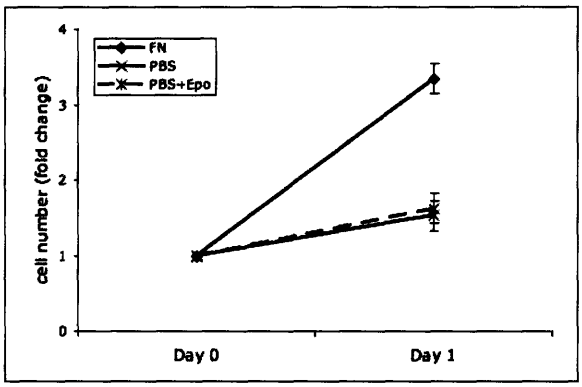

Figure 2-5: **TER119$^+$ cells exhibit fibronectin-dependent Epo-independent growth.** TER119$^+$ cells were cultured on fibronectin-coated or control wells in the absence of Epo. Only those cells cultured on fibronectin expanded; addition of Epo to the culture media (dashed line) had no effect on cell number.

Similarly, blocking the fibronectin-coated wells with increasing concentrations of an anti-fibronectin antibody abrogated expansion of TER119$^+$ cells, further indicating that fibronectin specifically mediates this phase of erythropoiesis (Figure 2-6). Taken together, these results suggest a two-phase model for erythroid expansion, where the presence of Epo in the first day of culture followed by the presence of fibronectin in the second day of culture are each essential to proper erythroid expansion.

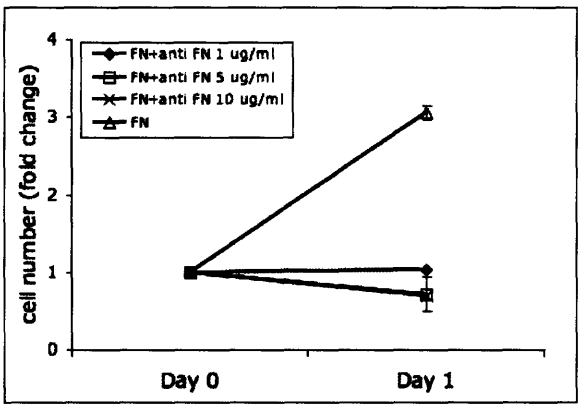

Figure 2-6: **Blocking fibronectin with antibodies blocks expansion of TER119$^+$ cells.** TER119$^+$ cells were cultured on fibronectin-coated wells that were pre-treated with increasing concentrations of anti-fibronectin antibody.

2.4 Role of integrins in erythropoiesis

2.4.1 Background

Given the observed biological significance of fibronectin on erythroid expansion, I was interested to determine the molecular mediators of this response. 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 (also known as VLA-5) mediates adhesion to the canonical RGDS sequence in the tenth type III repeat. There are two cell-binding sequences in the type III connecting segment (IIICS) that mediate adhesion to $\alpha_4\beta_1$ integrin (also known as VLA-4). The LDV sequence forms a high affinity binding site, while the REDV sequence forms a binding site with much lower affinity [26, 37] (Figure 2-7).

α_4 integrins appear to be essential for efficient differentiation and expansion of erythroid progenitors *in vivo* and *in vitro*. Deletion of α_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 α_4-null erythroid cells formed only small, pale colonies whereas wild-type cells transmigrated beneath the stroma, expanded, and formed large red colonies [4]. Early studies have

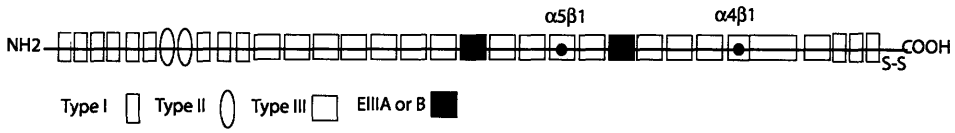

Figure 2-7: **Schematic diagram of fibronectin structure indicating integrin binding sites.**

shown that fetal liver erythroid cells express both α_4 and α_5 integrins and that these integrins mediate attachment of CFU-E to fibronectin and to stromal cells [65, 80, 81]. However, the biological significance of this adhesion was not yet known.

2.4.2 Integrin expression

I was interested in determining the molecular mediators of the observed fibronectin response, so I 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 relative expression of CD71 and TER119 (Figure 2-1). This system allowed me to examine expression of candidate fibronectin receptors at distinct phases in erythropoiesis that correlate with the two-phase model.

I used flow cytometry to determine fibronectin receptor expression on the surface of fetal liver erythroid progenitors. By co-staining E14.5 fetal liver cells with antibodies to CD71 and TER119 as well as a panel of integrin subunits, I determined that α_4, α_5 and β_1 are the most highly expressed integrins on these cells. Expression of α_2, α_v, β_2 and β_3 integrins was not distinguishable from the background fluorescence on erythroid progenitor R1 and R2 cells, a pooled population of progenitors I refer to as R1+R2 cells (Figure 2-8).

Expression of α_4, α_5 and β_1 integrins is downregulated as cells differentiate (Figure 2-9). R1+R2 cells express the highest levels of each integrin subunit, and the expression level in the R3, R4 and R5 populations progressively decreases. While R4 and R5 cells have completely lost expression of α_5 integrin, expression of α_4 and β_1

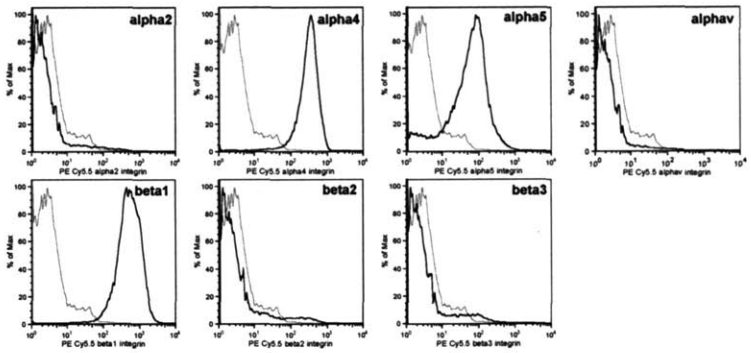

Figure 2-8: α_4, α_5 **and** β_1 **integrins are the most highly expressed integrin subunits on erythroid progenitor R1+R2 cells.** Fetal liver cells were stained with antibodies against CD71, TER119 and a panel of integrin subunits. Cells were gated into R1+R2 populations and integrin expression (dark trace) was compared to that of the secondary antibody alone (light trace).

integrin is clearly distinct from the background in these populations. Quantification of the mean fluorescence intensity of each integrin subunit in these populations is shown in Figure 2-10.

These data were further confirmed using quantitative PCR. Cells from each region were sorted and total RNA was isolated. RNA was reverse transcribed using random primers and amplified using the SYBR Green system. As shown in Figure 2-11, integrin message level decreases during erythroid differentiation. Data were normalized to 18S ribosomal RNA expression as well as to expression in the R1+R2 population.

Integrins can exist in active and inactive conformations, and only the active conformation is available for ligand binding and subsequent downstream signaling [27]. The monoclonal antibodies used above for integrin expression studies do not necessarily detect the active conformation. However, vascular cell adhesion molecule-1 (VCAM-1) is an $\alpha_4\beta_1$ ligand *in vivo* [18] and in its soluble form can be used in a functional assay of $\alpha_4\beta_1$ ligand binding [59]. To test the activity of $\alpha_4\beta_1$ integrins on erythroid progenitors, fetal liver cells were sorted into R1+R2 through R5 populations as described above. Cells were incubated with increasing amounts of soluble VCAM-1 in the presence of Mn^{2+} for 30 minutes at 4 °C. Unbound VCAM was removed by washing and cells were stained with an anti-VCAM antibody for quantitation by

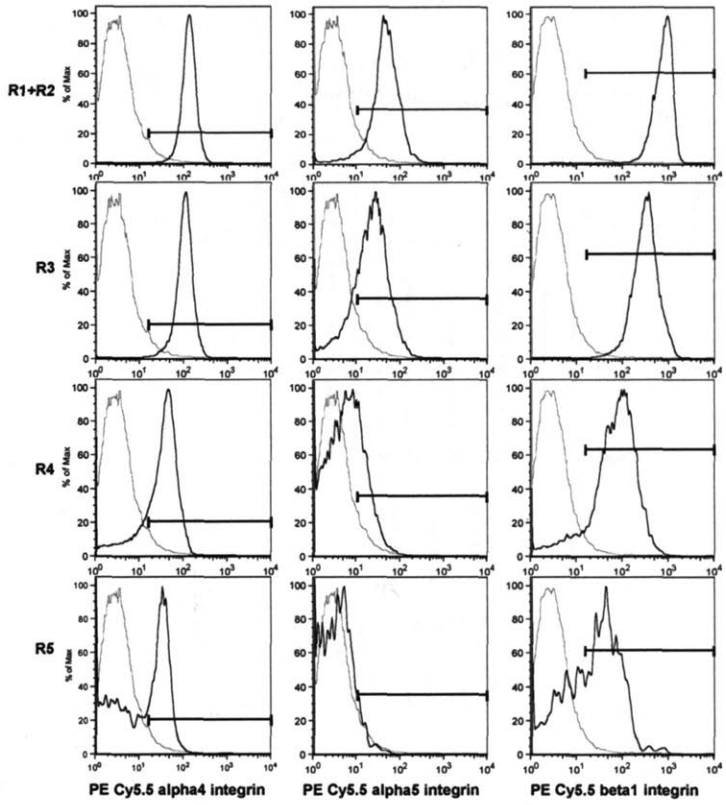

Figure 2-9: α_4, α_5 **and** β_1 **integrin expression is downregulated during erythroid differentiation.** Fetal livers were stained with antibodies against CD71, TER119 and each of α_4, α_5 and β_1 integrins. Cells were gated into regions as above and integrin expression within each population was compared. The dark trace represents integrin expression and the light trace is the background staining of the secondary antibody alone.

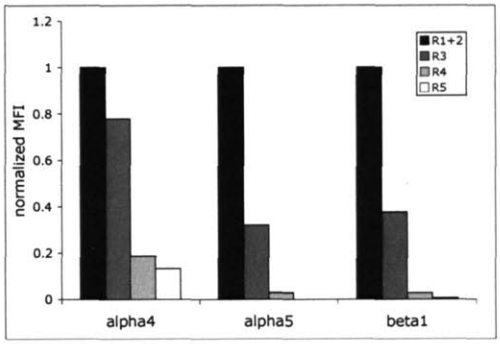

Figure 2-10: α_4, α_5 **and** β_1 **integrin expression is downregulated during erythroid differentiation.** Quantification of the data presented in Figure 2-9. Mean fluoresence values were determined and normalized to the R1+R2 population.

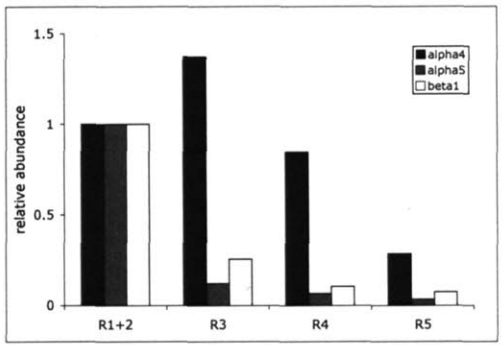

Figure 2-11: α_4, α_5 **and** β_1 **integrin message level is downregulated during erythroid differentiation.** Quantitative PCR was used to assess integrin expression levels in populations of sequentially differentiated erythroid cells. Early progenitor cells have high level of integrin message while differentiated cells have much lower levels.

flow cytometry. As shown in Figure 2-12, only the R1+R2 and R3 populations have significant VCAM-binding ability, indicating that the high $\alpha_4\beta_1$ integrin expression levels seen in these cells in Figure 2-9 corresponds to the active conformation.

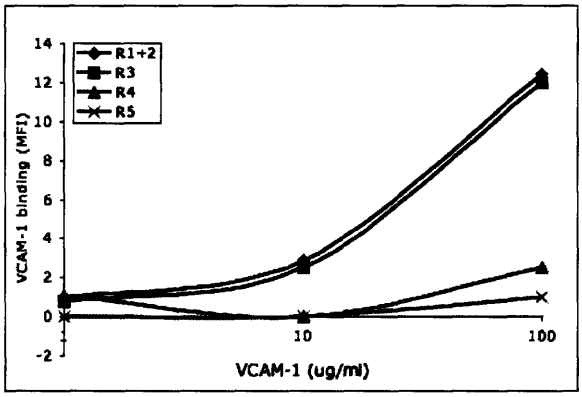

Figure 2-12: **R1+2 and R3 populations have significant VCAM-1-binding ability.** Sorted R1+2-R5 populations were incubated with increasing concentrations of soluble VCAM-1 in the presence of Mn^{2+}. Unbound VCAM-1 was removed by washing and cells were stained with a fluourescently labeled VCAM-1 antibody. VCAM-1 binding was quantitated by flow cytometry. Background binding of VCAM-1 in presence of EDTA was subtracted from each datum. VCAM-1 binding ability indicates expression of the active conformation of $\alpha_4\beta_1$ integrin.

2.4.3 Adhesion of erythroid progenitors

In order to test the functional role of loss of integrin expression on fetal liver erythroid cells, I adapted a quantitative cell-ECM adhesion assay for use with the fetal liver erythroid system (Appendix A). A centrifugation assay is ideal for this application because it is highly quantitative and repeatable while requiring no special equipment [25]. Using this assay, I demonstrated a progressive loss in adhesion to fibronectin during erythroid development (Figure 2-13). Sorted R1 to R5 cells were allowed to attach to plates coated with 10 μg/ml or 3 μg/ml human plasma fibronectin and then centrifuged at an acceleration of approximately 1000g. Whereas 75% of R1+R2 cells adhere to 10 μg/ml fibronectin under these conditions, the fraction of adherent cells decreases progressively in the R3 to R5 populations. Adhesion of all cell populations was greater on 10 μg/ml fibronectin than on 3 μg/ml. The progressive decrease in

adhesion on fibronectin parallels the loss of α_4, α_5 and β_1 integrin expression shown in Figure 2-9.

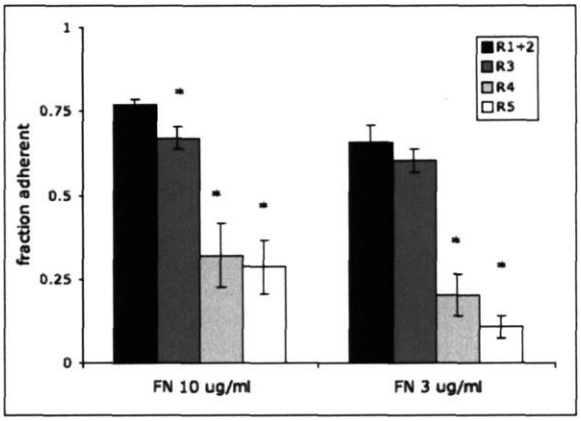

Figure 2-13: **Adhesion to fibronectin is correlated with integrin receptor expression.** Adhesion of R1+2, R3, R4 and R5 cells to either 10 μg/ml (left) or 3 μg/ml (right) human plasma fibronectin at an acceleration of approximately $1000g$. Cells from each population were sorted and allowed to adhere to fibronectin-coated plates for 30 minutes before the adhesion assay. Adhesion to fibronectin decreases as cells differentiate. Asterisks indicate statistical significance at the level of $p \leq 0.01$.

In order to test the role of specific integrin subunits in mediating adhesion to fibronectin, I used recombinant fibronectin fusion proteins obtained from Mariette Vogelezang, a collaborator in the Hynes lab. These recombinant proteins contain either the $\alpha_4\beta_1$ binding site (VRGD-), the $\alpha_5\beta_1$ binding site (Vo) or both binding sites (V) (Figure 2-14).

Cell adhesion assays were performed as above. As shown in Figure 2-15, adhesion to all fragments mirrors that on fibronectin, in that the fraction of adherent cells on V, Vo and VRGD- 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.

I then repeated the adhesion assay with the addition of function-blocking antibodies. Sorted R1+R2 cells were incubated with antibodies to α_4 or α_5 integrin before being added to pre-coated plates. When α_4 integrins were blocked on R1+R2 cells, adhesion to the $\alpha_4\beta_1$-binding fragment VRGD- was almost completely abrogated, while adhesion to the $\alpha_5\beta_1$-binding fragment Vo was unaffected (Figure 2-16).

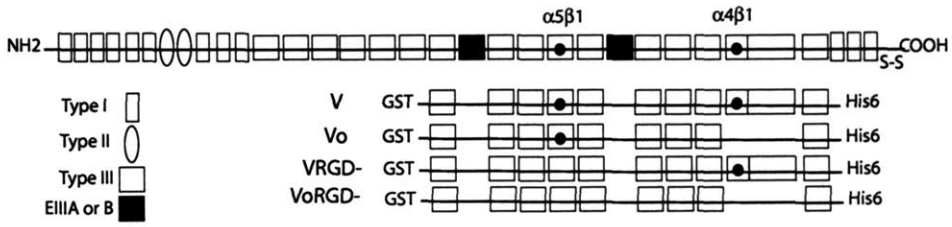

Figure 2-14: **Schematic diagram of recombinant fibronectin fusion proteins.** The V fragment presents both integrin binding sites. Vo presents just the $\alpha_5\beta_1$ integrin binding site and VRGD- presents just the $\alpha_4\beta_1$ integrin binding site. VoRGD- presents neither major integrin binding site. Figure adapted from Mariette Vogelezang.

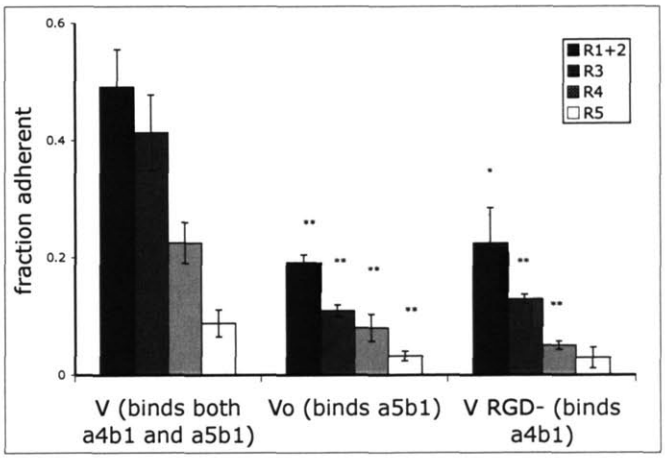

Figure 2-15: **Characterization of erythroid cell adhesion to V, Vo or VRGD substrates.** R1+2, R3, R4 and R5 cells were sorted and adhesion to 10 μg/ml substrates at an acceleration of $\sim 1000g$ was measured. Adhesion on the V fragment is similar to that on intact fibronectin. Adhesion of all populations was slightly greater on VRGD- than on Vo, while adhesion to V was the highest.

Similarly, blocking α_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.

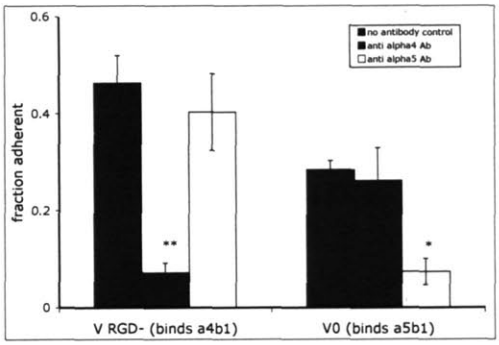

Figure 2-16: $\alpha_4\beta_1$ **and** $\alpha_5\beta_1$ **integrins mediate adhesion to different fibronectin domains.** Adhesion of sorted R1+2 cells to 10 μg/ml recombinant fibronectin fragments Vo or VRGD- with and without pre-treatment with function-blocking antibodies to α_4 and α_5 integrins. Two asterisks indicate statistical significance at the level of $p \leq 0.01$ and one asterisk indicates statistical significance at the level of $p \leq 0.05$.

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. Since fibronectin is a major extracellular matrix protein in both the fetal liver and the bone marrow, I 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 likely is crucial in triggering their release.

2.4.4 Role in two-stage model for erythroid expansion

I were interested to determine if both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins were responsible for the observed proliferative effects of fibronectin in the second phase of erythropoiesis.

To this end, I cultured TER119$^-$ erythroid progenitors on the various recombinant fibronectin fragments in Epo-containing media for one day and then in Epo-free media for a second day. Importantly, only those cultured on $\alpha_4\beta_1$-binding substrates V and VRGD- underwent a dramatic expansion between Day 1 and Day 2 similar to cells cultured on intact fibronectin (Figure 2-17). In contrast, cells cultured on $\alpha_5\beta_1$-binding fragment Vo exhibited a defect in cell expansion, indicating $\alpha_4\beta_1$-mediated adhesion to fibronectin is necessary for maximum numbers of terminal erythroid divisions.

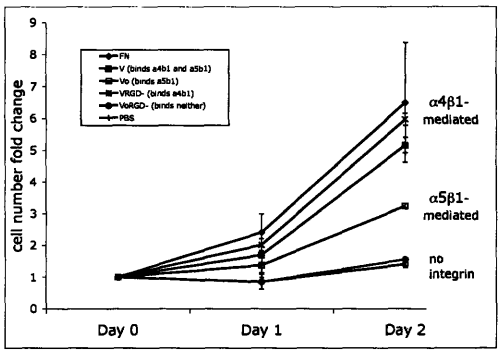

Figure 2-17: **$\alpha_4\beta_1$-mediated adhesion is required for expansion of differentiating fetal liver erythroid cells.** TER119$^-$ fetal liver erythroid progenitor cells were cultured on V, Vo, VRGD-, or VoRGD- substrates with Epo-containing media for one day and then in Epo-free media for a second day. Only $\alpha_4\beta_1$-binding substrates V and VRGD support an expansion in cell number similar to that seen on intact fibronectin.

I obtained the same result when I blocked integrin engagement with antibodies. TER119$^+$ cells were isolated from fetal liver and incubated with function blocking antibodies against α_4 or α_5 integrins for 15 minutes on ice. Cells were then added to fibronectin-coated or control plates in Epo-free media. As shown in Figure 2-18, blocking α_4 integrin blocked expansion significantly on fibronectin. Blocking α_5 integrin had a lesser effect that was not statistically significant.

Our two-phase model of erythroid expansion predicts that $\alpha_4\beta_1$ integrin engagement is only necessary during the second, Epo-independent phase. I tested this prediction by varying the extracellular matrix cues presented to the cells during the

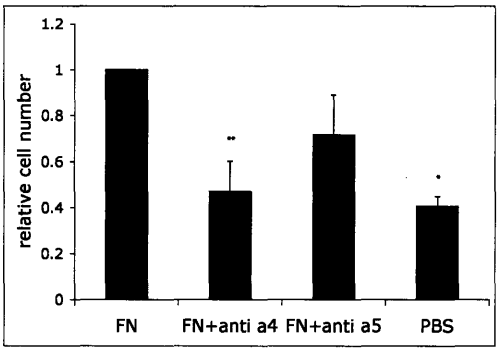

Figure 2-18: **Blocking α_4 integrin function blocks cellular expansion.** TER119$^+$ cells were isolated and incubated with function-blocking antibodies to either α_4 or α_5 integrin and then cultured on fibronectin-coated or control substrates for one day. Average normalized increase in cell number from three experiments are shown. Asterisks indicate significance at the level of $p \leq 0.05$.

first and second phases of culture. As seen in Figure 2-19, 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 (FN, VRGD-) 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 (Figure 2-20). Taken together, these results indicate that the proliferative effect of fibronectin in the second day of erythropoiesis is $\alpha_4\beta_1$ integrin-dependent.

2.5 Discussion

In this chapter I present data to establish a two-phase model for erythroid expansion. In this model, erythroid progenitor cell expansion is temporally regulated by the canonical erythropoietic growth factor Epo and the extracellular matrix protein fibronectin. Using an *in vitro* model of fetal erythropoiesis, I present evidence that erythroid progenitor cell expansion is proceeds in two distinct phases, the first governed by binding of Epo to its receptor and the second by engagement of $\alpha_4\beta_1$ integrin by fibronectin.

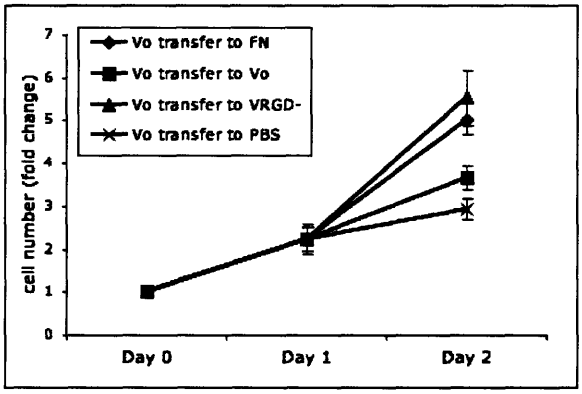

Figure 2-19: **Expansion of erythroid progenitors during the second day occurs only in conditions where $\alpha_4\beta_1$ is engaged.** TER119$^-$ cells were cultured on Vo substrates in Epo-containing media for the first day and then dissociated and transferred to wells coated with each of the various integrin-binding substrates in Epo-free media for a second day.

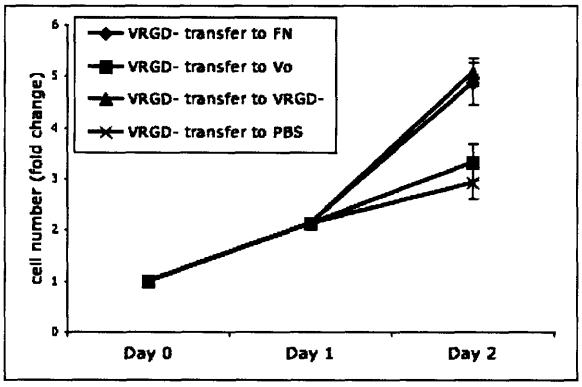

Figure 2-20: **Expansion of erythroid progenitors during the second day occurs only in conditions where $\alpha_4\beta_1$ is engaged.** TER119$^-$ cells were cultured on VRGD- substrates in Epo-containing media for the first day and then dissociated and transferred to wells coated with each of the various integrin-binding substrates in Epo-free media for a second day.

Erythropoietin and its specific receptor are crucial for promoting the survival, expansion, and differentiation of mammalian erythroid progenitors [62]. 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 [39]. Consistent with this, Epo receptors are lost as erythroid progenitors undergo terminal expansion and differentiation [94]. In the culture system for primary fetal liver erythroid progenitors, Epo is required only during the first day, when the progenitors undergo the initial ~ 2 divisions, and is not essential for the later stages of terminal cell expansion and differentiation. This raises the question of what extracellular signals, if any, these differentiating erythroblasts require to support terminal expansion, differentiation, and enucleation.

Although fibronectin is known to be the major extracellular matrix protein in the erythroid niche, its role in erythropoietic differentiation and survival was unknown. I used the established *in vitro* model of fetal erythropoiesis to systematically vary the extracellular matrix and growth factor cues presented to erythroid progenitors. My experiments indicate that withdrawal of Epo during the first day of the culture period leads to defects in cell expansion. In contrast, the presence or absence of Epo in the second day of culture had no effect on the extent of expansion of these more differentiated erythroid cells. Fibronectin plays a reciprocal role in that it has no effect on the expansion of early erythroid CFU-E progenitors but its absence on the second day of culture leads to a defect in expansion. These results establish that erythropoiesis is governed by an early Epo-dependent phase followed by a later fibronectin-dependent phase.

To determine the molecular mediator(s) of the observed fibronectin response, I measured integrin expression levels in erythroid progenitor cells; on these cells α_4, α_5, and β_1 were the mostly highly expressed integrins. Furthermore, I showed a progressive downregulation of α_4, α_5, and β_1 integrins over the three-day course of erythroid differentiation. A quantitative adhesion assay indicated that loss of integrin expression is correlated with loss of adhesion to 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 while the more differentiated reticulocytes must be released into the circulation. In this respect, α_4 and α_5 integrins play a similar role in that they both function as adhesion receptors. Since fibronectin is a major extracellular matrix protein in the bone marrow, I 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 likely is crucial in triggering their release.

By culturing erythroid progenitor cells on recombinant fibronectin fragments I demonstrated that only $\alpha_4\beta_1$ integrin engagement supports expansion on fibronectin-coated surfaces, indicating that α_4 integrins play an additional role in transducing signals from the extracellular matrix. Previous studies had identified $\alpha_4\beta_1$ and $\alpha_5\beta_1$ as the predominant integrins on erythroid progenitors [65], but here for the first time I distinguish between the roles of these two fibronectin receptors on erythroid cells.

As predicted by the two-phase model, $\alpha_4\beta_1$ integrin engagement is necessary only during the second phase of erythropoiesis, when erythroblasts undergo the final $\sim$ 2 cell divisions and enucleate. As shown in Figures 2-19 and 2-20, 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. This work thus 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, while in the second phase it functions to activate pathways necessary for erythroid expansion.

In other systems cytokine and integrin-mediated signals interact to direct cell behavior. For example, differentiation of mammary epithelia requires signals initiated by binding of prolactin to its receptor as well as simultaneous engagement of β_1 integrins by the basement membrane protein laminin [73]. Similar coordination is evident in neurogenesis, where simultaneous signals from FGF and β_1 integrins are necessary for neural stem cell maintenance and expansion [10]. 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.

The observed temporal separation in growth factor-mediated and integrin-mediated signaling can be thought of as both a consequence of the dramatic morphological and biochemical changes that take place during erythropoiesis as well as perhaps an indication. Microarray analysis of fetal liver erythroid cells at sequentially differentiated stages revealed that the most dramatic changes in the set of differentially expressed genes occurs between R2 and R3 cells (Hattangadi, Young and Lodish, unpublished data). Such genome-wide analysis provides further support for the two-stage model by indicating that early and late erythroid cells are in fact very different.

Integrin-mediated adhesion to the extracellular matrix initiates a diverse set of intracellular signaling pathways specific to each integrin dimer and cell type. Little is known about the precise downstream pathways activated following integrin receptor-ligand binding in erythroid cells. Exploring the signals that emanate from $\alpha_4\beta_1$ integrins in response to fibronectin binding and how these signals interact with those downstream of the Epo receptor is discussed in Chapter 3.

Chapter 3

Integrin Signaling in Erythropoiesis

3.1 Introduction

As presented in Chapter 2, integrin expression on fetal liver erythroid progenitor cells has a functional role in adhesion to extracellular matrix proteins as well as biological significance in supporting terminal erythroid expansion. In this chapter, I present my work investigating the mechanisms by which integrins mediate this behavior. In particular, I show that integrins promote survival of differentiating erythroid cells in the second phase of erythropoiesis by upregulation of the anti-apoptotic protein bcl-xl, much like the action of erythropoietin on immature erythroid progenitors in the first phase. In addition, I show that integrins are dispensable for erythroid differentiation, providing evidence to decouple these pathways. Finally, I show activation of canonical integrin signaling pathways downstream of fibronectin binding.

3.2 Cell cycle analysis

As seen in Figure 2-17, $\alpha_4\beta_1$ integrin engagement is necessary for expansion of erythroid cells. The total number of cells in a culture can increase over time by two mechanisms. One is an increase in cellular proliferation; the other is a decrease in

cell death. To explore the mechanism of $\alpha_4\beta_1$ integrin-mediated support of cellular expansion, I first tested the hypothesis that $\alpha_4\beta_1$ integrin engagement promotes entry into the cell cycle. First, TER119$^-$ cells were isolated from fetal liver and cultured on fibronectin or control substrate in the presence of Epo for one day. Cell cycle status was assessed using BrdU and PI staining. As shown in Table 3.1, the presence or absence of fibronectin in the first day had no effect of the percentage of cells in each stage of the cell cycle. However, withdrawal of Epo during this time had the effect of decreasing the percentage of cells in S phase.

Table 3.1: Cell cycle analysis of the first day of erythropoiesis

	Go/G1	G2/M	S
Time 0	51.4%	5.7%	42.3%
Day 1 FN	46.3%	4.3%	49.5%
Day 1 PBS	38.9%	8.1%	53.0%
Day 1 FN-Epo	52.0%	6.7%	41.3%

To test the cell cycle status of cells in the second phase of erythropoiesis, I isolated TER119$^+$ cells and cultured them without Epo on fibronectin or its various fragments. Cell cycle status was assessed at four hour time points. On fibronectin, the vast majority cells transitioned to Go/G1 over the 24 hour culture. As shown in Table 3.2, this pattern was generally the same on all substrates, including the uncoated control, indicating that the presence or absence of integrin engagement has no direct effect on cell cycle status. Furthermore, the use of an intracellular fluorescent dye to track cell division also showed no difference in the proliferative potential of cell cultured on different integrin-engaging substrates (data not shown). Thus the other remaining explanation for the cellular expansion seen in the presence of $\alpha_4\beta_1$ integrin engagement was that rather than promote expansion of erythroid cells directly via cell cycle entry, $\alpha_4\beta_1$ integrins prevent cell death.

Table 3.2: Cell cycle analysis of the second day of erythropoiesis

	Go/G1	G2/M	S
Time 0	44.5%	8.8%	46.7%
4h FN	54.4%	11.2%	34.3%
4h Vo	55.0%	11.8%	33.2%
4h VRGD	60.7%	6.9%	32.4%
4h PBS	53.2%	8.4%	38.4%
8h FN	65.6%	12.7%	21.8%
8h Vo	72.3%	11.6%	16.1%
8h VRGD	80.2%	6.7%	13.1%
8h PBS	65.0%	15.9%	19.1%
Day 1 FN	84.2%	4.1%	11.7%
Day 1 Vo	88.2%	3.3%	8.6%
Day 1 VRGD	90.6%	3.6%	5.8%
Day 1 PBS	89.0%	3.0%	8.0%

3.3 Integrin-mediated protection from apoptosis

3.3.1 Background

Apoptosis is a coordinated process of cell death by which excess cells are removed during development and growth. Distinct from necrosis, in which a cell dies due to acute injury, apoptosis, Greek for falling away, is a methodical, step-wise process where critical signaling proteins are digested, the nuclear membrane is disrupted and eventually the cell breaks apart into small fragments with are then phagocytosed. This process is orchestrated by a network of proteins referred to as the apoptosome, in which a series of initiator and executor proteins stimulate a cascade of competing pro- and anti-apoptotic signals [60, 63].

The role of Epo in preventing apoptosis of erythroid progenitors is well documented. Previous work in the Lodish lab established that signaling events downstream of the EpoR lead to an upregulation of the anti-apoptotic protein bcl-xl, and that this pathway is in part mediated by Stat5 [70]. The role of integrins in preventing apoptosis is also well characterized. In fact, anoikis, Greek for homelessness, is the specific form of apoptosis that results when cells are detached from the extra-

cellular matrix [19]. In many cases, integrin engagement leads to the generation of anti-apoptotic signals through the PI3-K and Akt pathways [60]. Because I found no direct effect of integrin engagement on cell cycle status, I investigated the role that integrins may play in survival of erythroid progenitors.

3.3.2 Annexin V

It is well known that Epo protects early erythroid progenitors from apoptosis [70], so I tested the hypothesis that fibronectin acts in a similar manner. First, I examined the roles of Epo and fibronectin in preventing apoptosis of early erythroid progenitors in the first phase of erythropoiesis using the annexin V binding assay. One of the early morphological changes during apoptosis is translocation of the plasma membrane phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane. When exposed in this manner, phosphatidylserine can bind annexin V, constituting the principle of the assay. I found that nearly 40% of TER119$^-$ cells cultured overnight on fibronectin in the absence of Epo were annexin V positive (Figure 3-1). When Epo was present in the culture medium, the percentage of annexin V positive cells dropped to 23.6%, confirming that Epo plays an anti-apoptotic 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 (Figure 3-1).

To test the role of Epo and fibronectin in preventing apoptosis during the second phase of erythropoiesis, TER119$^-$ progenitors were cultured overnight on uncoated substrates with Epo as above and then transferred to fresh fibronectin or control substrates in the absence of Epo. Four hours post Epo removal, 28.8% of the cells from PBS wells were annexin V positive, versus 17.1% of those on fibronectin (Figure 3-2), indicating that the presence of fibronectin during the second phase partially protects erythroid cells from apoptotic death.

I went on characterize the role of $\alpha_4\beta_1$ integrin in preventing apoptosis of differentiating erythroid cells. TER119$^-$ progenitor cells were cultured overnight on uncoated substrates in the presence of Epo and then transferred to fresh fibronectin

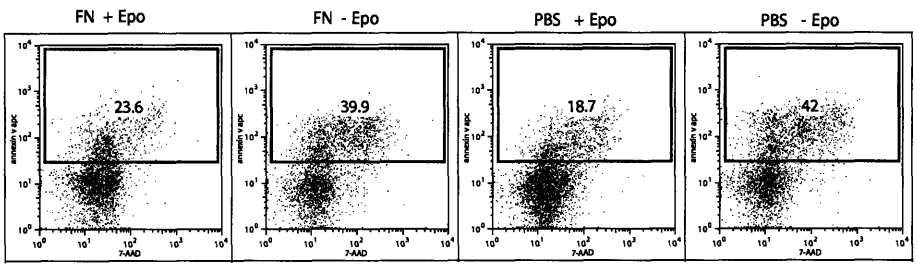

Figure 3-1: **Epo and fibronectin protect against apoptosis.** TER119$^-$ progenitor cells were cultured overnight on fibronectin or uncoated substrates in the presence or absence of Epo. AnnexinV binding (shown on the y-axis) was assayed via flow cytometry and the percentage of positive cells is indicated on each plot. The absence of Epo during the first day of culture leads to an increase the percentage of apoptotic cells, but the absence of fibronectin does not have an effect.

or uncoated wells in the presence or absence of function-blocking antibodies to α_4 and α_5 integrin. Blocking α_4 integrin increased the percentage of annexin V positive cells four hours post Epo removal whereas blocking α_5 integrin had little effect (Figure 3-2). Representative flow cytometry data are shown in Figure 3-2 and the normalized averages of 3 independent experiments are shown in Figure 3-3. Taken together, 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.

3.3.3 Bcl-xL

Bcl family proteins regulate apoptosis by either inducing or inhibiting the release of cytochrome c from the mitochondria into the cytosol. One of the mechanisms of apoptosis protection mediated by Epo is through upregulation of the anti-apoptotic protein bcl-xL [70], so I tested whether fibronectin protects against apoptosis in a similar manner in the second phase of erythropoesis. TER119$^-$ progenitors were cultured overnight on uncoated substrates in the presence of Epo and then serum-starved for one hour before being transferred to fresh fibronectin or control substrates in the absence of Epo as above. Cells were lysed at 5, 15 and 30 minutes and bcl-xL protein expression was assessed via Western blot. As shown in the representative

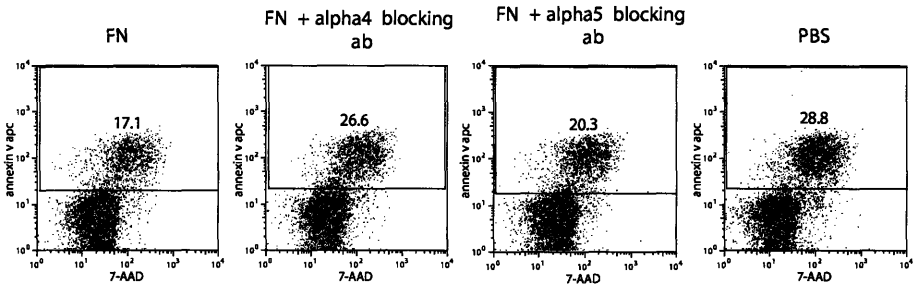

Figure 3-2: **Fibronectin protects from apoptosis in the second stage of expansion through $\alpha_4\beta_1$ integrin.** TER119$^-$ fetal liver cells were purified and cultured overnight on uncoated substrates in Epo-containing media. Cells were then dissociated, incubated with or without antibodies to α_4 or α_5 integrins and cultured in Epo-free media on fibronectin or control substrates. Four hours post-Epo removal, the cells were stained with annexin V (y-axis) and 7-AAD (x-axis) to assay for apoptosis. Data are representative of three independent experiments.

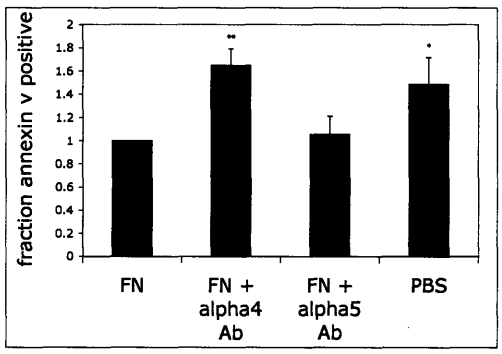

Figure 3-3: **Summary of apoptosis data.** The percentage of annexin V positive cells was normalized to that on fibronectin. Treatment with a function-blocking antibody to α_4 integrin significantly increases the percentage of apoptotic cells four hours post-Epo removal, while blocking α_5 integrin has no effect. Two asterisks indicate statistical significance at the level of $p \leq 0.01$ and one asterisk indicates statistical significance at the level of $p \leq 0.05$.

Western blot below (Figure 3-4), expression is distinctly higher in cells cultured on fibronectin than those cultured on control substrates. The Western blot in Figure 3-5 shows bcl-xl expression at 30 minutes in addition to a loading control, as represented by equivalent expression of an actin. Summary of these data are presented in Figure 3-6.

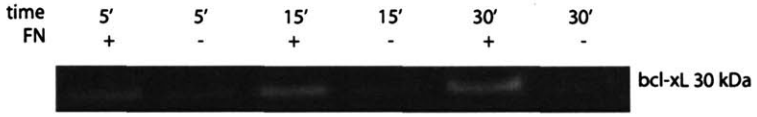

Figure 3-4: **Representative Western blot of bcl-xL protein expression.** TER119$^-$ progenitors were cultured overnight on uncoated substrates in the presence of Epo and then serum-starved for one hour before being transferred to fresh fibronectin or control substrates in the absence of Epo. Bcl-xL expression is distinctly higher in cells cultured on fibronectin than those cultured on control substrates.

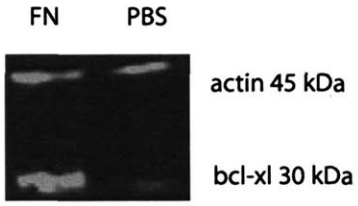

Figure 3-5: **Representative Western blot of bcl-xL protein expression with loading control.** Bcl-xL expression was assessed 30 minutes post-Epo removal. Bcl-xl expression distinctly higher in cells cultured on FN than those cultured on control substrates, whereas expression of actin is equivalent.

Treating cells with function-blocking antibodies to α_4 and α_5 integrins provided a means of analyzing the contribution of these molecules to bcl-xL upregulation. TER119$^-$ progenitors were cultured overnight on uncoated substrates in the presence of Epo and then serum-starved for one hour before being incubated with antibodies to α_4 or α_5 integrin. Cells were then transferred to fibronectin-coated wells in the absence of Epo. Blocking α_4 integrin resulted in abrogation of bcl-xl upregulation, while blocking α_5 integrin had a smaller effect (Figure 3-7). These data confirm the anti-apoptotic role of α_4 integrin in the second phase of erythropoiesis.

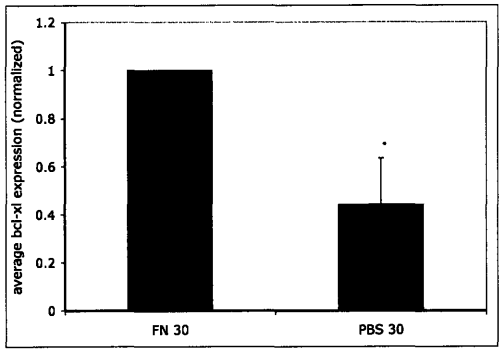

Figure 3-6: **Summary of bcl-xL Western blot data.** The ratio of bcl-xL expression on fibronectin to that on a control substrate at 30 minutes in four independent experiments were averaged. Asterisk indicates significance at the level of $p \leq 0.05$.

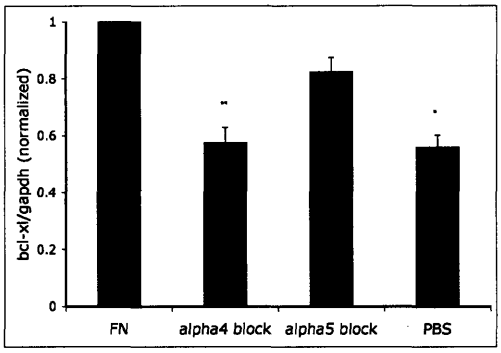

Figure 3-7: **α_4 integrin mediates bcl-xl upregulation.** TER119$^-$ progenitors were cultured overnight on uncoated substrates in the presence of Epo and then serum-starved for one hour before being incubated with antibodies to α_4 or α_5 integrin. Cells were then transferred to fibronectin-coated wells in the absence of Epo. Bcl-xL expression was assessed 30 minutes post-Epo removal via Western blot. Each bar is the average ratio of bcl-xl expression to GAPDH expression in two experiments.

Similar results were obtained via flow cytometry (Figure 3-8). One hour after Epo withdrawal, cells were fixed, permeabilized and stained with an antibody to bcl-xL. Bcl-xL expression in cells cultured on fibronectin was higher than those cultured on control substrates.

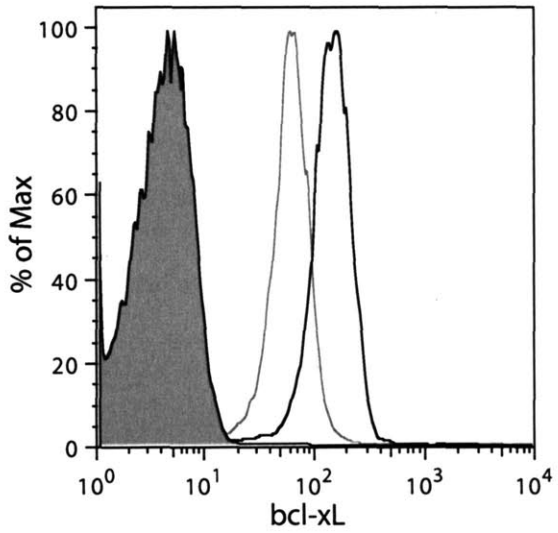

Figure 3-8: **Flow cytometric analysis of bcl-xL expression.** TER119$^-$ progenitors were cultured overnight on uncoated substrates in the presence of Epo and then transferred to fibronectin-coated or control substrates in the absence of Epo. One hour after Epo withdrawal, bcl-xL expression is distinctly higher in cells cultured on fibronectin (black trace) than those cultured on control substrates (grey trace). Background staining (filled histogram) was determined by staining of the secondary antibody alone.

Further confirmation of this effect came from quantitative PCR data (Figure 3-9). Cells were cultured as above overnight in the presence or absence of Epo. Total RNA was isolated, reverse transcribed and amplified using specific primers. As shown in the left side of Figure 3-9, the presence of Epo in the first phase of culture leads to upregulation of bcl-xL. Cells that were cultured with Epo overnight were then cultured in Epo-free media on fibronectin or control substrates. Total RNA was isolated at 15 minutes, one hour and four hour time points and reverse transcribed and amplified as above. At each time point, bcl-xL expression on fibronectin was normalized to expression of the 18S ribosomal subunit as well as to expression on the control substrate. As seen in Figure 3-9, relative expression of bcl-xL steadily

increased during culture on fibronectin, indicating that the presence of fibronectin in the second phase promotes cell survival much like the presence of Epo in the first phase.

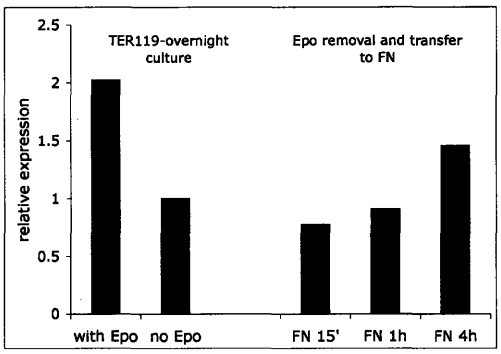

Figure 3-9: **Quantitative PCR analysis of bcl-xL expression.** To measure bcl-xl expression in the first phase of erythropoiesis (left side), TER119$^-$ progenitors were cultured overnight on uncoated substrates in the presence or absence of Epo overnight and bcl-xl expression was measured by Western blot. To measure bcl-xl expression in the second phase (right side), TER119$^-$ progenitors were cultured overnight on uncoated substrates in the presence of Epo overnight and then transferred to fibronectin-coated or control substrates in the absence of Epo. The presence of fibronectin in the second phase upregulates the anti-apoptotic protein bcl-xL much like the presence of Epo in the first phase.

3.3.4 Caspase-3

In order to show that integrins provide protection from apoptosis, I was interested to assay apoptotic proteins at a various stages in the pathway. Caspases are the executor proteins of apoptosis; in their active forms they enzymatically cleave a variety of cellular substrates. Caspase-3 is a key component of the caspase cascade in that in its active form it not only cleaves target proteins but also cleaves its own regulator, caspase-9, forming a positive feedback loop [60].

I used flow cytometry to measure the level of cleaved (active) caspase-3. TER119$^-$ cells were cultured overnight with Epo and then transferred to fibronectin or control substrates in Epo-free media. At the indicated time points, cells were dissociated,

fixed and permeabilized in order to stain with an intracellular antibody specific to cleaved caspase-3. As shown in Figure 3-10, cells cultured in the absence of fibronectin indicate a higher level of active caspase-3 expression, indicating a higher level of apoptosis. Again, these data add evidence to the model that integrin engagement in the second phase of erythropoiesis leads to increased cell survival.

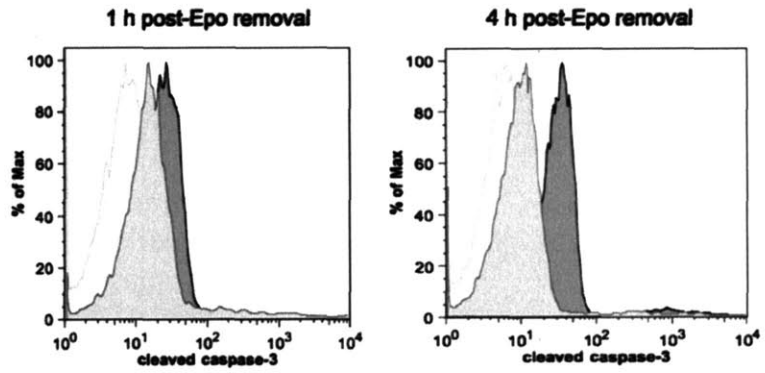

Figure 3-10: **Flow cytometry analysis of cleaved caspase-3 expression.** TER119$^-$ cells were cultured overnight with Epo and then transferred to fibronectin or control substrates in Epo-free media. At the indicated time points, cells were fixed and permeabilized and stained with an intracellular antibody specific to cleaved caspase-3. The absence of fibronectin in the second phase activates the pro-apoptotic protein caspase-3. In each plot, the dark-filled histogram represents the expression level in the absence of fibronectin, the medium-filled histogram the expression level in the presence of fibronectin, and the unfilled histogram the secondary antibody control.

3.4 Role in differentiation

Integrins play an important role in differentiation of many different cell types. For example, differentiation of mammary epithelia is dependent upon β_1 integrin-mediated adhesion to the extracellular matrix protein laminin [73] and fibronectin inhibits terminal differentiation of keratinoyctes via integrins as well [3]. Given the observed effects of $\alpha_4\beta_1$ integrin on erythroid cell expansion, I tested whether $\alpha_4\beta_1$ integrins had any effect on erythroid differentiation.

As shown in Figure 2-2, our fetal erythroid culture system is well suited to tracking differentiation *in vitro* using the cell surface markers CD71 and TER119. To test the

role of integrins in erythroid differentiation, TER119$^-$ cells were isolated and cultured on fibronectin and its fragments in the presence of Epo for 16-18 hours. After this time, the media was changed to Epo-free media and the culture was carried out for two more days. At each time point, cells were dissociated and stained with antibodies to CD71 and TER119 to assess the level of differentiation via flow cytometry. As shown in Figure 3-11, the pattern of CD71 upregulation, TER119 upregulation and subsequent CD71 downregulation is unchanged in the absence of fibronectin or $\alpha_4\beta_1$ integrin engagment. When Epo was absent from the culture media for the first phase, as shown in the right-most column in Figure 3-11, there is a significant defect in upregulation of CD71 during the first day. As previously suggested by Arroyo et al, these data provide direct evidence to decouple the pathways controlling erythroid differentiation and survival, in that cell survival is mediated by $\alpha_4\beta_1$ integrin, while differentiation is $\alpha_4\beta_1$ integrin-independent.

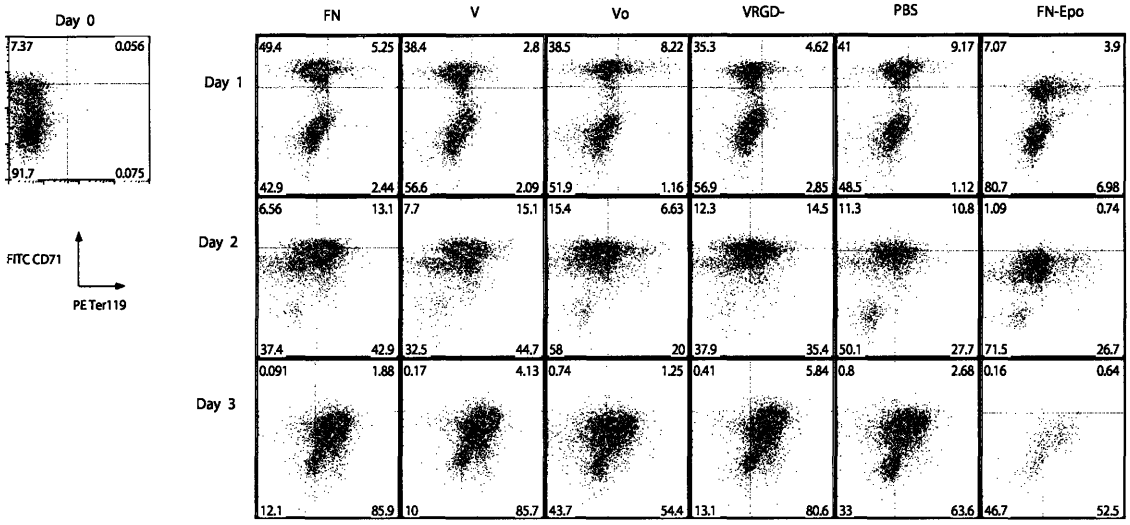

Figure 3-11: **Role of integrins in erythroid differentiation.** TER119$^-$ cells were isolated and cultured on fibronectin and its various fragments and CD71 and TER119 expression was analyzed using flow cytometry. The presence or absence of $\alpha_4\beta_1$ integrin engagement had no effect on erythroid differentiation, as indicated by similar staining patterns for cells cultured on Vo, VRGD- and PBS as on FN and V. Only in the absence of Epo (right-most column) is there a defect in differentiation.

3.5 Focal adhesion signaling

3.5.1 Paxillin and Pyk2

Formation of focal adhesions is one of the hallmarks of integrin signaling. These protein complexes are often centered around focal adhesion kinase (FAK), which forms linkages to cytoskeletal components in addition to activating downstream signaling pathways [87]. However, as suggested by others, I found that the closely related family member proline-rich tyrosine kinase-2 (Pyk2) rather than FAK itself is expressed in hematopoietic cells [5]. FAK and Pyk2 have 48% amino acid identity and share the same domain structure: a unique N-terminus, a protein tyrosine kinase domain, and two proline-rich regions at the C-terminus [5]. Quantitative real time PCR indicated expression of Pyk2 to be at least 3-fold greater than that of FAK in freshly isolated TER119$^-$ over four orders of magnitude input RNA amount (Figure 3-12).

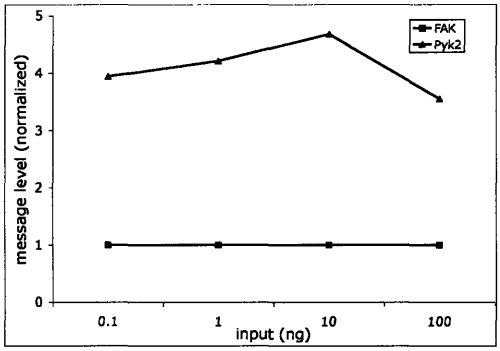

Figure 3-12: **Expression of Pyk2 and FAK in TER119$^-$ cells.** Quantitative PCR was used to assess Pyk2 and FAK expression in freshly isolated TER119$^-$. Expression of Pyk2 was at least 3-fold greater than that of FAK over four orders of magnitude input RNA amount. Data were normalized to expression of 18S ribosomal RNA as well as to FAK expression at each input RNA amount.

To assay Pyk2 activation, TER119$^-$ were isolated and cultured overnight in the presence of Epo. Cells were serum-starved for one hour and then transferred to fibronectin-coated or control wells in the absence of Epo. Cells were lysed at the indicated times and proteins were analyzed via Western blot. Membranes were blot-

ted with anti-phosphoPyk2 antibody as well as anti-GAPDH antibody as a loading control. As shown in Figure 3-13 Pyk2 phosphorylation increases with time in cells cultured on fibronectin, but less so on control substrates, indicating that the presence of fibronectin activates Pyk2. Quantitation and summary of these data in are shown in Figure 3-14).

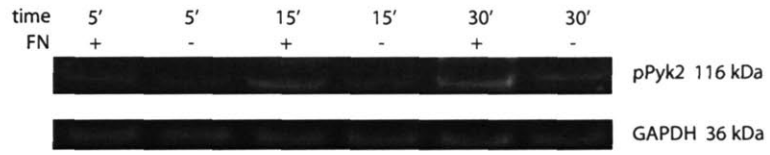

Figure 3-13: **Representative time course of Pyk2 phosphorylation in response to fibronectin.** TER119$^-$ cells were cultured overnight in the presence of Epo and then serum-starved for one hour and transferred to fibronectin-coated or control wells. Pyk2 activation was analyzed by Western blot with GAPDH as a control. The presence of fibronectin leads to Pyk2 activation.

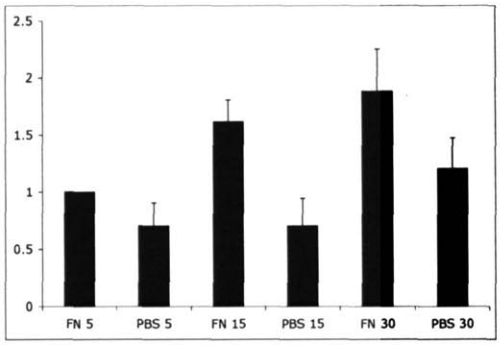

Figure 3-14: **Quantification of Pyk2 phosphorylation time course.** Each bar represents the average of the ratio of phosphorylated Pyk2 to GAPDH normalized to that on fibronectin at 5 minutes in two experiments. The presence of fibronectin leads to Pyk2 activation.

Blocking α_4 integrin interaction with fibronectin blocked Pyk2 activation (Figure 3-15). TER119$^-$ were isolated and cultured overnight in the presence of Epo. Cells were serum-starved for one hour and then incubated with function-blocking antibodies to α_4 or α_5 integrin and then transferred to fibronectin-coated or control wells in the

absence of Epo. Cells were lysed after 30 minutes and lysates were analyzed via Western blot. Membranes were blotted with anti-phosphoPyk2 antibody as well as anti-GAPDH antibody as a loading control. The ratio of phosphorylated Pyk2 to GAPDH is dramatically decreased when α_4 integrin binding to fibronectin is blocked while blocking α_5 integrin has little effect. These data indicate that activation of Pyk2 in the second phase of erythropoiesis is mediated by fibronectin binding to α_4 integrin.

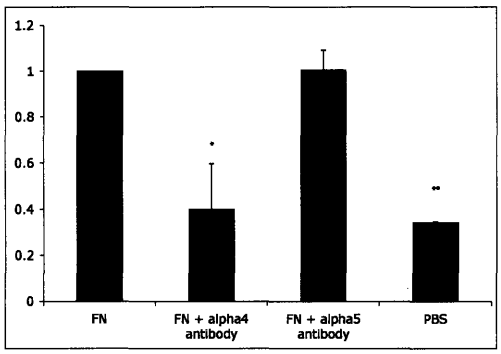

Figure 3-15: **Pyk2 activation is mediated by α_4 integrin.** TER119$^-$ cells were cultured overnight in the presence of Epo and then serum-starved for one hour and incubated with function-blocking antibodies to α_4 or α_5 integrin and then transferred to fibronectin-coated or control wells. Pyk2 activation was analyzed by Western blot. Each bar represents the average of the ratio of phosphorylated Pyk2 to GAPDH normalized to that on fibronectin in two experiments. Two asterisks indicate statistical significance at the level of $p \leq 0.001$ and one asterisk indicates statistical significance at the level of $p \leq 0.05$.

One of the roles of Pyk2 is to phosphorylate paxillin, a small adaptor protein that directly associates with the cytoplasmic tail of α_4 integrin [79]. Paxillin, through its numerous protein-binding domains, interacts with a variety of signaling molecules, thereby transmitting integrin-generated signals to the interior of the cell. One important biological consequence of α_4 integrin interaction with paxillin is the inhibition of cell spreading and migration [42].

An IP-Western approach was used to determine the role of paxillin in the integrin-dependent phase of erythropoiesis. TER119$^-$ were isolated and cultured overnight

in the presence of Epo. Cells were serum-starved for one hour and then transferred to fibronectin-coated or control wells in the absence of Epo. Cells were lysed and paxillin was immunoprecipitated from the lysates. Resulting proteins were analyzed via Western blot with anti-phosphopaxillin and anti-paxillin antibodies. As shown in Figure 3-16, phosphorylation of paxillin proceeds in a biphasic fashion (Figure 3-16). The data at 30 minutes were normalized and averaged, showing a significant decrease in the ratio of phosphorylated paxillin to total paxillin on control substrates relative to fibronectin (Figure 3-17). Taken together, these data establish that paxillin is activated in the presence of fibronectin in the second phase of erythropoiesis.

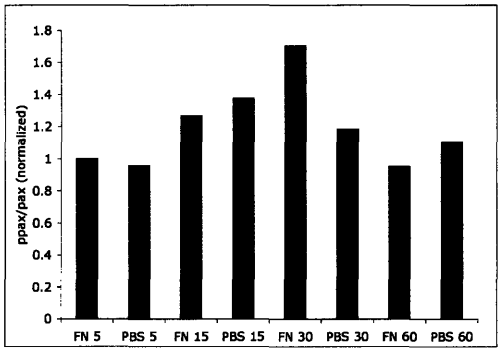

Figure 3-16: **Representative time course of paxillin activation.** TER119$^-$ cells were cultured overnight in the presence of Epo and then serum-starved for one hour and transferred to fibronectin-coated or control wells. Paxillin activation was analyzed using an IP-Western approach. Each bar represents the ratio of phosphorylated paxillin to total paxillin normalized to that on fibronectin at 5 minutes. The presence of fibronectin leads to biphasic paxillin activation.

Pyk2 and paxillin have also been shown to have a direct physical interaction [33]. In the second phase of erythropoiesis, the presence of fibronectin also stimulates binding of paxillin and Pyk2 (Figure 3-18). TER119$^-$ cells were cultured overnight in the presence of Epo and then serum-starved for one hour and transferred to fibronectin-coated or control wells. Paxillin was immunoprecipitated and proteins were analyzed via Western blot. As shown in Figure 3-18, phosphorylated Pyk2 co-immunoprecipitated with paxillin. Qualitatively, the level of phosphorylated

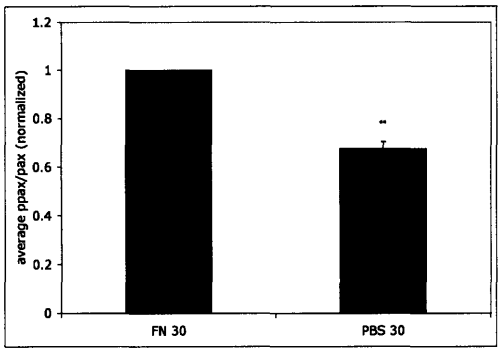

Figure 3-17: **Activation of paxillin in the second phase of erythropoiesis.** TER119$^-$ cells were cultured overnight in the presence of Epo and then serum-starved for one hour and transferred to fibronectin-coated or control wells. Paxillin activation was analyzed using an IP-Western approach. The ratio of phosphorylated paxillin to total paxillin at 30 minutes on control substrates was normalized to that on fibronectin. The presence of fibronectin leads to a significant increase in paxillin activation. Asterisks represent statistical significance at the level of p $\leq$0.001.

Pyk2 and phosphorylated paxillin was greater in the presence of fibronectin than on control substrates. Taken together with the data presented above, a model where fibronectin binding to $\alpha_4\beta_1$ integrin activates paxillin and Pyk2 and also stimulates their binding can be proposed.

3.5.2 Src-family kinases

I was also interested in exploring activation of Src-family kinases (SFKs) downstream of $\alpha_4\beta_1$ integrin in the second phase of eryhropoiesis. Upon integrin activation, some SFK family members are known to be activated and associated with focal adhesions [76]. SFKs have also been shown to play roles in the hematopoietic system. *Lyn-/-* adult bone marrow cells exhibited defects in production of TER119$^+$ erythroid cells as well as increased apoptosis and decreased cell cycle entry [31]. Thus I hypothesized that SFKs may play a role downstream of integrins in erythroid expansion or survival.

I used the Src-family kinase pharmacological inhibitor PP2 to explore the role of SFKs in erythropoiesis. PP2 inhibits the kinase activity of Src kinase as well as

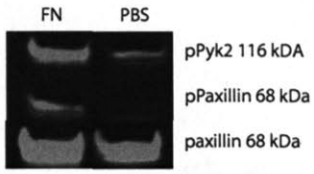

Figure 3-18: **Co-immunoprecipitation of paxillin and Pyk2.** TER119$^-$ cells were cultured overnight in the presence of Epo and then serum-starved for one hour and transferred to fibronectin-coated or control wells. Paxillin was immunoprecipitated and resulting proteins were analyzed by Western blot. Membranes were first blotted with phospho-paxillin and phospho-Pyk2 antibodies and stripped and reblotted with total paxillin antibody. On fibronectin but not control substrates, phosphorylated paxillin and phosphorylated Pyk2 co-immunoprecipitate. These blots are representative of two experiments.

other family members. TER119$^+$ cells were isolated from fetal liver and treated with increasing concentrations of PP2, PP3, the inactive analogue, or an equal volume of DMSO as an added control. Cells were then cultured on fibronectin coated plates for one day and cell number was assessed. As shown in Figure 3-19, PP2 blocks fibronectin-dependent expansion of erythroid cells in a dose-dependent manner.

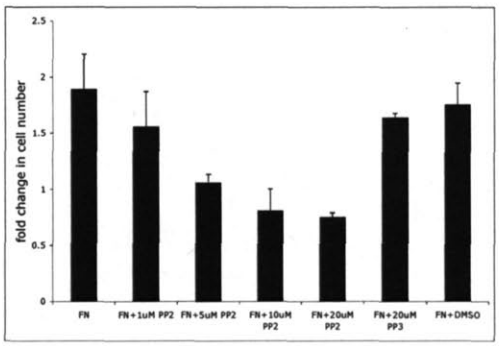

Figure 3-19: **Src-family kinase inhibitor PP2 blocks fibronectin-dependent exansion in a dose-dependent manner.** TER119$^+$ cells were treated with PP2, its inactive analogue PP3 or DMSO as a control and cultured for one day on fibronectin-coated plates. Each bar represents the average number of cells per well normalized to that in untreated cells.

The Src family is composed of many closely related family members, so it was

necessary to determine which family members were expressed in fetal liver erythroid cells. To do so, expression of SFK isoforms was assessed by real time PCR in both TER119$^-$ and TER119$^+$ cells (Figure 3-20). Hck was found to be highly expressed in TER119$^-$ cells and Lyn was highly expressed in both TER119$^-$ and TER119$^+$ cells. Thus these two family members so were chosen for further analysis.

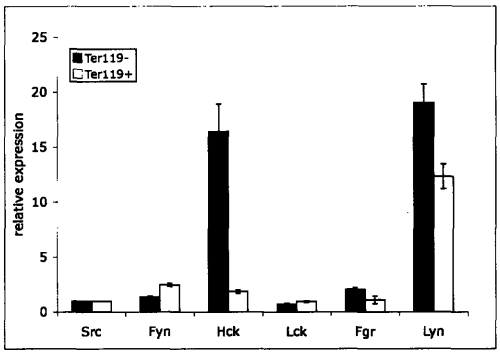

Figure 3-20: **Expression of Src-family kinases in fetal liver erythroid cells.** TER119$^-$ and TER119$^+$ cells were isolated and expression of SFKs was assessed using real time PCR. Hck was highly expressed in TER119$^-$ cells and Lyn was highly expressed in both TER119$^-$ and TER119$^+$ cells.

To determine the role of Hck and Lyn in fibronectin-dependent erythroid expansion, I undertook an immunoprecipitation-Western blot approach. TER119$^-$ cells were isolated from fetal liver and cultured overnight in the presence of Epo on uncoated plates. Cells were then serum-starved in the absence of Epo for one hour and transferred to fibronectin or uncoated wells. At the indicated time points, cells were lysed and proteins were analyzed via Western blot. Membranes were blotted with anti-phosphotyrosine and anti-Hck or anti-Lyn antibodies. As shown below, there was no significant difference in phosphorylation levels of Hck between treated (fibronectin) and untreated (PBS) cells (Figure 3-21). Although in the case of Lyn there was a decrease in relative phosphorylation at 5 minutes (Figure 3-22), the fact that Lyn kinase is activated by a combination of phosphorylation and dephosphorylation events make it difficult to interpret these data.

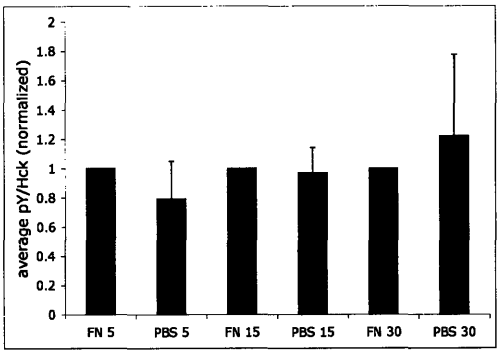

Figure 3-21: **Phosphorylation of Hck kinase during fibronectin-dependent erythroid expansion.** TER119$^-$ cells were cultured overnight in the presence of Epo and then serum-starved for one hour and transferred to fibronectin-coated or control wells in the absence of Epo. Cells were lysed at the indicated times and phosphotyrosine and total Hck levels were determined by Western blot. Data are the normalized averages of three experiments.

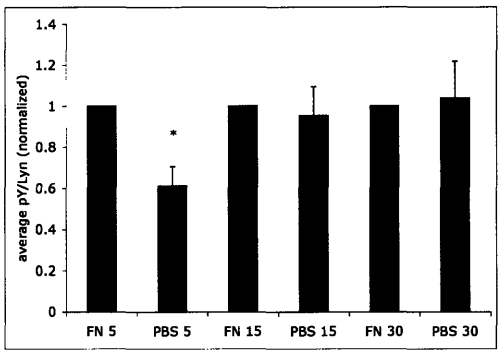

Figure 3-22: **Phosphorylation of Lyn kinase during fibronectin-dependent erythroid expansion.** TER119$^-$ cells were cultured overnight in the presence of Epo and then serum-starved for one hour and transferred to fibronectin-coated or control wells in the absence of Epo. Cells were lysed at the indicated times and phosphotyrosine and total Lyn levels were determined by Western blot. Data are the normalized averages of three experiments. Asterisk indicates significance at the level of $p \leq 0.05$.

3.6 Discussion

In this chapter I present data on the role of $\alpha_4\beta_1$ integrin-mediated signals in erythropoiesis. As shown in Chapter 2, $\alpha_4\beta_1$ integrin is necessary and sufficient for terminal erythroid expansion. The first phase of erythroid expansion is largely governed by binding of Epo to its receptor. The events downstream of EpoR activation are well characterized, and include auto-phosphorylation of receptor subunits, phosphorylation of JAK2 and STAT5 and subsequent translocation of STAT5 dimers to the nucleus [62]. Although $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins had been identified as important in the erythroid niche, virtually nothing about their biological significance or downstream signaling events was known.

3.6.1 Cellular proliferation and death

One important function of EpoR-mediated signals is to activate anti-apoptotic pathways in order to promote survival of erythroid progenitors. This is seen in upregulation of bcl-xl in the presence of Epo in the first phase of erythropoiesis [70]. In beginning to explore the mechanism by which $\alpha_4\beta_1$ integrin supports erythroid cell expansion, I first tested the hypothesis that $\alpha_4\beta_1$ integrin stimulates cellular proliferation. By analyzing the cell cycle status of cells cultured on various fragments, I determined that engagement of integrins in the second phase of erythropoiesis has no direct effect on cellular proliferation. However, the withdrawal of Epo in the first day of culture reduced the percentage of cells in S phase, indicating a direct effect on cellular proliferation.

Next, I focused on potential anti-apoptotic effects. While withdrawal of Epo during the first day of culture led to an increase in the percentage of apoptotic cells, the presence or absence of fibronectin during this time had no effect. However, in the second phase of erythropoiesis fibronectin does play an anti-apoptotic role. Furthermore, this effect is mediated by $\alpha_4\beta_1$ integrin. Blocking α_4 integrin led to an increase in the percentage of apoptotic cells, similar to culturing cells in the absence of fibronectin, while blocking α_5 integrin had a minimal effect on the level of apoptosis.

One mechanism of apoptosis protection by fibronectin binding was through upregulation of bcl-xl, much like the effect of Epo on early erythroid progenitors. Blocking α_4 integrin engagement with an antibody abrogated this effect. Taken together, these results establish that one of the roles of fibronectin in the erythroid niche is to protect differentiating erythroid cells from apoptosis, and that this behavior is specifically mediated by $\alpha_4\beta_1$ integrin.

Differentiation, in general, is associated with cell cycle arrest. In erythroid cells, however, one of the hallmarks of terminal differentiation is an expansion in cell number. Erythroid cell divisions can categorized into two types. Early erythroid progenitors undergo "renewal divisions" where the number of progenitors is doubled while maintaining differentiation potential. Later, erythroid cell divisions can be termed "differentiation divisions" where reduction in cell size resulting from cell division is a necessary step in terminal differentiation. There is evidence to suggest that these two types of cell divisions are marked by differing cell cycle characteristics as well as differing regulatory mechanisms [16, 36].

One question that follows from my work is the role that Epo and fibronectin may play in stimulating these different types of cell divisions. While my data suggests that integrin engagement in the second phase of erythropoeisis does not stimulate entry into the cell cycle, it could be that the types of cell divisions - as distinguished by the length of each phase and as well as activation of specific regulatory molecules - are in fact determined by integrin engagement.

The finding that integrin engagement does not directly induce cell cycle entry is important in that it highlights the distinction between cell proliferation and cell survival. Whereas the data suggest that Epo-mediated signaling directly stimulates proliferation and as well as survival, $\alpha_4\beta_1$ integrin seems to have only an anti-apoptotic role. One issue that is unclear, however, is whether the expansion seen in the second phase is driven by proliferative signals established by Epo in the first phase that continue in its absence in the second phase or merely a reduction in apoptosis mediated by $\alpha_4\beta_1$ integrins.

3.6.2 Differentiation

Interestingly, I found that integrin engagement is not essential for differentiation of erythroid progenitors, as measured by expression levels of CD71 and TER119, supporting the view that erythroid differentiation and expansion are decoupled and regulated by separate pathways [4]. Withdrawal of Epo during the first phase of erythropoiesis, and 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 EpoR 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 cell division and induction of erythroid-specific genes.

There appears to be a distinction in the requirement for $\alpha_4\beta_1$ integrins in fetal and adult erythropoiesis. While α_4 integrin-null fetal liver progenitors fail to develop into large colonies *in vitro*, faint red staining is visible, suggesting that proliferation but not differentiation is disrupted in the absence of α_4 integrin [4]. In an adult conditional knockout model, there was no reduction in the number of TER119$^+$ cells in the bone marrow of adult mice induced to lose α_4 integrin expression, but these animals exhibited an impaired response to anemia, with decreased numbers of reticulocytes and red blood cells [68]. This evidence that α_4 integrin is required for terminal erythroid proliferation during adult stress-induced erythropoiesis is consistent with my findings and thereby suggests that fetal and stress-induced erythropoiesis may be regulated differently from adult homeostatic erythropoiesis. Our *in vitro* model of fetal erythropoiesis allowed us to make direct, quantitative measurements of erythroid expansion and differentiation to show that these processes are in fact regulated separately.

3.6.3 Focal adhesions

Although the roles of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins in adhesion of erythroid cells has long been established, very little information about any downstream signaling events was known. By demonstrating a role for $\alpha_4\beta_1$ in providing protection from apoptosis via upregulation of bcl-xL, I have established that $\alpha_4\beta_1$ functions as a signaling receptor in addition to an adhesion receptor. In order to add support to that claim, I characterized some of the signaling events downstream of $\alpha_4\beta_1$ engagement by fibronectin in the erythroid niche.

Formation of focal adhesions are in many ways the hallmark of integrin signaling. Engagement of integrins by ligand binding leads to clustering of receptors on the cell surface as well as recruitment of signaling molecules in the cytoplasm. Typically, activation of FAK and other tyrosine kinases lead to activation of various signaling molecules including MAPK, Rac and PI3K. Paxillin is an important adaptor protein in the midst of this signaling network. Paxillin physically interacts with the cytoplasmic tail of α_4 integrin, and through its numerous protein binding domains, binds proteins that directly contribute to reorganization of the actin cytoskeleton [79]. Through this relationship with the cytoskeleton, integrin activation can result in formation of stress fibers and influence cell motility.

When initial Western blot experiments looking at FAK expression and activation in erythroid cells were unsuccessful, I began to focus on Pyk2, a closely related FAK family member whose expression had been documented in hematopoietic cells [15]. Via quantitative PCR, I determined that expression of Pyk2 is at least 3-fold greater than that of FAK over several orders of magnitude input RNA in TER119$^-$ cells. IP-Western blot studies indicated α_4 integrin-dependent activation of Pyk2 and paxillin upon fibronectin binding in the second phase of erythroid culture. In addition, the active forms of Pyk2 and paxillin co-immunoprecipitated in the presence of fibronectin. Although Pyk2 is known to phosphorylate paxillin, it cannot be explicitly be determined from the experiments presented here if binding precedes paxillin activation or if binding takes place only between active forms of the proteins. Taken together,

however, these data aid in characterizing the signaling behavior of $\alpha_4\beta_1$ integrin in erythroid cells.

One curious aspect of integrin signaling in erythroid cells is the role of the cytoskeleton. Erythroid cells are typically considered to be suspension cells in that they do not spread on tissue culture plastic or other substrates. Indeed, in my many adhesion experiments, I never observed any spread cells despite populations of strongly adherent cells. Paxillin is the key to this behavior. Binding of paxillin to the α_4 cytoplasmic tail inhibits cell spreading, as well as focal adhesion and stress fiber formation [42]. The biological significance of this behavior is that at the integrin-dependent stage of erythropoiesis, differentiating erythroid cells are preparing to leave the erythroid niche and enter the blood stream, and thus spreading on an extracellular matrix protein would be counterproductive. In addition, in other cell types a point mutation that distrupted integrin-paxillin binding markedly decreased cell migration [42, 64]. Further investigation of this mechanism in erythroid cells is needed, particularly in the process of egress of differentiated erythrocytes from the bone marrow, which is discussed further in Chapter 5.

3.6.4 Src-family kinases

Src-family kinases are protein tyrosine kinases activated following engagement of several different classes of cellular receptors, including integrins. Upon activation, some SFK are known to localize to focal adhesions and activate signaling molecules such as paxillin [76]. SFKs mutant animals have demonstrated hematopoietic and erythroid-specific defects, and specific SFK family members have been shown to provide protection from apoptosis in hematopoietic cells, which led to the hypothesis that they may play an important role in integrin-dependent erythropoiesis.

Treatment of TER119$^+$ cells with a SFK inhibitor led to a dose-dependent inhibition of cell expansion. Quantitative PCR expression profiling studies indicated that Lyn and Hck were the most highly expressed members in fetal liver cells, so they were chosen for further study. The IP-Western approach utilized, however, did not indicate that either of these kinases is specifically activated by the presence of

fibronectin during integrin-dependent erythropoiesis. One important caveat is that the kinase activity of these proteins is regulated by a combination of phosphorylation and dephosphorylation events, so assaying for total phosphotyrosine, as above, may not provide an accurate measure of protein activation. A fluorometric kinase assay was also used to assess activity but the differences in activity level between fibronectin-stimulated and control samples was too small to be significant (data not shown).

The evidence from other groups that Lyn plays a role in erythropoiesis is very strong. *Lyn -/-* erythroblasts displayed a defect in upregulation of TER119 as well as decreased expansion capacity [31]. In addition, *Lyn -/-* erythroid cells had decreased levels of GATA-1 and Stat5, key erythroid transcription factors [29]. While I cannot conclude from the experiments presented above that Lyn or Hck are activated in response to fibronectin binding during integrin-dependent erythropoiesis, further experiments are needed before a definitive statement can be made. In particular, all of the studies indicating a role for Lyn in erythropoiesis were done with knockout mice; it could be that the reagents available to study Lyn activation directly are not sensitive enough to detect the relevant differences. Exploration of integrin-dependent erythropoiesis in Lyn knockout mice is certainly one area of future work.

3.6.5 Conclusion

In this chapter, I present data to establish that $\alpha_4\beta_1$ integrin functions as a *bona fide* signaling receptor in the second phase of erythropoiesis. One of its major functions is to prevent differentiating erythroid cells from apoptosis through upregulation of bcl-xl, much like the action of Epo binding to its receptor on early erythroid progenitors. $\alpha_5\beta_1$ integrin does not play a similar survival role, consistent with the idea that it functions only as an adhesion receptor. In addition, the signals downstream of $\alpha_4\beta_1$ integrin in erythroid cells capture many of the hallmarks of canonical integrin signaling, including activation of the focal adhesion kinase protein Pyk2 and cytoskeletal adaptor protein paxillin. Linking these signals to cellular behavior is a focus of future work, as discussed in Chapter 5.

Chapter 4

Role of Integrins in Hematopoietic Stem Cell Maintenance

4.1 Introduction

Hematopoietic differentiation is a unidirectional process. Once an HSC receives the appropriate differentiation cues, it follows a path of progressive lineage commitment until it reaches its terminal phenotype. At each developmental stage, a unique combination of signals from cytokines, stromal cells and the extracellular matrix converge to determine cell fate. The contribution of cytokines to this milieu are traditionally the most well studied, but recent progress in defining the stem cell niche has focused the field on the role of stromal cells and, to a lesser extent, extracellular matrix. One of the aims of my thesis was to explore the role of ECM in directing HSC self-renewal and differentiation decisions. This chapter presents my work on identifying highly expressed integrins on purified populations of HSCs and characterizing the role of those integrins in mediating HSC adhesion to ECM proteins.

4.2 ECM in the HSC niche

The extracellular matrix in the bone marrow serves two functions. The first is a structural role, where the complex three dimensional network created by the vari-

ety of ECM molecules expressed in the bone marrow serves to trap and concentrate soluble factors, protecting them from proteolytic cleavage, and also to attract specific cell types to interact with each other. However it is clear that ECM molecules themselves can also present important developmental cues in many tissues, including the hematopoietic system. Here I summarize the roles of three bone marrow ECM molecules in hematopoietic development.

4.2.1 Tenascin-C

Tenascin-C is a large disulfide linked extracellular matrix molecule that plays an important role in morphogenesis. Tenascin-C is expressed at epithelial-mesenchymal junctions during development, playing an especially important role in neurogenesis, skeletogenesis and vasculogenesis. In the adult, it is expressed during processes such as wound healing, nerve regeneration and tissue involution [30]. Tenascin-C has a hexabrachion structure, with each monomer comprised of several functional domains, including EGF-like repeats, a series of fibronectin type III domains and a fibrinogen globe at the terminal knob. Tenascin-C can have both adhesive and anti-adhesive effects on cells, and may also stimulate proliferation in hematopoietic cells [69]. The $\alpha_v\beta_3$ and $\alpha_9\beta_1$ integrins are known to function as receptors for tenascin-C.

The expression pattern of tenascin-C in developing embryos suggests a specific interaction with HSCs. In day 34 human embryos, tenascin-C expression was found in a specific region of the ventral mesoderm beneath the dorsal aorta [46]. This staining was found only in the portion of the mesoderm closest to rounded $CD34^+$ cells within the dorsal aorta, while other ECM proteins such as fibronectin showed more uniform expression both around the dorsal aorta and in more rostral and caudal sections. This morphological polarity suggests that tenascin-C may interact with developing HSCs in a way that induces or preserves the mechanism of asymmetrical divisions that lead to stem cell self renewal, as in *Drosophila* spermatogenesis [89].

Suppression of hematopoietic activity in tenascin-C knockout mice provides further evidence of an important role in stem cell maintenance. The colony forming capacity of bone marrow cells from tenascin-C knockout mice was significantly lower

than that of normal mice [50]. Furthermore, bone marrow stromal cells derived from the knockout mice had a decreased ability to support hematopoietic cells derived from normal mice, as measured by cell number (cobblestone assay) and colony forming ability of the nonadherent cells. The addition of soluble tenascin-C rescued the supportive ability of tenascin-C deficient stromal cells in a dose-dependent manner. Though by no means definitive, these studies suggest a close interaction between tenascin-C and hematopoietic cells in a way that helps to maintain stem cell activity.

4.2.2 Collagen

Collagens are the most abundant proteins in the animal kingdom, and thus comprise the extracellular matrix of nearly every tissue [43]. The structure of collagen consists of three coiled chains wound in a characteristic triple-helix. Collagen type I was found to be one of the major ECM components of bone marrow [49]. Immunofluorescence studies indicated high expression of collagen I in both compact bone as well as trabecular bone, where HSCs have been shown to reside [93]. Collagen I is also the major ECM protein secreted by osteoblasts, further indicating a potential role for this particular matrix protein in regulation of stem cell self-renewal. Integrins of the β_1 subfamily including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ are known to function as collagen receptors, although $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the most widely expressed.

In a recent study of the role of the calcium-sensing receptor (CaR) in the HSC niche, the Scadden group found that while CaR knockout mice had normal numbers of HSCs, the retention of these cells in the bone marrow was impaired [2]. CaR knockout mice had fewer HSCs in the bone marrow but an abnormally high number in the circulation, suggesting a defect in localization to the endosteum. The authors show that the knockout cells also have impaired adhesion to collagen type I, thereby indicating that as a mechanism for retention of HSCs in the endosteal niche.

4.2.3 Laminin

Laminins are extracellular matrix glycoproteins that comprise all basement membranes. Laminins are heterotrimers comprised of varying α, β, and γ chains. Five α, three β, and three γ chains have been identified and so far 12 of the theoretically possible 45 different heterotrimers have been clearly identified [78]. Laminin-1, made of α_1, β_1, and γ_1 chains, is the first discovered and most well characterized form. All laminin isoforms have the same basic cross-shaped structure, with three short arms and one long arm, with molecular weight around 820 kDa. Integrins of the β_1 ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_9\beta_1$) and α_v ($\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_8$) subfamilies are reported to function as laminin receptors, though with differing specificities and tissue distribution [7].

The localization pattern of laminin isoforms within the bone marrow suggests a close interaction with developing hematopoietic cells at different stages. Immunofluorescence staining with monoclonal antibodies to different laminin chains revealed that the α_4 chain was expressed in intersinusoidal spaces in adult mouse bone marrow, suggesting that laminins containing the α_4 chain such as laminin-8 may be the preferred isoform interacting with HSCs [24]. A developmentally regulated shift in the localization of the α_5 chain was also observed. Whereas the α_5 chain is expressed in the arteriolar walls and subendothelial basement membrane in the adult, its expression is restricted to the arteriolar walls in the bone marrow of the newborn. This postnatal shift could suggest a role for α_5 chain-containing laminins such as laminin-10 and laminin-11 in trafficking of mature blood cells across the sinusoidal membrane and hence an adhesion preference for more differentiated cells over HSCs.

4.3 HSC Purification Strategies

The history of hematopoietic stem cell purification from bone marrow is essentially that of finding a needle in a haystack, as HSCs occur at a frequency of about 1 in 100,000 bone marrow cells [95]. The development of the spleen colony assay by Till and McCulloch in 1961 provided the first technique to quantify stem cell behavior *in*

vivo. The initial study was done by injecting whole bone marrow cells intravenously into lethally irradiated recipients and counting the number of proliferating colonies seen on the spleen, termed CFU-S [77].

The earliest efforts to separate stem cells were based on physical characteristics such as size and density. Using velocity sedimentation, the observation was made in 1969 that smaller bone marrow cells were more likely to produce secondary CFU-S, thought to be the result of proliferation of primitive cells. Later, building upon the finding that quiescent cells were smaller than proliferating cells, cell-cycle active drugs were used to isolate quiescent cells as purified populations of HSCs. At this time, in the mid-1970s, the best possible purification led to a 30-fold enrichment in CFU-S activity [57].

In the late 1970s, the development of new technologies to separate cells in addition to more sensitive techniques to assess their function *in vivo* completely revolutionized stem cell science. The fluorescence-activated cell sorter (FACS) enabled researchers to analyze and collect individual cells on the basis of their cell-surface markers. In 1977 the competitive repopulation assay was introduced as a more sensitive assay of stem cell activity than the CFU-S [1]. In this assay, now the gold standard for quantification of *in vivo* stem cell behavior, purified populations of stem cells are injected intravenously into lethally irradiated recipients. The donor cells are marked genetically and can be distinguished from recipient cells by highly specific monoclonal antibodies. Using FACS analysis, the contribution of the donor cells to the blood lineages of the recipient mouse can be assessed at short and long times post transplant and the purity of the starting population can be calculated using Poisson statistics.

Over the next 20 years, several cell surface proteins were identified as stem cell markers on the basis of their ability to enrich bone marrow populations for the most primitive cells. By the early 1990s, the most well accepted stem cell population was the so-called KTLS population, selected by the phenotype of c-Kit$^+$ (stem cell factor receptor), Thy-1.1^{low} (CD90, a thymocyte marker), lineage$^-$ (markers for differentiated cells including B cells, T cells, granulocytes, platelets and erythrocytes) and Sca-1$^+$ (stem cell antigen 1). This population yields a 2000-fold enrichment over

whole bone marrow, as measured by the competitive repopulation assay [57]. Of importance is the fact most of these stem cell markers were empirically determined, and that with the exception of c-kit, the receptor for stem cell factor, expression of these markers has not been functionally linked to stem cell maintenance.

In addition to cell surface markers, supravital stains have also been used to isolate HSCs. The two most commonly used stains are Hoechst 33342, used to separate a side population of bone marrow cells highly enriched in stem cell activity [21] and the mitochondrial dye rhodamine-123 [72]. Using a combination of previously established cell-surface markers, supravital stains, and endoglin, a stem cell marker identified in the Lodish lab, the purity of bone marrow derived HSC populations has been narrowed to 1 in 3 [12].

More recent advances in HSC purification have come from the use of the SLAM family of proteins as HSC markers [34]. This purification strategy takes advantage of a family of receptors that are differentially expressed among functionally distinct progenitor cells. Using gene expression profiling, highly purified HSCs were identified as $CD150^{+}CD244^{-}CD48^{-}$ while more committed progenitors are $CD244^{+}CD150^{-}CD48^{-}$ and the most restricted progenitors are $CD48^{+}CD244^{+}CD150^{-}$. Competitive reconstitution analysis indicated that 1 in 2.1 $CD150^{+}CD48^{-}$Sca-1^{+}lineage c-kit^{+} cells were true long-term repopulating HSCs, the best purification to date.

4.4 Integrin Expression

4.4.1 Identifying highly expressed integrins

I decided to use the endoglin^{+} Sca-1^{+} rhodamine^{low} HSC phenotype for my studies because at the time it represented the most pure population of stem cells. These cells are purified from bone marrow by first gating a Sca-1 and endoglin double positive population. Of these cells, the bottom 1% of rhodamine low or negative cells were further gated. The frequency of these cells in whole bone marrow is roughly 1 in 100,000 [12]. A sample of the flow cytometry gating schemes is shown in Figure 4-1.

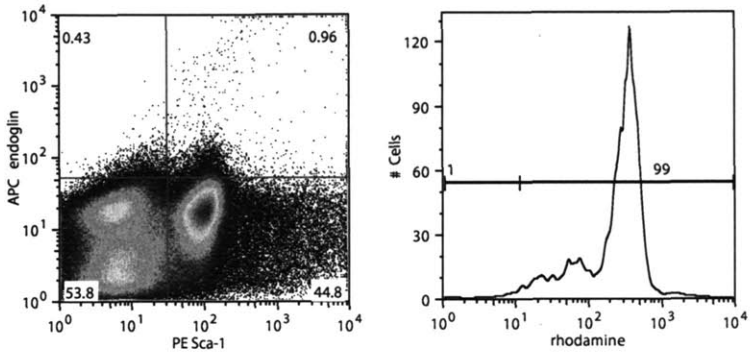

Figure 4-1: **Flow cytometry gating scheme for sorting Sca-1$^+$ endoglin$^+$ rhodamine^{low} HSCs.** After gating on live bone marrow cells, Sca-1 and endoglin double positive cells are gated (left panel). Of this population, the bottom 1% of rhodamine low or negative cells are further gated (right panel).

To asses integrin expression on these cells, I used a flow cytometry strategy similar to that used to assess integrin expression on fetal liver erythroid progenitors. In this case, bone marrow cells were isolated and stained for the HSC markers as described above. Cells were also incubated with biotinylated primary antibodies against a panel of integrin subunits and then with PE Cy5.5- conjugated streptavidin. Cells were gated into a Sca-1$^+$ endoglin$^+$ rhodamine^{low} population, and PE Cy5.5 fluorescence was examined within this population. Cells that had PE Cy5.5 expression two orders of magnitude above the background staining of the secondary antibody alone were scored as positive (Figure 4-2).

Due to limiting numbers of cells, a subset of these highly expressed integrin subunits were chosen for further analysis. I decided to focus on expression of α_2, α_v, α_9, β_1 and β_3 integrins because these subunits were not only differentially expressed in HSC and WBM populations, but also because they form receptors for extracellular matrix proteins relevant to the HSC niche. More specifically, $\alpha_2\beta_1$ is a receptor for collagen type I and both $\alpha_v\beta_3$ and $\alpha_9\beta_1$ bind to tenascin-c and laminins.

Integrin expression was further analyzed by real time PCR. Sca-1$^+$ endoglin$^+$ rhodamine^{low} cells were sorted and total RNA was isolated. Because of the very small number of cells used (on order of thousands) in each experiment, carrier RNA

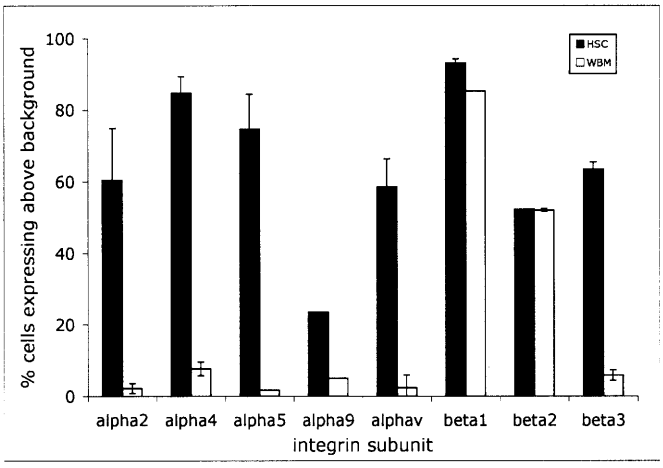

Figure 4-2: **Expression of integrin subunits on purified HSCs and whole bone marrow.** Flow cytometry was used to compare integrin expression on Sca-$^+$ endoglin$^+$ rhodamine^{low} HSCs to that on unfractionated bone marrow. Expression of integrin subunits two orders of magnitude above the background staining of the secondary antibody alone was scored as positive.

was used in the isolation. The amount of carrier RNA was then subtracted from the total RNA concentration prior to reverse transcription. Primers to each integrin subunit were generated and primer efficiency was calibrated against genomic DNA samples (data not shown). As shown in Figure 4-3, α_v, α_9, β_1 and β_3 integrins are all highly expressed relative to whole bone marrow. Expression of α_2 integrin was four orders of magnitude greater in HSCs than in WBM. In order to accurately represent the very high expression level of α_2 integrin in HSC, in Figure 4-4, α_2 expression is shown relative to other highly expressed integrin subunits.

4.4.2 Focus on α_2 integrin

The very high expression level of α_2 integrin on HSCs relative to whole bone marrow as well as to other highly expressed integrin subunits led me to further focus on this particular subunit. Using a strategy previously used in the Lodish lab [92], I examined α_2 integrin expression among lineage$^-$ Sca-1$^+$ endoglin$^+$ and lineage$^-$ Sca-

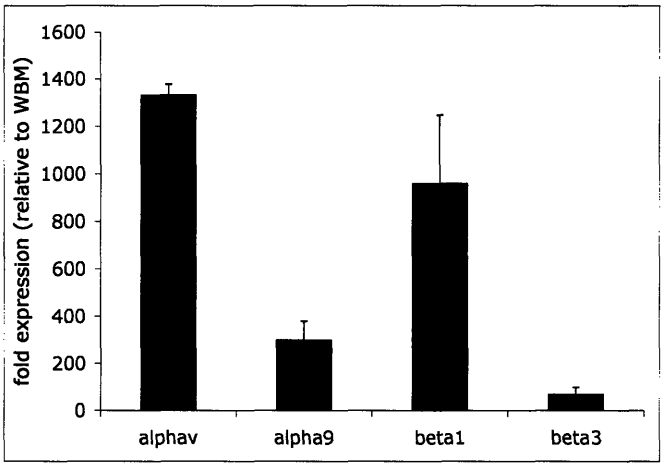

Figure 4-3: α_v, α_9, β_1, **and** β_3 **integrins are highly expressed on purified HSCs.** Quantitative PCR was used to assess integrin expression on Sca-$^+$ endoglin$^+$ rhodamine^{low} HSCs. Expression levels were normalized to expression of 18S ribosomal RNA as well as to unfractionated bone marrow. 2 ng total RNA input was used in each case.

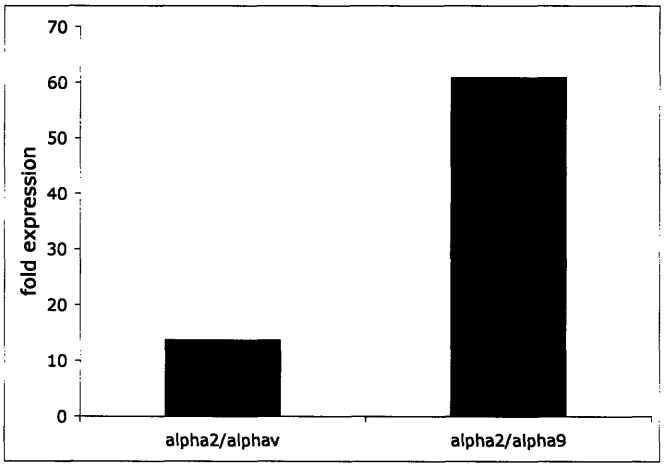

Figure 4-4: α_2 **integrin is highly expressed on purified HSCs.** Quantitative PCR was used to assess integrin expression on Sca-$^+$ endoglin$^+$ rhodamine^{low} HSCs relative to other highly differentially expressed integrins. α_2 integrin expression on HSCs is an order of magnitude greater than α_v and α_9 integrin.

1^+ endoglin$^-$ populations. Lineage$^-$ cells are a population of progenitor cells depleted for mature cells of each of the myeloid, lymphoid and erythroid lineages. Previous work has shown that all of the long term repopulating ability is found within the lineage$^-$ Sca-1^+ endoglin$^+$ population [12], so I compared integrin expression in this population with that in lineage$^-$ Sca-1^+ endoglin$^-$ via quantitative PCR. As shown in Figure 4-5, the lineage$^-$ Sca-1^+ endoglin$^+$ population has 7-fold higher α_2 integrin expression relative to the lineage$^-$ Sca-1^+ endoglin$^-$ population, indicating that α_2 integrin is expressed on purified HSCs with long term repopulating ability.

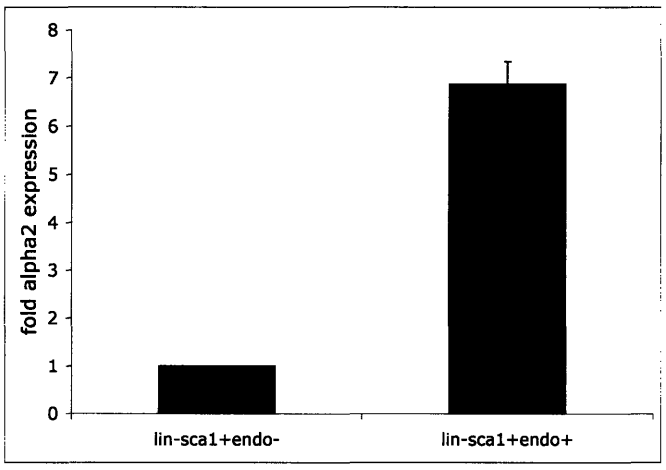

Figure 4-5: **Expression of α_2 integrin in lineage$^-$ Sca-1^+ endoglin$^+$ and lineage$^-$ Sca-1^+ endoglin$^-$ populations.** Quantitative PCR analysis indicated that α_2 integrin is highly expressed on lineage$^-$ Sca-1^+ endoglin$^+$ cells relative to lineage$^-$ Sca-1^+ endoglin$^-$ cells.

I also used flow cytometry to further analyze α_2 integrin expression. Bone marrow cells were stained with antibodies to lineage markers, endoglin, Sca-1 and α_2 integrin. The lineage$^-$ Sca-1^+ population was gated and α_2 integrin expression was compared in the endoglin$^-$ and endoglin$^+$ subpopulations among these cells. The frequency of these cells is very low in total bone marrow, so hundreds of millions of total bone marrow cells must be analyzed in order to collect data on a large enough population of HSCs. The flow cytometer data acquisition software program that is used in

conjunction with the MoFlow3 Cytometer does not have the ability to save files with more than one million data points so in order to be able to analyze very large populations of cells, I saved the flow cytometer data in small truncations. Each one of these subpopulations represents on order hundreds of HSCs. As shown in the histograms in Figure 4-6, in every case expression of α_2 integrin is greater in the lineage$^-$ Sca-1$^+$ endoglin$^+$ population than in the lineage$^-$ Sca-1$^+$ endoglin$^-$ population. These data indicate preferential expression of α_2 integrin on HSCs over other hematopoietic progenitor cells.

4.5 HSC-Extracellular Matrix Adhesion

In order to determine the functional significance of integrin expression on HSCs, I adapted a centrifugal force detachment assay to measure HSC adhesion to extracellular matrix proteins. (Appendix A)

Because of the focus on $\alpha_2\beta_1$ integrin, as described above, I was interested to test adhesion of HSCs to collagen type I, an $\alpha_2\beta_1$ ligand and also a major ECM protein in the bone marrow niche. As discussed in Appendix A, optimal centrifugation speeds were determined using whole bone marrow and fetal liver cell populations. Next, I tested HSC-adhesion to a range of collagen concentrations. Freshly isolated Sca-1$^+$ endoglin$^+$ rhodamine^{low} cells (on order hundreds/well) were added to plates coated with 1, 10 or 100 μg/ml collagen type I in 100 μL serum-free media. After 30-60 minutes incubation at 37 °C, the cells were the photographed, inverted and centrifuged and then photographed again. The fraction of adherent cells was quantitated using ImageJ image analysis software. The data indicate a direct relationship between collagen concentration and HSC adhesion (Figure 4-7). The middle collagen coating concentration of 10 μg/ml was chosen for all further experiments.

I tested the role of $\alpha_2\beta_1$ integrin in mediating HSC-collagen adhesion by incubating cells with 20 μg/ml α_2 integrin function-blocking antibody before the adhesion assay. As shown in Figure 4-8, the majority of HSCs were adherent to 10 μg/ml collagen I after detachment at 1000g. In five independent experiments, this adhesion was

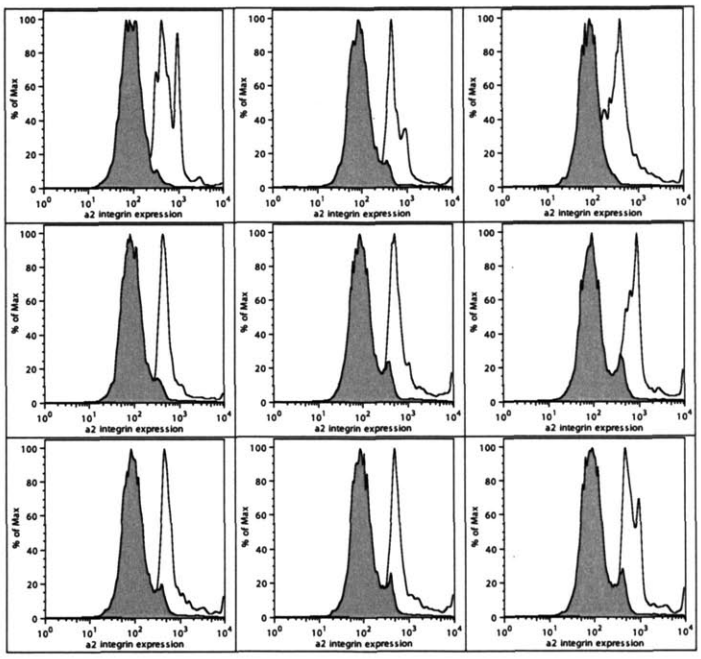

Figure 4-6: **Expression of α_2 integrin in lineage$^-$ Sca-1$^+$ endoglin$^+$ and lineage$^-$ Sca-1$^+$ endoglin$^-$ populations.** Flow cytometry analysis indicated that α_2 integrin expression is greater on lineage$^-$ Sca-1$^+$ endoglin$^+$ cells than on lineage$^-$ Sca-1$^+$ endoglin$^-$ cells. In each plot, the filled histogram represents α_2 integrin expression on lineage$^-$ Sca-1$^+$ endoglin$^-$ cells and the unfilled histogram is expression on lineage$^-$ Sca-1$^+$ endoglin$^+$ cells. Each histogram represents data from on order hundreds of lineage$^-$ Sca-1$^+$ endoglin$^+$ cells and thousands of lineage$^-$ Sca-1$^+$ endoglin$^-$ cells, representing the relative frequencies of these populations in bone marrow.

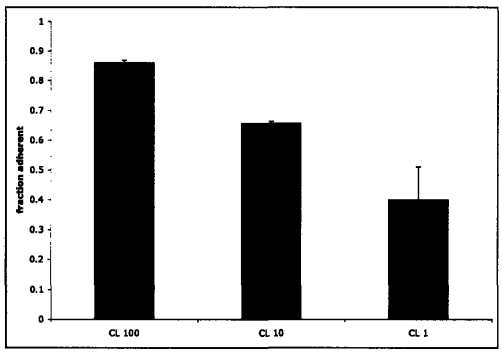

Figure 4-7: **Adhesion of Sca-1$^+$ endoglin$^+$ rhodamine^{low} HSCs to collagen type I at 1000*g*.** Adhesion of Sca-1$^+$ endoglin$^+$ rhodamine^{low} HSCs to 1, 10 and 100 μg/ml collagen type I was measured using a centrifugal force detachment assay. There is a direct relationship between collagen concentration and HSC adhesion. Each bar represents the average of average of 2-3 experiments.

significantly reduced in the presence of the α_2 integrin antibody. Because of the variation in the basal level of adhesion, likely due to the inherent impurities in the stem cell population and the very small numbers of cells utilized, these data were normalized and averaged. As shown in Figure 4-9, addition of α_2 integrin blocking antibody significantly reduced the level of HSC adhesion to collagen type I. Together, these data show that $\alpha_2\beta_1$ integrin mediates HSC adhesion to collage type I.

4.6 Discussion

Hematopoietic stem cells develop in highly specific microenvironments where a combination of signals from soluble factors, stromal cells and extracellular matrix come together to support self-renewal and differentiation, the definitive processes of any stem cell. While the contributions of soluble factors and stromal cells have traditionally been the focus of studies of the HSC niche, extracellular matrix-derived signals are only now being studied. To begin to build a knowledge base of ECM-HSC interactions, I focused on the role that integrins play in the HSC niche.

Despite major advances in the past decade, the precise molecular definition of a

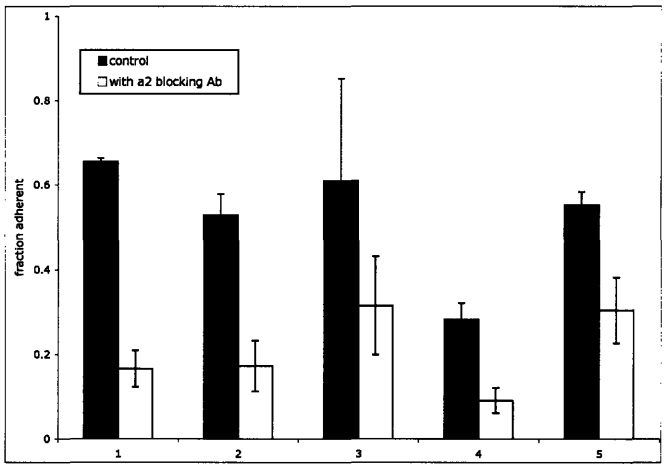

Figure 4-8: **Adhesion of Sca-1$^+$ endoglin$^+$ rhodamine^{low} HSCs to collagen is mediated by α_2 integrin.** Addition of 20 μg/ml α_2 integrin function-blocking antibody blocked adhesion of Sca-1$^+$ endoglin$^+$ rhodamine^{low} HSCs to 10 μg/ml collagen at 1000g in 5 experiments.

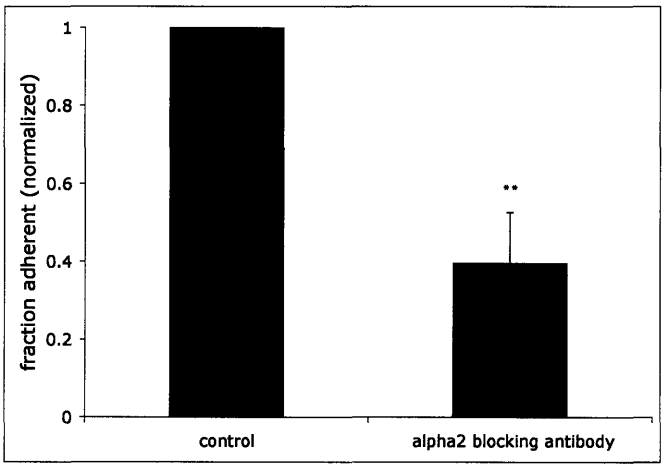

Figure 4-9: **Summary of HSC adhesion to collagen.** The presence of α_2 integrin function-blocking antibody partially abrogates HSC adhesion to collagen. Asterisks indicate significance at the level of p $\leq$0.01.

hematopoietic stem cell is still a relatively open question in stem cell biology. The combination of supravital dyes and cell surface markers has led to the isolation of increasingly purified populations, but the lack of a functional significance of these markers is a major hindrance. In addition, the inability to definitively know the stem cell potential of a given population without transplantation is another challenge. Because integrins have a very clear functional role in mediating adhesion and can also function as signaling receptors, identification of those integrins that are highly expressed on stem cells and the biological significance of that expression could result in the discovery of a functional marker in addition to yielding new insights about stem cell regulation.

Using flow cytometry and quantitative PCR, I identified α_2 integrin as an integrin subunit that is highly differentially expressed on HSCs versus whole bone marrow. Furthermore, its expression on rhodamine^{low} Sca-1$^+$ endoglin$^+$ cells is greater than even other highly differentially expressed integrins. $\alpha_2\beta_1$ integrin forms the major receptor for type I collagen, which is secreted by osteoblasts. Given the identification of osteoblasts as important regulators of the HSC niche, I hypothesized that the high expression of $\alpha_2\beta_1$ integrin on HSCs serves to mediate adhesion to collagen and thus either bring HSCs in close contact with osteoblasts or transmit self-renewal signals itself or both.

To test this hypothesis, I first measured adhesion of highly purified HSC populations to collagen and other ECM proteins. I found that adhesion to collagen was relatively higher than to other ECM proteins. Furthermore, there was a direct relationship between HSC adhesion and collagen concentration. Treating HSCs with a function-blocking antibody to α_2 integrin significantly reduced the level of adhesion to collagen.

An important next step to verify that α_2 integrin is a *bona fide* HSC marker is to do a competitive repopulation experiment. The Weissman group has already done this experiment, transplanting α_2^+ lineage$^{-/lo}$ Thy1.1^{lo} Sca-1$^+$ c-kit$^+$ and α_2^- lineage$^{-/lo}$ Thy1.1^{lo} Sca-1$^+$ c-kit$^+$ HSC populations into irradiated recipients [83]. By analyzing the contribution of the donor cells to each of the hematopoietic lineages

up to 21 weeks post transplant, they determined that α_2^+ lineage$^{-/lo}$ Thy1.1^{lo} Sca-1$^+$ c-kit$^+$ represent a short term repopulating phenotype. Their overall conclusion is that α_2 integrin selectively marks a subset of primitive HSCs which retains multilineage repopulating potential but exhibits reduced self-renewal capacity.

The critical factor in analyzing their work is that the antibody they used to detect α_2 integrin expression and to sort HSCs for transplantation is a function-blocking antibody. This has several implications for their results. First, the lack of long term repopulation in the α_2^+ population could be a result of a defect of these cells to home to and/or engraft in the appropriate niche. A short term homing assay done by the same group using the same function-blocking antibody to α_2 integrin indicates that there is no homing defect [82]. Lineage$^{-/lo}$ Thy1.1^{lo} Sca-1$^+$ c-kit$^+$ GFP$^+$ cells were incubated with and without the function-blocking α_2 integrin antibody and injected into irradiated recipients. Three hours later, bone marrow was harvested and the frequency of GFP$^+$ cells was determined using flow cytometry. Their data show a similar frequency of GFP$^+$ cells in control and antibody-treated animals, indicating that blocking α_2 integrin on HSCs does not lead to a homing defect to the bone marrow. However, this experiment does not preclude the possibility of an engraftment defect. Indeed, as my data show, α_2 integrin mediates adhesion of HSCs to collagen, which is present in the bone marrow niche. Distinct from homing or engraftment, α_2 integrin could also have an explicit role in the stem cell niche by transmitting signals necessary for self-renewal. Given this understanding of the Weissman work, it cannot be concluded that α_2 integrin-expressing HSCs are not true long term repopulating stem cells.

The proper experiment to determine the role of α_2 integrin in stem cell self-renewal is a transplant experiment in which populations of highly purified HSCs expressing α_2 integrin are injected with and without treatment with a function-blocking antibody. Although this experiment will not be able to distinguish between engraftment and intrinsic self-renewal effects, a positive result will motivate further investigation.

In conclusion, the main point of this chapter is that α_2 integrin is highly expressed on highly expressed populations of HSCs, and mediates adhesion to collagen type I,

an important matrix protein in the bone marrow niche. A definitive conclusion regarding the repopulating ability of α_2 integrin-expressing HSCs cannot be drawn until a competitive repopulation transplant is performed with HSCs with fully functioning α_2 integrins. Other future experiments regarding the role of integrins in the HSC niche are discussed in Chapter 5.

Chapter 5

Future Directions

5.1 Integrin-mediated signal transduction in erythropoiesis

5.1.1 Coordination of erythropoietin- and $\alpha_4\beta_1$ integrin-mediated signals

The work presented in Chapter 2 and 3 establishes a two-phase model for regulation of erythropoiesis by growth factor and integrin-mediated signals. While coordinated signaling by growth factors and integrins together control cell behavior in a number of other contexts, my finding is unique in that signals appear to dominate in temporally restricted regimes, with the early steps of erythropoiesis regulated by binding of Epo to its cognate receptor and the later steps by engagement of $\alpha_4\beta_1$ integrin by fibronectin. Determining the biological significance of such regulation is one area of further exploration.

An open question is this area is how Epo signaling in the first phase leaves erythroid cells in a state receptive to integrin signaling in the second phase. My finding that engagement of $\alpha_4\beta_1$ in the first phase is not sufficient to support terminal erythroid cell expansion, despite the presence of Epo, indicates either that the signals generated by $\alpha_4\beta_1$ integrin in early erythroblasts are short lived or that the proper

signals are not generated in those cells. These hypotheses could be tested by comparing $\alpha_4\beta_1$ integrin-mediated signals in early erythroblasts and later, differentiating cells. One challenge is that survival of early erythroblasts in the absence of Epo is compromised, so in the early cells, Epo-mediated signals and $\alpha_4\beta_1$ integrin-mediated signals will necessarily be intertwined. By comparing the signaling profile of early erythroblasts with Epo alone, it might be possible to begin to determine the contribution of integrins at this phase.

Systematic testing of the various signaling molecules involved in Epo- and $\alpha_4\beta_1$ integrin signaling is required to parse these pathways. While the traditional immunoblot method is certainly feasible for this type of study, one of its limitations is that it can give only average, population-level results. In this system, the input cells, the early erythroblasts derived from fetal liver, are not a completely homogeneous population and likewise their responses to stimulation by Epo, in terms of differentiation and expansion, are heterogeneous as well. By developing flow cytometric assays for key signaling molecules in the pathways of interest - for example, phosphorylated JAK2, STAT5 or Akt - single-cell level resolution can be achieved [56]. Furthermore, by coupling these types of assays with CD71 and TER119 staining, the differentiation state of each cell can be correlated with signaling molecule expression. This could provide a powerful tool to examine signal transduction in erythropoisis at higher resolution. Other systems-level analyses using microarrays could also provide useful information on the set of genes stimulated by Epo and $\alpha_4\beta_1$ integrins at different stages.

5.1.2 Hsp70 and apoptosis

One interesting aspect of caspase-3 activation is that it occurs during normal erythropoietic differentiation as well as during apoptosis [90]. During apoptosis induced by Epo starvation, one of the targets of activated caspase-3 is GATA-1, a key erythroid transciption factor. During normal erythroid differentiation, however, GATA-1 remains uncleaved by caspase-3 and is thus able to activate transcription of erythroid genes. It was recently established that the heat shock protein Hsp70 co-localizes with GATA-1 in the presence of Epo to protect it from cleavage and thus promote cell

survival. [61]

Because of the similarities between Epo-mediated and integrin-mediated protection form apoptosis, one area of ongoing work is to test the hypothesis that integrin engagement in the second phase of erythroid culture also leads to Hsp70 protection of GATA-1. Ribeil et al used unfractionated human umbilical cord blood cells cultured in a cytokine cocktail in order to generate erythroid progenitors, a system very different from the fetal liver cells that I use. Therefore it is first necessary to repeat their finding that the presence of Epo leads to Hsp70 mediated protection of GATA-1 by immunoprecipitating GATA-1 from TER119$^+$ cells cultured in the presence and absence of Epo and assessing Hsp70 expression by Western blot. Preliminary evidence shows that this is indeed the case in the fetal liver system (data not shown). Next, this same experiment will be done in cells in the second phase of erythropoiesis cultured on fibronectin-coated or control substrates. Finally, in order to link the association of Hsp70 and GATA-1 to downstream apoptotic behavior, the nuclear export inhibitor leptomycin B can be used to sequester Hsp70 in the nucleus in apoptotic conditions. Thus it is predicted that the in absence of fibronectin, which would normally lead to apopotosis, administration of leptomycin B would result in greater cell survival.

5.1.3 Egress of differentiated erythrocytes from bone marrow

Under normal homeostatic conditions, the bone marrow is the site of erythropoiesis in the adult. There is a large body of work characterizing the numerous biochemical and morphological changes that take place during the course of erythropoietic differentiation, in addition to mechanistic data on the signals underlying these processes, of which this thesis is a part. Once erythroid cells have terminally differentiated and enucleated, they must egress the bone marrow in order to enter the circulation. Surprisingly, there is a dearth of knowledge of the mechanism and regulation of this process.

One of the conceptual motivations for this thesis was the idea that adhesive inter-

actions on hematopoietic cells are dynamic, responding to the unique needs of cells at distinct developmental stages. As discussed in Chapter 2, one possible biological explanation for the downregulation of integrin subunits on differentiated erythroid cells is to minimize adhesive interactions as cells prepare to enter the circulation. In parallel, there is new evidence that Epo signaling upregulates expression of the sialomucin podocalyxin during differentiation and that circulating reticulocytes retain podocalyxin expression [66]. Podocalyxin has been shown to have an anti-adhesive role in some contexts, and decrease in its expression level leads to abnormal retention of reticulocytes in the bone marrow. Together these data provide support for the conceptual framework posed above. Identifying other genes that are differentially expressed between differentiated erythroid cells and early erythroblasts may provide other clues in this continuum.

The study of what role integrins may play in transendothelial migration of differentiated red blood cells in the bone marrow is a natural extension of my work. Of the many changes that take place during erythroid differentiation, modulation of mechanical properties is critical to bestowing the differentiated erythrocyte with the elasticity needed to traverse narrow capillaries [85]. Through their connections to the cytoskeleton, integrins display exquisite inside-out and outside-in signal transduction capabilities. Likewise, they have a noted role in mechanotransduction in many other systems [32]. Although their expression is downregulated during the course of erythropoisis, the most differentiated population of fetal liver cells, the R5 cells, still do express $\alpha_4\beta_1$ integrins above background level, and it is possible that signaling pathways initiated by these receptors on these cells or perhaps even in earlier stages play a role in regulating transendothelial migration.

5.2 Role of integrins in HSC maintenance and differentiation

5.2.1 Endosteal niche

As discussed above, osteoblasts have recently been identified as important regulators of the HSC niche *in vivo* [9, 93]. However the mechanism by which they do so has not yet been determined in any detail. One potential mechanism is that membrane-bound Jagged-1 on the surface of osteoblasts activates the Notch signaling pathway in HSCs [9]. Notch signaling regulates many developmental cell fate decisions, including stem cell self-renewal *in vivo* and *in vitro* [13]. Genetically increasing the number of osteoblasts *in vivo* led to increased expression of Jagged 1, which led to a concomitant increase in the level of activated Notch in HSCs. Moreover, blocking Notch cleavage with a γ-secretase inhibitor reduced the ability of stromal cells to support HSC activity [9]. Given the data presented in Chapter 4 showing that α_2 integrin specifically mediates adhesion of HSCs to collagen, one possible model for the role of α_2 integrin in the osteoblastic HSC niche is that through adhesion to collagen, it brings HSCs in a position to interact with membrane-bound Jagged-1 on osteoblasts.

Evidence supporting this model comes from recent studies of calcium-sensing receptor (CaR) knockout mice. The Scadden group found that while CaR knockout mice had normal numbers of HSCs, retention of these cells in the bone marrow was impaired [2]. CaR knockout mice had fewer HSCs in the bone marrow but an abnormally high number in the circulation, suggesting a defect in localization to the endosteum. The authors show that the knockout cells also have impaired adhesion to collagen type I, thereby indicating that as a mechanism for retention of HSCs in the endosteal niche

There are several ways to test this hypothesis. First, as discussed in Chapter 4, the logical next step is to transplant α_2^+ HSCs with and without pre-treatment with an α_2 integrin function-blocking antibody into irradiated recipients and monitor the contribution of the donor cells to each of the hematopoietic lineages over a period of

6 months. In the meantime, *in vitro* experiments exploring the role of α_2 integrin in the osteoblastic niche could be conducted. As shown by Calvi et al, co-culture of purified HSCs with osteoblasts supports expansion of LTC-ICs, possibly though activation of Notch signaling in HSCs. This experiment could be repeated in the presence of function-blocking antibodies to α_2 integrin and expansion of LTC-ICs and Notch activation could be measured.

Another possible connection between integrins and Notch signaling comes from primary neural stem cells, where activation of β_1 integrin leads to cleavage of the Notch intracellular domain and subsequent translocation to the nucleus [11]. This suggests that a similar mechanism could be at play in the HSC niche, where adhesion of HSCs via α_2 and potentially other β_1 integrins could lead to additional activation of the Notch signaling pathway. One way to test this hypothesis is to assay for Notch activation in adherent and non-adherent populations of HSCs. Similarly, Notch activation could be assayed after treatment with function blocking antibodies for β_1 or other integrins. Finally, the presence of Mn^{++} cations is known to activate integrins. A simple test would be to incubate stem cells in the presence and absence of Mn^{++} and then assay for Notch activation.

5.2.2 Vascular niche

Recent models of the HSC niche provide for dynamic interactions with bone marrow components. While the endosteal niche may be the preferred microenvironment for quiescence, the vascular niche may be better suited for differentiation and mobilization [38]. Much is unknown about the vascular niche, in particular at what stage cells take residence there and at what stage they transition to a new niche. The work presented above has focused on the role of integrins the endosteal niche in self-renewal, but there could also be a role for integrins in the vascular niche as well.

Rolling of hematopoietic progenitor cells along bone marrow endothelium is mediated by selectins as well as by interactions between $\alpha_4\beta_1$ integrins and VCAM-1 [47]. However, little is known about what downstream signaling pathways are activated by this interaction, and what role they play in HSC differentiation. Furthermore,

the vascular niche is rich with cytokines and their effects are likely modulated by integrin signaling as well. For example, stromal-derived factor 1 (SDF-1) is required for efficient homing of circulating HSCs, as well as for transdendothelial migration [55], and it also increases adhesion of hematopoietic progenitor cells to VCAM-1 [54].

In particular, integrin-mediated interactions with laminins may be an important mechanism for differentiation or trafficking of HSCs in the vascular niche. As discussed in Chapter 4, laminins are expressed in arteriolar walls of the bone marrow. By careful identification of the cells present in the vascular niche and characterization of the set of integrins they may express, a model for how these interactions may influence differentiation can be constructed.

5.3 Discussion

Hematopoietic stem cells hold great promise for the future treatment of disease. Despite decades of progress, however, there are still many challenges before the full therapeutic potential of these cells can be realized. Studying the interactions of hematopoietic cells with their local microenvironments is one promising avenue of inquiry. Precise characterization of the "adhesome" - the set of genetic, morphologic and biochemical changes mediated by adhesion to the extracellular matrix - of hematopoietic cells can lead to design of better *ex vivo* expansion systems as well as uncover fundamental properties of self-renewal and differentiation decisions.

Appendix A

Development of Centrifugal Force Detachment Assay

A.1 Introduction

Because of the fundamental importance of cell-matrix adhesion there are a number of different adhesion assays reported in the literature. Aside from substrate preparation and incubation times, which are specific to system under study, the main experimental parameters are the methods of cell detachment and quantification. The simplest detachment method is to aspirate the media and nonadherent cells with a pipette. Although these wash assays provide a quick, qualitative assessment of cell adhesion, they are limited by a lack of sensitivity and repeatability, due to the application of uneven, unknown forces. The importance of flow in many physiological conditions led to protocols using shear forces to detach cells. A variety of devices including a parallel plate flow chamber, a spinning disk apparatus and a high throughput microfluidic chamber have been used to quantify cell adhesion [45].

These methods, though highly quantitative, require specific equipment, which can be expensive. A method that resolves the drawbacks of the wash assay and flow assay is the centrifugation assay [25]. The use of a centrifuge to detach cells ensures uniformity and repeatability, while maintaining simplicity and requiring no specialized equipment. In addition, the normal force experienced by the cells can be calculated,

and many conditions can be tested at once using multiwell plates. Quantification of the adherent and nonadherent cells can also be done in several different ways. In addition to manual counting, fluourescence and spectrophotometric methods can also be used to quantitate cells.

I adapted the centrifugation assay for the specific application of quantitating adhesion of hematopoietic cells to matrix proteins. The primary challenge was optimizing assay conditions for use with a very small number of cells, as necessitated by HSC studies. In addition, the adhesive properties of a given population of cells are relative to the assay context. The major determinants of adhesion, substrate concentration and detachment force, create many possible assay environments wherein cell behavior may be very different. While by no means an exhaustive sampling of the full experimental space, data generated in assay development and optimization are presented below.

A.2 Fetal Liver Adhesion Studies

One important assay parameter to be determined was the detachment force applied to the cells. The centrifugal force detachment assay applies a centrifugal force to populations of adherent cells. Experimentally, the applied force can be varied by varying the centrifugal speed according to the following relationship:

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

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

where V is the volume of the cell, ρ_{cell} is the density of the cell, ρ_{medium} is the density of the medium, RCF is the relative centrifugal force, ω is the centrifugal velocity and r_c is the radius of the centrifugation.

After observing a developmentally regulated change in integrin expression on fetal liver erythroid cells, I was interested in measuring adhesion of these cells to fibronectin. I first tested the adhesion of the R1+R2 and R5 populations cells to one concentration of fibronectin under a range of detachment forces. The R1+R2

population had the highest integrin expression and was thus predicted to be more adhesive than the R5 population, which had low integrin expression. At low and high centrifugal detachment forces, there was no little difference in adhesion of the two populations (Figure A-1). It is likely that the adhesion seen at low detachment force is due to nonspecific factors, while the application of high force is enough to sever all attachments to the substrate. At the intermediate level of $1000g$, the detachment force is enough to detect differences between the most and least adhesive populations while at the same time leaving some dynamic range on the low end of adhesion.

As further illustration of this point, the effect of function-blocking antibodies was tested at two centrifugal accelerations. At $1000g$, it was possible to discern a clear abrogation in adhesion of all cell populations (Figure A-2). At $1800g$, however, the effect of the antibody on R4 and R5 populations was obscured by the very high detachment force. Therefore $1000g$ was used in all further adhesion assays.

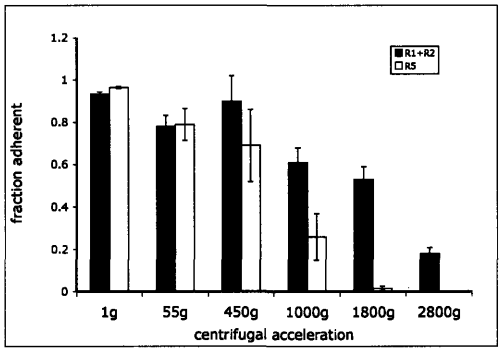

Figure A-1: **Effect of detachment force on adhesion of fetal liver erythroid cells.** R1+R2 and R5 populations of fetal liver cells were sorted and their adhesion to 10 μg/ml fibronectin was assessed at a range of centrifugal accelerations correlating to a range of detachment force. Application of low and high detachment force did not detect differences in adhesion of the two cell populations, but application of intermediate force resulted in statistically significant differences in adhesion. One asterisk indicates significance at the level of $p \leq 0.05$ and two asterisks indicate significance at the level of $p \leq 0.01$.

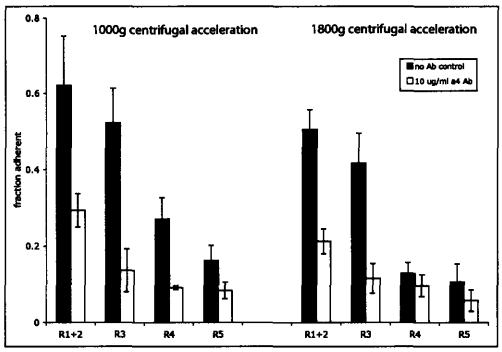

Figure A-2: **Effect of function-blocking antibody on adhesion of R1+R2 cells.** R1+R2 cells were incubated with 10 μg/ml function-blocking antibody against α_4 integrin and adhesion to 10 μg/ml fibronectin was assessed at two centrifugal accelerations correlating to different detachment force. At the high detachment force, the low level of adhesion of R4 and R5 cells obscures the effect of the antibody. Lower detachment force allows for a dynamic range in even the least adhesive populations.

A.3 Bone Marrow Adhesion Studies

The very small number of purified HSCs that could be readily obtained presented a challenge in testing a large number of adhesion conditions. Some preliminary studies were carried out using freshly isolated unfractionated bone marrow cells as a close substitute.

Once a protocol was determined, I tested adhesion of purified HSCs to two concentrations of fibronectin, laminin 10/11 and tenascin-C. The data in Figure A-3 shows that the assay technique is sensitive enough to detect differences in HSC adhesion to substrates at various concentrations. However, more optimization is needed to acquire biologically meaningful data for each of these substrates. For example, the range of fibronectin concentrations tested here is too high, as indicated by a lack of difference in adhesion over one order of magnitude concentration. For laminin and tenascin, however, the data indicate that the low concentration is within the dynamic range.

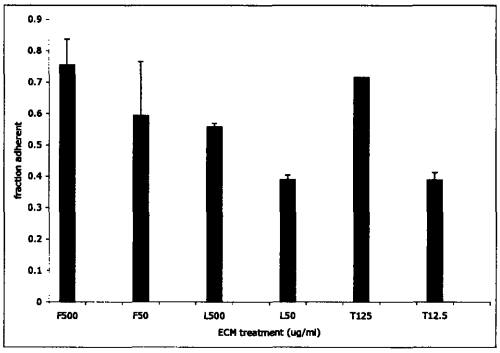

Figure A-3: **Adhesion of HSCs to fibronectin, laminin and tenascin.** Sca-1^+ endoglin$^+$ rhodamine^{low} cells were sorted from bone marrow directly into plates pre-coated with the indicated concentrations of fibronectin (F), laminin10/11 (L) and tenascin-C (T). Fraction of cells adherent after centrifugal acceleration at $1000g$ is shown. The range of fibronectin concentrations is too high, but the low concentrations of laminin and tenascin appear to be within the dynamic range. These data are the averages of 2-3 experiments, each done in triplicate.

Appendix B

Materials and Methods

B.1 Chapter 2

Animals C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at the Whitehead Institute animal facility.

Fetal liver erythroid progenitor cells C57BL/6 E14.5 fetal livers were dissected into Hanks Balanced Salt Solution containing 2% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 10mM HEPES at pH 7.4 (called HBSS+) at a concentration of 2 livers per ml. Liver tissue was disaggregated by vigorous pipetting followed by passage though a 70 μm cell strainer. Cells were blocked with a 1:50 dilution of ChromPure Rat IgG (Jackson Laboratory) and labeled with a 1:200 dilution of PE TER119 and FITC CD71 antibodies (Pharmingen, San Diego, CA) for 15 minutes on ice. Cells were washed and resuspended in HBSS+ and labeled with 1 μg/ml propidium iodide for cell sorting on a MoFlow3 Cell Sorter. (Becton Dickinson)

***in vitro* erythroid culture** Day 14.5 fetal livers were suspended in PBS+2%FBS at a concentration of 2 fetal livers/ml. Cells were incubated with 1:50 rat IgG for 15 minutes on ice, followed by 1:100 biotinylated anti-Ter119 antibody. (Pharmingen). Cells were washed, resuspended at the same concentration and further labeled with 1:10 tetrameric streptavidin complex (StemCell Technologies) for 15 minutes, followed by 60 μl magnetic colloids per milliliter of cells (StemCell Technologies) for 15

minutes. TER119$^-$ cells were purified using a StemSep magnetic column (StemCell Technologies) according to manufacturers instructions. Cells were then seeded into 24-well plates coated overnight with 0.5 ml of 20 μg/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 hours, they were cultured in "Day 1" media, which consists of Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 15% FBS (StemCell Technologies, Vancouver, BC), 1% BSA (StemCell), 10 μg/ml recombinant human insulin (Sigma), 200 μg/ml recombinant human holo-transferrin (Sigma), 10^{-4} M β-mercaptoethanol, 1% penicillin/streptomycin, 2 mM glutamine and 2 U/ml erythropoietin (Amgen, Thousand Oaks, CA). After 16-18 hours, the medium was changed to "Day 2" media, which consists of IMDM supplemented with 20% FBS (Invitrogen), 10^{-4} M β-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 minutes at 37 °C. Cells were counted using a hemocytometer and then stained with 1:200 FITC CD71 and PE TER119 for 15 minutes on ice. Cells were washed and then analyzed used a Becton Dickinson FACS Calibur flow cytometer. FACS data were analyzed using FlowJo 6.0. (Tree Star, Inc., Ashland, OR)

In experiments where the substrate was varied from Day 1 to Day 2, cells were cultured as above for the first day, dissociated with PBS containing 5 mM EDTA and 2% FBS for 5 minutes at 37 °C, counted using a hemocytometer, and added to fresh plates coated with 20 μg/ml human plasma fibronectin or Vo or VRGD- recombinant fusion proteins in Day 2 media. After 24 hours in culture, cells were dissociated and counted as described above.

In experiments utilizing TER119$^+$ cells, E14.5 fetal livers were blocked and labeled with biotinylated TER119 antibody as above. Cells were then washed, resuspended in HBSS+ and incubated with streptavidin-coupled microbeads (Miltenyi Biotec, Auburn, CA) according to manufacturers instructions. TER119$^+$ cells were isolated on an autoMACS column (Miltenyi Biotec) using the Possels program.

Recombinant fibronectin proteins Recombinant fibronectin fusion proteins

were expressed in E.coli grown to late log phase and induced with 1mM isopropyl-b-D-thiogalactopyranoside for 4 hours. Cells were harvested by centrifugation and stored at -80 °C in PBS containing a cocktail of protease inhibitors (Sigma). Bacterial pellets were thawed and the buffer was adjusted to 50 mM sodium phosphate pH 8.0, 10 mM imidazole, 300 mM NaCl. Pellets were incubated with 2 mg/ml lysozyme and DNase for 30 minutes at 4 °C, lysed using a French press, brought to 1% Triton-X100, and centrifuged at 20,000g for 20 minutes. Supernatants were incubated with TALON resin (Clontech), washed in 50 volumes of wash buffer (50mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole), and proteins were eluted with 2 volumes of elution buffer (wash buffer adjusted to 250 mM imidazole). Proteins were dialysed against CAPS buffer (20 mM CAPS, pH 11.0, 150 mM NaCl). The final protein concentrations were determined by UV absorption and protein sizes were confirmed on SDS-PAGE.

Integrin expression C57/Bl6 E14.5 fetal livers were dissected and stained as above with the addition of a 1:100 dilution of the following biotinylated anti-integrin antibodies, all from Pharmingen: α_2 (DX5), α_4 (9C10), α_5 (5H10-27), α_v (RMV-7), β_1 (Ha2/5), β_2 (C71/16) and β_1 (2C9.G2). Cells were washed, resuspended and stained with 1:1000 streptavidin PE Cy5.5 (Caltag Laboratories, Burlingame, CA) for 15 minutes on ice. Cells were washed, resuspending in HBSS+ and labeled with 1 μg/ml propidium iodide for FACS analysis. Data was collected on a MoFlow3 Cell Sorter (Becton Dickinson) and analyzed with FlowJo 6.0.

For quantitative PCR analysis, E14.5 fetal liver cells from R1-R5 populations were sorted and total RNA was isolated using an RNeasy Micro Kit. (Qiagen, Valenica, CA) RNA was reverse-transcribed using SuperScript II RT (Qiagen) using random primers. SYBR Green real-time PCR was performed on an Applied Biosystems Prism 7000 machine with primers designed using Primer Express 2.0 (Table B.1).

Adhesion assays 96-well half-area plates (Costar, Corning, NY) were pre-coated with various concentrations of human plasma fibronectin (Sigma-Aldrich, St. Louis, MO) diluted in PBS overnight at 4 °C and washed twice with PBS before cell seeding. 5000 freshly sorted fetal liver R1-R5 erythroid progenitors were resuspended in 100

Table B.1: Quantitative PCR Primers for α_4, α_5 and β_1 integrins.

Gene	Forward Primer	Reverse Primer
α_4	CGTGCGAGACATCCTTACCC	CCAAGGTGGTATGTGGCCTC
α_5	GGACTTCCCATCTGACTCCG	CTGGCAAGACCAAAGATGCAT
β_1	CTGCTGGTGTTTTCCACGG	TTTCCCATCTCCAGCAAAGTG
18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

μl Alpha Modified Essential Medium (Gibco, Carlsbad, CA) and added to each well. Cells were incubated at 37 °C for 30 minutes. 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 in for 5 minutes. Each well was photographed and the number of cells 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. Each condition was carried out in triplicate for each adhesion experiment.

The force experienced by the cells is given by:

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

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

where V is the volume of the cell, ρ_{cell} is the density of the cell, ρ_{medium} is the density of the medium, RCF is the relative centrifugal force, ω 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 μg/ml of the following function blocking antibodies prior to cell seeding: α_4 clone PS/2 (Serotec, Raleigh, NC), α_5 clone 5H10-27 (Pharmingen) and β_1 clone Ha2/5 (Pharmingen). Cells were stained for 15 minutes at 4 °C.

Statistical Analysis Significance levels for adhesion and apoptosis data were determined using the Students t-test algorithm in Microsoft Excel using a two-tailed distribution. (Redmond, WA) In cases where the same samples were subject to

different treatments, a homoscedastic two-tailed distribution was used. In cases where different samples were subject to the same treatment, a heteroscedastic two-tailed distribution was used.

VCAM-1 binding assay R1+2 through R5 populations were sorted as described above. Cells were washed with 5 mM EDTA in PBS and then incubated with increasing amounts of VCAM-1-k (RD Systems, Inc) in the presence of 1mM $MnCl_2$ for 30 minutes at 4 °C. Unbound VCAM-1-k was removed by two washes (also in the presence of 1mM $MnCl_2$) and cells were incubated with 1:100 FITC-conjugated goat anti-mouse anti-VCAM-1 antibody (Caltag) for 30 minutes at 4 °C. BInding was quantitated on a FACS Calibur Flow Cytometer. At least 10,000 cells were analyzed for each value plotted. Background binding, determined by incubating cells from each population with each concentration of VCAM-1-k in the presence of 5mM EDTA, was subtracted for each value.

B.2 Chapter 3

Cell Cycle Analysis Cell cycle analysis was performed by pelleting cultured cells at each time point and resuspending them in 500 μl HBSS+. Cells were fixed with the addition of 1.5ml ice cold ethanol for one hour at 4 °C. Cells were then washed two times at a high centrifugation rate in order to avoid the loss of flocculent cells. Cells were then incubated in 500 μl of the following staining solution for three hours at 4 °C: 3.8 mM sodium citrate, 50 μg/ml propidium iodide and 0.5 μg/ml RNAse A (Qiagen). Data were then collected with a FACS Calibur flow cytometer and analyzed using ModFit LT(Verity Software House).

Apoptosis Assay In order to measure apoptosis, $TER119^-$ cells were isolated and cultured as above on uncoated surfaces in the presence of Epo for 16-18 hours. At this time, cells were dissociated as above, washed and incubated with 10 μg /ml function blocking antibodies to either α_4 (Serotec, Raleigh, N.C, clone PS/2) or α_5 (Pharmingen, 5H10-27) integrin for 15 minutes on ice. The cells were then transferred to fresh fibronectin or control substrates in Epo-free Day 2 media. Four hours after

Epo-removal, cells were dissociated and stained with annexin V-APC (BD Pharmingen) and 7-AAD (BD Pharmingen) according to manufacturers instructions. Bcl-xL expression was assayed by fixing and permeabilizing the cells and then staining with 1:500 antibody to bcl-xL (Cell Signaling Technology, clone 54H6) for 15 minutes at room temperature followed by 1:1000 AlexaFluor 647 secondary for 15 minutes at room temperature. (Molecular Probes, Carlsbad, CA) Expression data were then collected on a FACS Calibur flow cytometer and analyzed using FlowJo. Cells were gated based on FSC and SSC properties before then analyzing annexin V and 7-AAD levels.

Bcl-xl Expression TER119$^-$ cells were isolated as above and cultured in Day1 media on uncoated surfaces overnight. Cells were then dissociated and serum starved in IMDM+1%BSA for one hour before being transferred to fresh fibronectin or uncoated wells. At the indicated time points, cells were lysed in modified RIPA buffer (50 nM Tris pH 7.4, 250 nM NaCl, 2nM EDTA, 0.5% NP-40) containing protease inhibitors (Complete mini (Roche), 4mM NaF, 4mM Na_3VO_4) for 20 minutes at 4 °C. Lysates were ultracentrifuged at 13,000g for 10 minutes at 4 °C and then analyzed via SDS-PAGE. Membranes were blocked with Odyssey Blocking Buffer (Li-COR Biosciences, Lincoln, NE) for one hour and then incubated with 1:1000 bcl-xL antibody clone 54H6 (Cell Signaling Technology, Danvers, MA) and 1:1000 actin antibody I-19 (Santa Cruz Biotechnology) overnight. Proteins were visualized using the Odyssey infrared imaging system (LI-COR Biosciences).

For flow cytometry analysis, cultured cells were dissociated with PBS with 5 mM EDTA and 2% FBS and pelleted. Cells were fixed in 3.7% formaldehyde for 15 minutes at room temperature and washed once. Cells were permeabilized in ice cold methanol for 30 minutes on ice. After washing, cells were resuspended in 100 μl HBSS+ and incubated with 1 μl bcl-xL antibody (same as above) for 1 hour at room temperature. After washing, cells were incubated with 1:100 AlexaFluor 647 anti-rabbit antibody (Molecular Probes). Background staining was determined by incubation with the secondary antibody alone.

For quantitative PCR analysis, the primers shown in Table B.2 were used:

Table B.2: Quantitative PCR Primers for bcl-xl.

Gene	Forward Primer	Reverse Primer
bcl-xL	ATGGACTGGTTGAGCCCATC	CCCGGTTGCTCTGAGACATT
18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

Caspase-3 Caspase-3 expression was assessed by flow cytometry. Cells were dissociated, fixed and permeabilized as above. Each sample was resuspended in 100 μl HBSS+ and stained with 0.5 μl cleaved caspase-3 antibody C92-605 (Pharmingen) for 30 minutes at room temperature. Cells were washed once and stained with 0.5 μl Alexa 647 secondary antibody in 100 μl HBSS+ for 30 minutes at room temperature.

Paxillin and Pyk2 For quantitative PCR analysis, the primers shown in Table ?? were used:

Table B.3: Quantitative PCR Primers for FAK and Pyk2.

Gene	Forward Primer	Reverse Primer
FAK	CCTTCCTGTCCCTCCTACCC	GGTGGCATGCAAAGGAAGA
Pyk2	GCATGTGGGAGATCCTCAGC	TTTCGAGCCAGAAGAAAGGC
18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

Pyk2 activation was assessed by Western blot. Cells and lysates were prepared as above. Membranes were blotted with an anti-phosphoPyk2 antibody (Tyr 402) from Cell Signaling. GAPDH (6C5, Research Diagnostics, Inc) was used to normalize Pyk2 expression.

Paxillin activation was assessed via immunoprecipitation and Western blot. Lysates were prepared as above and pre-cleared with 25 μl protein A beads for 1 hour at 4 °C. Pre-cleared lysates were incubated with 4 μl paxillin antibody (2452, Cell Signaling) overnight at 4 °C. Proteins were bound to 25 μl protein A beads for 1 hour at 4 °C and eluted as above. Membranes were blotted with anti phospho-paxillin antibody (Tyr118, Cell Signaling) and anti paxillin antibody. For Pyk2 and paxillin co-immunoprecipitation experiments, 4 μg Pyk2 antibody form Upstate was used.

SFKs

To test the effect of SFKs on erythroid cell expansion, increasing concentrations of the SFK inhibitor PP2 and its inactive analogue PP3 were incubated with TER119$^+$ cells for 5 minutes at room temperature before being added to fibronectin coated plates. Equal volumes of DMSO were used as an added control.

For quantitative PCR analysis, the primers shown in Table B.5 used:

Table B.4: Quantitative PCR Primers for SFKs.

Gene	Forward Primer	Reverse Primer
Src	CAAGGTGCCAAATTCCCCAT	TCGGTCAGCAGAATCCCAAAG
Fyn	TCACTTTCCATCCGTGATTGG	GGCCCGCGTTGTGATATAGTA
Hck	CAACAAGCACACCAAAGTGGC	ACCAGTTTGTCATGCTGCAGC
Lck	ATCCCACGATTCAGCGCTT	GCCCCAACATCAGACATCCTA
Fgr	TGGAACTGCAGCACAAAGGTG	TGCCTCAGCAGCTTCATGATC
Lyn	CCAACCTCATGAAGACCTTGC	ACCCTTAGCCATGAACTCGGT
18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

For SFK immunoprecipitations, 4 μg Hck antibody 06-833 (Upstate) and 4 μg Lyn antibody 2732 (Cell Signaling) were used.

B.3 Chapter 4

HSC staining Integrin Expression Bone marrow cells were stained with the same anti-integrin antibodies as above. In addition, an α_9 antibody was obtained from Dean Sheppard at UCSF.

For quantitative PCR analysis, the primers shown below (Table B.5) were used in addition to the ones noted above:

Adhesion Assay In order to block α_2 integrin, 10μg/ml of function-blocking antibody Ha1/29 (BD Pharmingen) was used.

Table B.5: Additional Quantitative PCR Primers for integrin subunits.

Gene	Forward Primer	Reverse Primer
α_2	GCATCTCTGACCTGATCCTGG	GAGTTTGAATGGCTGGCAGC
α_v	AGAGGCAAGAGCGCAATCC	AACGTCTCGGTCCACAGCAG
α_9	GGTCTTCGAGGCCTTGCAC	CACCCCACAACGTAGCCAC
β_2	AGCAGTCCTTTGTCATCCGG	GCACGGTCACTGTATCCGTG
β_3	TCACCAATATCACCTACCGGG	GAGAGTCCCACGGTCCTGG
18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

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