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Stress Erythropoiesis Model Systems

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Abstract

Bone marrow steady-state erythropoiesis maintains erythroid homeostasis throughout life. This process constantly generates new erythrocytes to replace the senescent erythrocytes that are removed by macrophages in the spleen. In contrast, anemic or hypoxic stress induces a physiological response designed to increase oxygen delivery to the tissues. Stress erythropoiesis is a key component of this response. It is best understood in mice where it is extramedullary occurring in the adult spleen and liver and in the fetal liver during development. Stress erythropoiesis utilizes progenitor cells and signals that are distinct from bone marrow steady-state erythropoiesis. Because of that observation many genes may play a role in stress erythropoiesis despite having no effect on steady-state erythropoiesis. In this chapter, we will discuss in vivo and in vitro techniques to study stress erythropoiesis in mice and how the in vitro culture system can be extended to study human stress erythropoiesis.

Keywords

Stress erythropoiesis; Hypoxia; Anemia; In vitro culture; Bone marrow transplant; Phenylhydrazine induced acute hemolytic anemia; Methylcellulose colony assays

1 Introduction

Under most conditions bone marrow steady-state erythropoiesis is able to maintain erythroid homeostasis; however, there are times when bone marrow erythropoiesis is inhibited or is unable to maintain erythrocyte numbers. At these times an alternative pathway for erythropoiesis, stress erythropoiesis, is activated [1]. It functions at times of anemic stress and produces a wave of new erythrocytes to maintain tissue oxygenation until steady-state erythropoiesis can recover [2–4]. It is best understood in the mouse where it is extra-medullary. Stress erythropoiesis is observed during development in the fetal liver and in adults in the spleen and liver [5,6]. The features that define stress erythropoiesis are the use of signals and progenitor cells that are distinct from bone marrow steady-state erythropoiesis. Some of the signals utilized are not associated with erythropoiesis, but are commonly observed in stem cell-mediated tissue regeneration, for example in the intestinal epithelium [7]. Among these signals are bone morphogenetic protein 4 (BMP4), Hedgehog (HH), and growth and differentiation factor 15 (GDF15), which function in concert with stem cell factor (SCF) and erythropoietin (Epo) [3,4, 8, 9]. The progenitor cells in stress erythropoiesis are also distinct from steady-state erythropoiesis. Steady-state erythroid progenitors have been shown to develop from a defined hierarchy of myeloid differentiation which results in the development of erythroid committed progenitors from megakaryocyte/erythroid progenitors (MEPs) [10, 11]. In contrast, our analysis has demonstrated that stress

erythroid progenitors in the spleen are derived directly from short-term reconstituting hematopoietic stem cells (ST-HSCs, CD34+Kit+Sca1+Lin-), which migrate to the spleen [2]. Once in the spleen, these cells become committed to the stress erythroid fate and undergo an initial expansion stage where cells maintain their stem cell characteristics, but are unable to differentiate. The increase in serum Epo concentration causes the progenitor cells to transition to committed erythroid progenitors that will form BFU-E colonies when plated in methylcellulose media. During the differentiation stage progenitor cells continue to proliferate and undergo terminal erythroid maturation [9]. This developmental pathway is associated with a change in cell surface markers as well as changes in the growth factors required (Fig. 1). The experimental readout of stress erythropoiesis in vitro is the stress BFU-E. Unlike steady-state BFU-E formation that requires two signals Epo and a second signal, a burst promoting activity or BPA, stress BFU-E require only Epo. Colony assays done in media containing Epo only quantitate mature stress BFU-E. A second readout measures maximal stress BFU-E potential, which measures immature stress BFU-E that do not respond to Epo only media but are induced to form stress BFU-E when additional growth factors are present in the media. Maximum stress BFU-E potential can be measured by plating cells in media containing BMP4 + SCF + Epo at 1% oxygen [3, 8]. By assaying cells using both experimental conditions, one can delineate defects in stress BFU-E formation at different stages of development. An important distinction between stress erythropoiesis and steady-state erythropoiesis is that steady-state BFU-E do not respond to BMP4. Another feature of stress erythropoiesis is that it is not continuous like steady-state erythropoiesis. It occurs in a coupled wave of expansion, transition, and differentiation [2,9]. Afterward, new cells need to migrate into the spleen to start the cycle again. These properties are characteristic of a stress response pathway, where stress induces a short-term biochemical or cellular response designed to alleviate that stress until the homeostatic machinery can resume and maintain homeostasis.

Because of these unique properties, many of the genes involved in stress erythropoiesis have little or no effect on steady-state erythropoiesis [4, 12, 13]. Therefore, the roles of different genes in stress erythropoiesis must be directly assayed under conditions that model stress erythropoiesis. Over the years, we have utilized two in vivo assays for stress erythropoiesis that complement one another and allow us to test all stages of stress erythropoiesis. Below, we describe the analysis of donor-derived erythroid short-term radioprotection immediately following bone marrow transplant as a method to study all the stages of stress erythropoiesis [2]. This technique allows for the analysis of the migration of progenitor cells to the spleen, the expansion of early stress progenitors, their transition to differentiation, and the terminal differentiation of new erythrocytes, as all of these events occur prior to stem cell engraftment and the establishment of donor-derived steady-state erythropoiesis. In addition, we have adapted the well-known protocol for phenylhydrazine-induced acute hemolytic anemia as a means to study the expansion and differentiation of stress progenitors already resident in the spleen [3]. Coupled with these in vivo assays, we have developed an in vitro culture system that recapitulates the development of stress erythroid progenitors. It uses growth factors that are known to be present in the spleen and whole unfractionated bone marrow as its starting material. By manipulating the culture conditions, we can maintain and expand cells in the early expansion stage and then by adding Epo and growing cells at low oxygen, we can

induce the transition and differentiation stage. We can quantitate the ability of the cells to generate stress progenitors by plating for stress BFU-E colony formation in methylcellulose assays. In addition, this culture protocol generates functional early stress erythroid progenitors that can be isolated by flow cytometry sorting and transplanted into irradiated recipient mice to test their ability to provide erythroid short-term radioprotection. The ability of the in vitro culture system to recapitulate stress erythropoietic development can also be extended to the culture of human bone marrow to analyze human stress erythropoiesis [9].

2 Materials

2.1 Phenylhydrazine Treatment

1. 8–10 week old mice. This protocol is designed for C57BL/6 mice (*see Note 1*). All mice should be approximately the same weight (for example 24–26 g).
2. Phenylhydrazine: Calculate the concentration of phenylhydrazine needed for a dose of 100 mg/kg in 100 μ L of sterile PBS. The solution must be made fresh immediately prior to use and protected from light.

2.2 Stress BFU-E Assays

All the cytokine stock solutions are stored frozen until diluted in media for use in assays.

1. Methylcellulose media with 3 U/mL Epo (Stem Cell Technologies), stored frozen in 1.8 mL aliquots. BMP4 and SCF are also added for assays to assess maximum stress BFU-E potential (*see Subheading 3.2, step 11*).
2. BMP4: 10 ng/ μ L (Used at a final concentration of 15 ng/mL).
3. SCF: 50 ng/ μ L (Used at a final concentration of 50 ng/mL).
4. Insulin from bovine pancreas (1000 $\times$ stock): 10 mg/mL (dissolved in H₂O, pH = 3).
5. Bovine holo-transferrin (100 $\times$ stock): 20 mg/mL.
6. Ciprofloxacin HCL (1000 $\times$ stock): 10 mg/mL.
7. Iscove's Modified Dulbecco's Media (IMDM) base medium: 500 mL IMDM containing 2 mM L-Glutamine and 25 mM Hepes buffer, 5 g Bovine Serum Albumin (BSA), 3.5 μ L β -mercaptoethanol. Filter sterilize.
8. Final IMDM (per 100 mL): 85 mL IMDM base medium (from **item 8**), 15 mL Fetal bovine serum (FBS), 1 mL 100 $\times$ transferrin, 0.1 mL 1000 $\times$ Insulin, 0.1 mL 1000 $\times$ Ciproflaxin.
9. Trypan blue.
10. Red Cell Lysis Buffer: 8.26 g Ammonium chloride (NH₄Cl), 1.0 g Potassium bicarbonate (KHCO₃), 0.037 g EDTA. Add water to total of 1 L of solution. Filter sterilize before use. It can be stored for up to 6 months.
11. Benzidine stock solution: 0.3 g benzidine, 9 mL acetic acid, 1 mL H₂O. Avoid light, store in a foil-covered tube.

12. Acid Benzidine stain: 5 parts H₂O, 1 part H₂O₂ (Fresh), 1 part benzidine stock solution (from **item 10**). Avoid light, store in a foil-covered tube.

2.3 Bone Marrow Transplant and In Vitro Culture of Stress Erythroid Progenitors from Bone Marrow

1. Acidified water: Add glacial acetic acid to double distilled water until pH 2.5–3.0.
2. Epo stock solution: 10 U/ μ L.
3. BMP4 stock solution: 10 ng/ μ L.
4. SCF stock solution: 50 ng/ μ L.
5. GDF15 stock solution: 10 ng/ μ L.
6. Shh stock solution: 25 ng/ μ L.
7. Stress erythropoiesis expansion media (SEEM): Final IMDM (*see* Subheading 2.2, **item 8**), 15 ng/mL BMP4, 30 ng/mL GDF15, 25 ng/mL Shh, 50 ng/mL SCF.
8. Stress erythropoiesis differentiation media (SEDM): SEEM containing 3 U/mL Epo.

3 Methods

3.1 Phenylhydrazine-Induced Acute Hemolytic Anemia

Phenylhydrazine (PHZ) induces erythrocyte lysis by membrane oxidation leading to acute hemolytic anemia [14]. This technique is used to examine the expansion and differentiation of stress erythroid progenitors resident in the spleen. We have developed a protocol that utilizes a single dose of PHZ (100 mg/kg mouse) which induces an acute anemia with reproducible kinetics of recovery. In approximately 24 h, 50% of the erythrocytes are lysed, and the mice will develop anemia. The stress erythropoiesis response occurs over a 7 day period. Control C57BL/6 mice fully recover their hematocrits in 5–7 days.

1. Weigh mice. Concentration of PHZ depends on weight of mice. All mice should be approximately of the same weight (*see* **Note 1**).
2. Dissolve PHZ in sterile PBS. Dose is 100 mg/kg and total volume given per mouse is 100 μ L.
3. Inject 100 μ L of PHZ subcutaneously.
4. Typical analysis of anemic mice would include complete blood counts during the recovery period (*see* **Note 2**). Euthanize mice on different days posttreatment and analyze the expansion of stress erythroid progenitors in the spleen by stress BFU-E colony assays (*see* Subheading 3.2 below). Analysis of gene expression in the spleen or bone marrow may also be performed using established techniques.

3.2 Stress BFU-E Assay

Stress erythroid progenitors can be quantitated by using methylcellulose colony assays. These assays are similar to those used to analyze bone marrow steady state but differ in the growth factors required and in the culture conditions [8]. Unlike steady-state BFU-E, stress BFU-E can form when cultured with Epo alone. These stress BFU-E are considered mature stress BFU-E. In addition, we also plate cells in media containing BMP4 + SCF + Epo at 1–2% O₂. Under these conditions the number of stress BFU-E increases because these signals allow for the maximum stress BFU-E potential to be measured. We typically do both conditions when analyzing mutant mice.

1. To isolate splenocytes for colony assay, sacrifice mouse and harvest the spleen, removing as much connective tissue as possible.
2. Place spleen in 1 mL of sterile PBS, and mash the spleen with the flat end of a syringe until you have a homogeneous suspension.
3. Pass cell suspension through a 70 μ M filter into a 50 mL collection tube. Centrifuge the cells and collect the pellet.
4. Add 10 mL of cold red cell lysis buffer and incubate on ice for 5 min.
5. Add 10 mL PBS after incubation to stop cell lysis.
6. Spin cells at $1700 \times g$ for 5 min to pellet.
7. Remove the supernatant and resuspend cells in Final IMDM (see Subheading 2.2, **item 8**). For a normal spleen, 1 mL is sufficient but this volume should be increased in the case of larger spleens (i.e., 5 mL is more appropriate for mice treated with phenylhydrazine. Cells from phenylhydrazine-treated spleens tend to stick to each other. If necessary filter cells through a cell strainer to remove any small clumps.).
8. Count live cells using a hemocytometer after staining with trypan blue.
9. Thaw methylcellulose aliquots (for colony assays done in a 12- well plate, aliquots should be 1.8 mL).
10. Add 4×10^5 cells (enough for four wells).
11. There are two possible cytokine conditions that can be used. Condition 1 is for mature stress BFU-E, and contains 3 U/mL Epo only. Condition 2 is for maximum stress BFU-E potential and for this condition the media contains 15 ng/mL BMP4, 50 ng/mL SCF, and 3 U/mL Epo.
12. For plating Colony Assays, cells, cytokines, and IMDM should be added to methylcellulose for a total volume of 2.0 mL to allow for 500 μ L/well. The volume of cells, cytokines, and IMDM should equal 200 μ L.
13. Once cells and cytokines are added to methylcellulose media, vortex briefly to mix. The viscosity of the media does not allow for pipetting.
14. Plate 0.5 mL/well of methylcellulose mixture in a 12-well plate using a 16 g needle and a thin 1 mL syringe (see **Note 3**). Each condition should be plated in

triplicate. This will give 1×10^5 cells/well. *For best results, begin plating in the center and spiral outward to evenly disperse the methylcellulose. If there are uncovered portions of the well, tilt the plate until the entire well is covered.

15. Let methylcellulose sit until the bubbles have risen to the top (approximately 15 min).
16. Any large bubbles should be popped before plates are put in the incubator since they will affect colony formation and will not pop on their own.
17. Incubate at 37 °C for 5–7 days at 20% O₂ for Epo only Stress BFU-E, or at 1% O₂ for cells plated in media containing BMP4 + SCF + Epo for maximum stress BFU-E potential (*see Note 4*).
18. Stress BFU-E colonies can be easily visualized and counted by staining with acid benzidine stain, which reacts with hemoglobin to form a dark blue color (*see Note 5*). Carefully add enough stain to each well to cover methylcellulose medium without disturbing the colonies (approximately 500 µL/well is sufficient). Stain acts instantly and colonies should be counted immediately. Acid benzidine stain should be made fresh and used immediately. BFU-Es will be a dark and distinct blue color from the benzidine stain.

3.3 Bone Marrow Transplant

A second in vivo method to study stress erythropoiesis is to analyze the erythroid recovery in the immediate post-transplant period. Donor-derived stress erythropoiesis generates a wave of new erythrocytes that maintains the recipient mouse until stem cells engraft and begin donor-derived steady-state erythropoiesis [3]. Bone marrow transplant (BMT) has a few advantages over PHZ-induced acute anemia. It allows one to analyze the initial migration to the spleen and expansion stress erythroid progenitors. BMT is compatible with flow cytometry. Mutant donor cells can be transplanted into wild-type control recipients and vice versa so that cell intrinsic defects and microenvironment defects can be analyzed. Below is the standard transplant protocol that is used at The Penn State University.

1. Acidified water—Animals to be irradiated should be placed on acidified water (pH 2.5–3.0) from 1 week prior to 2 weeks post irradiation.
2. Antibiotic treatment: Prior to irradiation animals must be treated with antibiotic with the intent to eliminate *Pseudomonas aeruginosa* from the gastrointestinal tract. The antibiotics are selected by the investigator in consultation with the Attending Veterinarian and are specified in the IACUC Protocol. Directions for preparation and administration of the antibiotic are provided by the Attending Veterinarian. The following list contains alternatives for antibiotic treatment (concentrations are in drinking water): 1–1.2 mg/mL Gentamycin, 1.1 mg/mL Neomycin, 1.1 mg/mL Neomycin and 1000 U/mL Polymyxin B sulfate, 0.17 mg/mL Baytril, or between 0.65 and 1.6 mg/mL Sulfamethoxazole (Sulfadiazine) with Trimethoprim. This last drug comes as a mixture of the two drugs and the dose is for the mixture.

3. Three different approaches can be used for irradiating mice, depending upon the mouse strain and whether full reconstitution is needed. For the single exposure protocol, mice are irradiated with 800–950 rads (exact dose is strain dependent, we commonly use 950 rads for C57BL/6 mice) at a rate of approximately 60–80 rads/min. For split exposure, the total dose is delivered in two 5–6 min periods separated by 3 h. The split dose is often used with mice that might have defect in erythroid recovery after transplant. Strains highly sensitive to irradiation such as SCID mice receive a reduced total exposure dose of 350–400 rads. The reduced exposure can also be used when full reconstitution is not needed, such as adoptive transfer of mature cells or transfer of leukemia stem cells.
4. Transplant procedure: Bone marrow donors are euthanized with CO₂ for marrow collection. Bone marrow cells are collected in sterile media, and injected into the tail vein or retro-orbital sinus of the irradiated recipients. The number of cells will depend on the experiment and will range from 2×10^2 to 5×10^6 in 150–200 μ L. Although we routinely transplant $1\text{--}5 \times 10^5$ cells into C57BL/6 recipients up to 5×10^6 cells can be transplanted (*see Note 6*).
5. Whole body irradiation damages the bone marrow and potentially the GI tract leading to increased susceptibility to opportunistic infections. Although successful bone marrow transplant should be protective, the transfer will occasionally be unsuccessful. Potential signs of complication are nonspecific and may include the following: Poor body condition/weight loss; rough coat, inactivity, hunched posture; and death without prior signs of illness. Animals need to be monitored daily by the investigator following irradiation and bone marrow transplant. Mildly affected animals (rough coat, inactive, hunched posture) but still in good body condition are monitored for further deterioration. More severely affected animals displaying obvious weight loss and poor body condition (body condition score of 2 minus) are removed from the study. A veterinarian may need to be consulted for possible diagnostic testing.
6. Transplanted mice can be analyzed in a number of ways (*see Notes 7 and 8*). Complete blood counts and reticulocyte assays can be done. In addition mice that constitutively express GFP (for example C57BL/6-Tg(UBC-GFP)30Scha/J) (15) could be used so that the donor or the recipient is GFP+. The usual dose of $1\text{--}5 \times 10^5$ unfractionated bone marrow cells leads to erythroid recovery in approximately 16–20 days.

3.4 In Vitro Analysis of Stress Erythropoiesis

1. We have developed an in vitro culture system that recapitulates the signals and microenvironment present in the spleen. This system uses unfractionated bone marrow cells as starter material. During culture, the cells form a microenvironment that supports the expansion and development of stress erythroid progenitors that can be assayed in stress BFU-E assays or transplant assays. The culture can be done in two ways. The first way is to include all factors necessary for the expansion and differentiation of cells, which includes

BMP4, Shh, SCF, GDF15, Epo and the culture is performed at 1–2% O₂. The production of stress BFU-E can be assayed on different days of culture. Alternatively, the culture can be separated into an expansion phase and a differentiation phase, which mimics the development in vivo. For this type culture, cells are first grown in stress erythropoiesis expansion media (SEEM), which contains BMP4, Shh, GDF15, and SCF. The cells are cultured in this media for 5–7 days at 20% O₂. The production of stress BFU-E is minimal in these culture conditions. The second stage of culture is a switch to stress erythropoiesis differentiation media (SEDM). In addition to the growth factors in SEEM, SEDM contains Epo and the cells are grown at 1–2% O₂. The production of stress BFU-E can be assayed from days 1–5 of SEDM culture. In addition, the two-stage culture system can allow investigators to examine the expansion of early stress progenitors characterized by the expression of CD34+CD133+Kit+Sca1+ cell surface markers. This analysis can be extended to examine the transition of these early cells to more mature cell populations which lose the expression of CD34, CD133, and Sca1, as described previously [9]. This culture system can be readily adapted to human cells. We purchase whole unfractionated bone marrow cells for these experiments, but unfractionated cord blood will also work. Purified subsets like CD34+ cells do not work in these cultures. When culturing human cells, obviously human growth factors need to be used and we usually purchase fetal calf serum that has been tested for human cultures.

1. For a single-stage culture: Isolate cells from bone marrow and culture at a concentration of 1×10^6 cells/mL in SEDM for 5–7 days at 1–2% O₂. Cells can be harvested any time after 24 h of culture depending on the experiment and plated for stress BFU-E as described above or analyzed by flow cytometry as previously described.

2. For a two-stage culture: First stage—Expansion of early progenitors: Isolate cells from bone marrow and culture at a concentration of 1×10^6 cells/mL in SEEM for 5–7 days at 20% O₂. At this stage, early stress progenitors (CD34+CD133+Kit+Sca1+ and CD34–CD133+Kit+Sca1+ cells) can be isolated by flow cytometry and analyzed (*see Note 9*).

Second stage transition to differentiation: After first stage culture, count cells and shift to SEDM at a concentration of 1×10^5 cells/mL for 1–5 days at 1% O₂. When changing the media, return the cells in SEDM to the same dish that contains the adherent microenvironment.

3 Notes

1. This dose of PHZ was designed using C57BL/6 mice. The kinetics of the response is well characterized (for example *see ref. 3*). The kinetics in other mouse strains is different, so it is always important to have a strain specific wild-type control. For some mutant mice, 100 mg/kg mouse is too strong a dose and results in lethality. If we encounter lethality we test mice at a 75% and a 50% dose, so mice survive the treatment and the phenotype in stress erythroid progenitors can be analyzed.

2. PHZ treatment can complicate flow cytometry. In our hands, stress erythroid progenitors from mice treated with PHZ exhibit high levels of autofluorescence, which make analysis of cell populations difficult.
3. Our use of 12-well plates is designed to conserve methylcellulose and other reagents. The assays can be done in 6-well plates, but in that case the number of cells should be increased to $0.5\text{--}1 \times 10^6$ cells per well in 1–1.5 mL of media. Methylcellulose is viscous. We always set up enough media and cells for four wells so that each well of the triplicate set receives the full 0.5 mL of media and cells. When plating in a 12-well dish we always fill at least three wells with sterile distilled water, which prevents the methylcellulose media from drying out during the culture.
4. Hypoxic culture can be done in several ways. The most economical is a hypoxia chamber (an example is the modular incubator chamber from Billups-Rothenberg). Plates of cells are placed in the chamber, the chamber is flushed with a custom gas mixture (we use 94% N₂, 5% CO₂, and 1% O₂) and then the chamber is placed in the incubator. The hypoxia chamber is flushed with gas daily during the culture period. The second option is the use of a chamber that fits in an incubator, but independently controls CO₂ and O₂ levels (an example of this system is the Biospherix Proox C21 CO₂/O₂ controller and C-Chamber for hypoxia culture). The third option is an incubator that controls both CO₂ and O₂ levels.
5. Benzidine staining is very efficient. If the stain does not work, the most likely problem is old H₂O₂. When counting colonies, stain wells as you count them. The stain will dilute and fade with time. Also once you start staining wells on a plate you have to count the whole plate. If you place a plate with stained wells in the incubator the cells will die in the other wells.
6. The radiation dose and number of cells needed for transplant are optimized for C57BL/6 mice. Other strains may be more or less tolerant to radiation doses and number of donor cells. We routinely transplant with 1×10^5 cells, which is on the lower end of doses. At that dose we feel that we have a sensitized screen for defects in erythroid short-term radioprotection. If you encounter lethality at that dose, increase the dose of donor cells to 5×10^5 or 1×10^6 . At the higher doses of cells, more mutant mice will survive so that analysis will be possible. Similarly, using a split dose of irradiation can improve the survival of recipient mice.
7. We use a number of genetic methods to mark donor versus recipient cells. The classic method is to use C57BL/6 (CD45.2+) and congenic B6.SJL mice that carry the CD45.1 allele as donors and recipients. In addition, mice that constitutively express GFP (for example C57BL/6-Tg(UBC-GFP) 30Scha/J) [15] could be used so that the donor or the recipient is GFP+.
8. For the analysis of mice following transplant, we use complete blood counts. Investigators should be aware that if you continuously sample blood from the

recovering animals that is an anemic stress. Control transplanted mice are not affected by the blood sampling, but for some mutant donor transplants the added stress is enough to cause transplant failure. To circumvent that problem you can euthanize mice at each time point or you can set up several cohorts where the blood sampling occurs less frequently; for example, four cohorts with sampling every other day will only require two blood samples per mouse over the 16 day recovery period.

9. It is normal for cell numbers to decrease in the cultures. The progenitor cells not supported by the growth factors are lost by apoptosis. By day 3 of the initial culture in SEEM, the numbers of cells will begin to increase. Hypoxic cultures can be done as described in **Note 4**, but the investigator should realize that under hypoxic conditions cells grow faster. Care should be given so that the cultures do not over-grow the media.

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Fig. 1.
Schematic of stress erythropoiesis. The cell surface markers of the progenitors at each stage of development are shown, and growth factors required at each stage are indicated below