Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors

Running title: Steroids regulate human erythropoiesis in vitro

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Footnotes:
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Supported by the Austrian Science Foundation (FWF; to HB and EWM) and the Herzfelder-Family Foundation (EWM).
The online version of the article contains a data supplement.

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Total word count: **4930** (of 5000 allowed)
Abstract

Outgrowth, long term self renewal and terminal maturation of human erythroid progenitors derived from umbilical cord blood in serum-free medium can be modulated by steroid hormones. Homogenous erythroid cultures, as characterized by flow cytometry and dependence on a specific mixture of physiological proliferation factors were obtained within 8 days from a starting population of mature and immature mononuclear cells. Due to previous results in mouse and chicken erythroblasts, the proliferation-promoting effect of glucocorticoids was not unexpected. Surprisingly, however, androgen had a positive effect on the sustained expansion of human female but not male erythroid progenitors. Under optimal conditions, sustained proliferation of erythroid progenitors resulted in a >10^9-fold expansion within 60 days. Terminal erythroid maturation was significantly improved by adding human serum and thyroid hormone (T3) to the differentiation medium. This resulted in highly synchronous differentiation of the cells towards enucleated erythrocytes within 6 days, accompanied by massive size decrease and hemoglobin accumulation to levels comparable to those in peripheral blood erythrocytes. Thus, obviously, different, ligand-activated nuclear hormone receptors massively influence the decision between self renewal and terminal maturation in the human erythroid compartment.

183 words (of 200 allowed)
Introduction

Hematopoietic development within the erythroid lineage requires a delicate balance between the opposing effects of proliferation-promoting factors maintaining the renewal capacity of immature erythroid progenitors, and differentiation-inducing factors required for successful terminal maturation of erythroid progenitors into red blood cells\(^1\). In adult humans, erythroid differentiation produces about \(2 \times 10^{11}\) (roughly 20g) red cells per day, representing the most vigorous proliferation process in the entire body. Since low numbers of erythrocytes are immediately life-threatening, erythropoiesis needs regulatory mechanisms temporarily altering the balance towards enhanced proliferation, to cope with stress situations such as low oxygen levels at elevated altitudes (hypoxia) or massive blood loss.

\textit{In vivo}, both renewal and maturation of human erythroid progenitors proceed in parallel in the bone marrow. It has thus been difficult to assess the contribution of particular signaling pathways and their deregulation during aberrant depletion of these progenitors, or their death upon differentiation in anemia, versus unscheduled progenitor proliferation (as occurring in leukemogenesis). Most information was obtained from established cell lines and –more recently– primary animal cell models of chicken and mouse in defined media\(^1\)\(^-\)\(^3\). The latter approach even allowed to analyze cells from genetically modified mice\(^4\)\(^-\)\(^6\). For sustained proliferation, both, avian and murine primary erythroblasts require co-operation between erythropoietin (Epo), stem cell factor (SCF) and glucocorticoids (dexamethasone; Dex). Involvement of Epo and SCF was expected from their contribution to proliferation/survival of erythroid cells\(^7\)\(^-\)\(^9\), whereas the stress hormone Dex massively prolonged progenitor proliferation (from 10 to 30 days in chicken, 7 to 20 days in mouse), at the same time reducing their rate of spontaneous differentiation. Our idea that this might point to specific roles for glucocorticoids in stress erythropoiesis could be verified \textit{in vivo} using mutant mice carrying a dimerization-defective glucocorticoid-receptor (GR-dim). In contrast to wild-type mice, GR-dim mice could not respond to hypoxia or experimental anemia with increased erythroblast renewal\(^10\).

Interestingly, activated leukemia oncogenes utilize the same signaling pathways driving transient renewal during stress erythropoiesis to induce permanent proliferation as well as differentiation arrest in leukemia. The \(v\)-ErbB oncogene product of avian erythroblastosis virus (AEV) or endogenous mouse \(c\)-ErbB can substitute for ligand activation of EpoR plus c-Kit, the SCF receptor\(^11\)\(^,\)\(^12\). On the other
hand, GR can be replaced by v-ErbA, the second AEV oncogene, a constitutively repressing variant of TRalpha/c-ErbA\textsuperscript{13}.

These observations indicated a need for corresponding studies in human erythroblasts under \textit{in vivo}-like conditions. Although cooperation between Epo, SCF and Dex was shown to induce renewal of human erythroblasts\textsuperscript{14}, the use of serum-containing media limited the usefulness of these culture conditions with respect to cell numbers and homogeneity of cells obtained. Human erythroid cell lines such as K562, yielding unlimited cell numbers, are even less useful, since they proliferate in the absence of physiological regulators and thus compromise mechanistic studies. Finally, techniques to expand erythroid progenitors from multipotent ones in two- or three-step protocols\textsuperscript{15} yield cell populations which undergo sustained proliferation for $< 2$ weeks, accompanied by high rates of spontaneous differentiation. Thus, contributions of individual exogenous factors to self renewal versus maturation were difficult to assess by these approaches.

We addressed this problem by systematically exploring the renewal- and differentiation requirements of erythroid progenitors from umbilical cord blood. Cord blood has received a lot of attention as a source for hematopoietic stem cells (mainly CD34+) as an alternative to bone marrow stem cells in transplantation medicine\textsuperscript{16-19}. Furthermore, cord blood is a source of multipotent stem cells, which may replace human ES cells in future forms of therapy\textsuperscript{20} and highly enriched in committed hematopoietic progenitors, including those of the erythroid lineage\textsuperscript{21}.

Here, we describe thoroughly optimized long-term serum-free culture conditions, allowing expansion into mass cultures of highly homogenous human erythroid progenitors derived from umbilical cord blood. Cells could be expanded routinely for more than 45 days, undergoing up to 35 population doublings, which approaches the \textit{in vitro} life-span of primary fetal human fibroblasts. In these experiments, we confirmed the requirement of glucocorticoids (Dex)\textsuperscript{14} for sustained proliferation. More importantly, human erythroblasts exhibited a gender-specific promotion of proliferation via androgen. Finally, we optimized induction of synchronous terminal differentiation, generating fully mature, enucleated erythrocytes, which required thyroid hormone (T3) in synergy with Epo/Ins (insulin; Ins) and (an) as yet unidentified factor(s) from human serum.
Materials and methods

Isolation and culture of mononuclear cells from cord blood

Umbilical cord blood (normal, full-term deliveries; informed consent; General Hospital, Vienna, Division of Gynecological Endocrinology) was delivered in 20U/mL heparin. Approval was obtained from the Department of Medical Biochemistry, Institute of Molecular Pathology and Department of Gynecological Endocrinology institutional review boards for these studies. Erythrocytes were removed by mixing blood with ery-lysis buffer (1/25 v/v; 89.9g ammonium-chloride, 10g KHCO₃, 0.37g EDTA) and several washes with ice-cold PBS until the cell pellet appeared white. For initial expansion, 5x10⁶ cells/mL were cultivated in serum-free medium (StemSpan™; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with Epo (2U/mL, Erypo Janssen-Cilag AG, Baar, Switzerland), the synthetic glucocorticoid dexamethasone (Dex; 1µM, Sigma, St. Louis, MO), IGF1 (40ng/mL, Promega, Mannheim, Germany), stem cell factor (SCF; 100ng/mL, R&D Systems, Minneapolis, MN) and lipids (40µg/mL, cholesterol-rich lipid mix, Sigma). Homogenous cultures of erythroid progenitors established after several days were kept in the same medium at 2x10⁶ cells/mL by daily partial medium changes. Proliferation kinetics and size distribution of cells were monitored daily using an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany); cumulative cell numbers were calculated as described. Proliferating erythroid progenitors could be frozen and thawed with high efficiency (>90%).

Differentiation induction

To induce terminal differentiation, proliferating erythroblasts were washed in ice-cold PBS and re-seeded at 2x10⁶ cells/mL in StemSpan™ supplemented with Epo (10U/mL), insulin (Ins; 4x10⁻⁴ IE = 10ng/mL, Actrapid HM, Bagsvaerd, Denmark), the Dex antagonist ZK112993 (3µM)²³, iron-saturated human transferrin (1mg/mL; Sigma), and 3% human male serum (Sigma). Differentiating erythroblasts were maintained at 2-4x10⁶ cells/mL by daily cell counts and partial medium changes.

Cell morphology, histological staining and hemoglobin determination

Erythroblasts at various differentiation stages were cytocentrifuged onto glass slides and stained with histological dyes and benzidine for hemoglobin as described²⁴.
quantitate hemoglobin, 50µl aliquots of cultures were analyzed in triplicate by photometry after normalization for cell number and volume as described25.

**Apoptosis assay**
Cells (2x10⁶) were fixed in 0.5mL 8% para-formaldehyde in PBS for 30min at room temperature and stored in 70% ethanol at -20°C. Cells were washed in PBS and incubated in 45µl labeling- plus 5µl enzyme solution (fluorescein-dUTP; TdT; In situ Cell Death Detection Kit, Roche) for 1h at 37°C. Cells were washed once, re-suspended in PBS and subjected to flow cytometry to determine percentages of TUNEL positive cells.

**Flow cytometry**
Erythroblasts (1x10⁶) were washed twice with PBS plus 1% fetal calf serum (FCS) and stained with fluorescently labeled antibodies against CD15 (FITC=fluorescein-isothiocyanate; PharMingen, San Diego, CA, 555401), CD19 (FITC; PharMingen, 555412), CD116 (FITC; PharMingen, 554532), GPA (GlycophorinA; FITC; PharMingen, 559943), CD8 (PE=phycoerythrin; PharMingen, 555367), CD33 (PE; PharMingen, 30945X), CD38 (PE, PharMingen, 555460), CD45RA (PE; PharMingen, 555489), CD56 (PE; PharMingen, 555516), CD71 (transferrin receptor; PE; PharMingen, 555537), CD117 (c-Kit, the stem cell factor receptor; PE; PharMingen, 555714), CD3 (ECD=phycoerythrin-Texas Red; Coulter-Immunotech, Marseille, France, IM2705), CD45 (ECD; Coulter-Immunotech, IM2710), CD4 (PC5=phycoerythrin-cyanin 5.1; Coulter Immunotech, IM2636), CD14 (PC5; Coulter-Immunotech, IM2640) and CD34 (PC5; Coulter-Immunotech, IM2648). Surface marker expression was analyzed by flow cytometry (FacScan or LSR, Becton Dickinson), quantitation of cell populations positive for individual markers was performed with the Paint-a-gate software package (Becton Dickinson).

DNA profiles were determined in a PAS-III flow cytometer (Partec, Germany) after staining cellular DNA with 6µM 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Percentages of cells in specific cell cycle phases were calculated using a software package of the same manufacturer26.

**Density gradient purification of hematopoietic cells**
Density gradient centrifugation (>10x10^6 cells, to optimize the yield) was used to remove dead and spontaneously differentiating cells. Cultures were transferred into tubes with 2mL lymphocyte separation medium (Eurobio, Les Ulis Cedex B, France, 1,077g/mL). After centrifugation (600g, 7min; immature progenitors band between the phases, dead and differentiating cells are in the pellet) erythroblasts were transferred into another tube, counted, washed with PBS and re-cultivated.
Results

In vitro mass culture of human erythroid progenitors from umbilical cord blood

Human erythroid progenitors were expanded from umbilical cord blood after selective lysis of mature erythrocytes (Material and Methods) in serum-free medium (StemSpan™) by using a combination of Epo, SCF, Dex (synthetic glucocorticoid) and IGF1, some of which are typical for stress erythropoiesis (Table 1A). From day 3 on, a small population of expanding erythroid progenitors became visible in cytospins, which was clearly detectable in an electronic cell analyzer (CASY) around day 6 as an increasing population of large, non-adherent cells (~12µm diameter; Figure 1). From day 10 onwards, morphologically homogenous erythroid progenitors could be expanded into mass cultures (Figure 1), which proliferated exponentially for over 30 days, demonstrating a clear capacity for long-term self renewal. At regular intervals, more mature or apoptotic cells were removed by density gradient centrifugation. After 27-30 days, the majority of progenitors were undergoing gradual proliferation arrest before eventually undergoing apoptosis. During the entire expansion, a measured 4000-fold increase in cell numbers was obtained (Figure 2A, circles; see also Supplementary Figure 1).

In experiments to further improve the medium composition (Table 1A), lipids (40µg/mL; cholesterol-rich) reproducibly extended the period of sustained proliferation from 30 to >45 days (in two experiments up to 60 days), resulting in another three logs of cumulative cell number obtained. This corresponds to a total of 3 million-fold cell expansion (Figure 2A, squares). Extrapolating from the data on cell numbers and proliferation rates back to day 0, an apparent, overall increase in cell number of 10^8- to >10^9-fold was obtained. This corresponds to 30-35 generations, almost approaching the known in vitro life-span of primary human skin fibroblasts. Unless explicitly stated otherwise, all further experiments were performed in medium supplemented with lipid from day 0 on, although no differences in cell phenotype or proliferation were observed upon inclusion of lipid at any time between day 0-18. Of several additional cytokines and hormones tested for their potential to improve progenitor cell outgrowth, none proved effective (Table 1A), with the exception of androgen (see below).

During the last third of the expansion period, the cultures showed a gradual increase of cells in G1 plus a simultaneous decrease in percentage of S-phase cells (Figure 2B, analysis of DNA content by flow cytometry; duration of G2/M nearly
unchanged). This effect is somewhat overestimated by the presence of non-cycling cells arising through spontaneous differentiation, despite repeated density gradient purification of the immature progenitors.

**Long-term expansion of human erythroblasts: continuous requirement for Epo, SCF and Dex for proliferation and differentiation arrest.**

To verify that the cultures remained dependent on each of the proliferation factors required for erythroid progenitor expansion and/or survival during the entire expansion period, cell batches pre-grown for 18 days in complete medium were switched to media lacking either single or all factors. The requirement for Epo, SCF and/or Dex was then assessed by measuring cell numbers (Figure 3A) and rates of apoptosis by TUNEL assays (Material and Methods; Figure 3B). In our hands, omission of IGF-1 did not decrease proliferation rates within 8 days, consistent with findings in murine erythroblasts. Since, however, IGF-1 was previously reported several times to support long-term survival of myeloid progenitors as well as committed erythroid cells, the media used in further experiments were nevertheless supplemented with IGF1.

As expected, erythroblasts exposed to medium without factors stopped proliferating within 24 to 48 hours. After 2 days, more than 75% and after 4 days essentially all cells had died from apoptosis (Figure 3B). Glucocorticoid receptor signaling (GR) was abolished by removal of Dex plus addition of the glucocorticoid antagonist ZK. Under these conditions, cells rapidly ceased to proliferate; >50% were apoptotic after two days, confirming the strict requirement for this hormone for renewal. Omitting Dex from the medium alone caused only partial effects, in line with findings in several nuclear receptors including GR that antagonist is required to enforce an inactive receptor conformation and releasing receptor-bound ligand. The glucocorticoid levels present in serum-containing media make it difficult or impossible to observe Dex effects. However, addition of the ZK antagonist showed that human erythroblasts require Dex also for expansion in serum-containing media. In the absence of Epo or SCF all progenitors disintegrated within 6 days, but proliferation arrest and apoptosis were delayed in the absence of Epo as compared to SCF, similar to previous findings in chicken and mouse. Taken together, cultured human erythroblasts remain strictly dependent on Epo, SCF and Dex throughout their extended period of exponential proliferation.
Cell surface marker expression indicates synchronous, gradual establishment of cells committed to the erythroid lineage

Next, we determined cell type composition of cord blood-derived cultures during early and later phases of expansion, using surface markers for different lineages and immature progenitors. First, we analyzed markers for immature and mature erythroid cells. High CD117 (c-Kit) expression levels are typical for erythroid progenitors (besides expression on very immature progenitors)\(^9,36\). Freshly established cord blood cultures (day 1) exhibited very low CD117 levels, which steadily increased, reached a maximum with almost 100% positive cells around day 14 (Figure 4A) and then gradually declined to 50% CD117\(^{high}\) cells at day 35. This suggested that proliferating progenitors gradually increased c-Kit expression from an immature to a more mature erythroid progenitor phenotype, in line with a similar decrease in CD45 (data not shown). This marker for immature cells of most leukocyte lineages is down-regulated during erythroid maturation\(^37\). Gradual maturation of the cultured erythroblasts was also indicated by the continuous increase of CD71\(^{high}\) cells (transferrin receptor, up to 100% at day 35, Figure 4A,B). CD71 shows maximal expression in differentiating erythroid cells but disappears on mature erythrocytes. Similarly, glycophorin A (GPA), a protein abundant on more mature erythroid cells\(^38\), resembled CD71 in its gradual increase during the whole culture period. These patterns of changes in marker distribution were reproducible for several samples from individual cord blood donors.

The above data show that the initial population of mononuclear cells isolated from cord blood and used to initiate erythroblast cultures contained very low levels of erythroid progenitors. The most prominent sub-populations of fresh cord blood isolates were mature granulocytes (40-70%), T lymphocytes (7-15%), monocytic cells (5-8%) and other more immature progenitors as seen from corresponding marker analysis (lymphoid cells: CD3, CD4, CD8, CD19; CD45RA; natural killer cells: CD56; granulocytic cells: CD15; monocytes: CD14; CD11b). Changes in sub-population distribution over time suggested that non-erythroid cell types persisted for several days (Figure 4B, data on CD3, CD14, CD19; Supplementary Table 1 for other CD antigens mentioned) but did not proliferate under our culture conditions, thus being gradually diluted out by the rapidly proliferating erythroid progenitors.

To determine the abundance of multipotent progenitors in the cord blood cultures, CD33 (myelomonocytic progenitors), CD34 (multipotent progenitors) and CD38
expression (immature lymphoid progenitors) was quantitated in parallel. Starting from low levels (<0.5%; similar to values reported for freshly prepared cord blood\(^{39}\)), the percentage of CD34+ cells increased 10-fold up to 4% (Figure 4B) by day 6. In absolute numbers, however, this corresponded to a slight decrease from about 10^6 to 5x10^5 cells while during the same time the total population of non-erythroid mononuclear cells was declining about 40-fold (from 3x10^8 to 8x10^6 cells). The majority of the CD34+ cells (>85%) was derived from the myelomonocytic compartment, as indicated by CD33/CD34 double staining. The increase in CD34+ cells peaked around day 6, well preceding the maximum in CD33 expression (>60% positive cells at day 11) and CD117 (day 14). This argues for persistence of multipotent progenitors during the first 10-14 days, which then gradually developed into progenitors of the erythroid lineage.

**Androgen enhances proliferation of female but not male human erythroblasts**

Due to their predominantly gender-specific actions, the potential of sexual steroids to modulate human hematopoietic development in vitro has not been addressed so far. However, disturbances in sex steroids contribute to a number of diseases such as anemia\(^{40}\). In chicken, estrogen and glucocorticoid receptor ligands (Dex) both contribute to long-term self renewal of erythroid progenitors\(^{41,42}\) and – consequently – interfere with terminal maturation\(^{43}\).

These observations prompted us to expand human erythroblasts in the presence of various other hormones besides Dex. While estrogen and progesterone had no effects (Table 1A), dihydro-testosterone (an androgen) significantly enhanced the proliferation rate of cells from certain cord blood donors but not from others. One way to interpret this puzzling behavior was that androgen might act in a gender-specific manner. Thus, erythroid progenitors from cord blood of male and female newborns were expanded in the presence or absence of dihydro-testosterone. As a control, the androgen antagonist cyproterone-acetate (Cpa)\(^{44}\) was included (Figure 5; see also Supplementary Figure 2⁹).

Dihydro-testosterone had no effect on the proliferation of “male” cells over 25 days of culture. However, for “female” erythroblasts a significant and reproducible enhancement in proliferation was observed with dihydro-testosterone. The antagonist Cpa reduced the testosterone-induced, increased proliferation of female cultures back to the level of untreated controls but not below, while again it had no effect on male cultures (Figure 5). This indicates that the androgen effect was specific and
argues against unspecific cross-talk between different steroid hormone-dependent signaling pathways.

**Induction of synchronous terminal differentiation**

Most established erythroid cell lines to study terminal erythroid differentiation suffer from drawbacks like un-physiological stimuli required to initiate maturation, asynchronous progress into differentiation, low levels of hemoglobin synthesized and a failure to enucleate. Recently, we established conditions for efficient terminal maturation of primary and permanently proliferating (p53-deficient) mouse erythroblasts by removing proliferation factors (Epo, SCF, Dex) and exposing the cells to differentiation conditions (Epo , Ins, iron saturated transferrin, ZK). Initially, we tried to adapt these protocols to terminally differentiate homogenous cell populations of cultured human erythroid progenitors pre-expanded for 14 days. Differentiation media consisted of serum-free medium (StemSpan TM) used for expansion plus factors/hormones to be tested (Table 1B).

To follow the progress of terminal maturation, proliferation rate, volume decrease (measured in a cell analyzer) and hemoglobin accumulation (quantitative photometric assay, see and Materials and Methods) were monitored daily. In addition, cyto-centrifugation followed by histological staining was applied for phenotypic characterization, again to assess hemoglobinization but also to detect enucleated erythrocytes at the end of maturation. Differentiation with the factor mix used for mouse cells did not yield satisfying results. Rather than differentiating, the cells underwent apoptosis at high frequency and accumulated little hemoglobin (Figure 6A-E). Systematic variation of multiple differentiation medium components to improve terminal maturation of immature progenitors was ineffective (Table 1B). In contrast, addition of normal human serum combined with Epo /Ins was found to strongly improve synchronous differentiation.

Like chicken and mouse erythroblasts, human erythroid progenitors continued to divide upon differentiation initiation but only for 1-2 divisions, as compared to 4-5 in chicken and 3-4 in mouse (Figure 6A). Accompanying these “differentiation divisions”, cell size decreased from about 660 to 380fl. Hemoglobin accumulation started from very low levels (Figure 6C,E) –confirming the low rate of spontaneous differentiation in the renewing cells– and steadily increased more than 10-fold within 6 days. Hemoglobin levels reached ~35% (11.5±1.0pg; n=5) of the value determined for peripheral human erythrocytes (31.6±2.4pg; n=5). Although
cells “differentiated” in the absence of human serum had a stronger size decrease (Figure 6B), this was due to massive cell death as indicated by broad size distribution, the extensive presence of cell debris, and the lack of hemoglobin (Figure 6C,D,E). Cytospins after 6 days of differentiation plus serum exhibited morphological changes and staining for hemoglobin consistent with successful maturation (Figure 6E). The differentiation-promoting effect of serum was further confirmed by cultivating erythroblasts under standard proliferation conditions plus 3% serum, indeed observing reduced proliferation and enhanced spontaneous differentiation (Supplementary Figure 3’). Finally –due to the gender specific proliferation effect– the influence of testosterone on differentiation of female versus male erythroid progenitors was tested: no effects were observed (data not shown).

Terminal erythropoiesis was also assessed on the basis of the cell surface markers c-Kit (CD117; characteristic for proliferating erythroid progenitors), transferrin receptor (CD71) and glycophorin A (GPA, both markers for mature cells). As expected, erythroblasts differentiating in the presence of serum showed a downregulation of c-Kit to background levels and a further induction of GPA (from 70% to 100% positive cells; Figure 6F). CD71 expression at the cell surface was slightly down-regulated, agreeing with our earlier data showing a partial redistribution of CD71 to intracellular compartments in differentiating avian and murine cells46; Lobmayr and Mullner, unpublished). While regulation of CD71 and GPA was similar in cells differentiating in the presence or absence of serum, c-Kit levels did not decrease when serum was absent (Figure 6F, upper left panel). This was in striking contrast to data from murine erythroblasts, where removal of SCF plus Dex is sufficient to induce loss of c-Kit and terminal differentiation, provided the cells are protected from apoptosis3. This indicates that human erythroid progenitors require (a) human serum factor(s) for terminal differentiation, which is not required in mouse cells, pointing to an apparent difference between murine and human erythropoiesis.

**Thyroid hormone (T3) improves terminal maturation of human erythroblasts**

One possibility to explain the above serum effects could be that human and murine erythroid cells differ in their responsiveness to thyroid hormone (T3). Since lack of T3 in humans is associated with anemia47,48 and its addition strongly accelerates terminal differentiation of chicken erythroblasts13,49, we analyzed the effect of T3 on human erythroid differentiation, together with several other hormones (Table 1B).
T3 further accelerated the rate of hemoglobin accumulation and the abundance of enucleated cells, which by morphological criteria were indistinguishable from mature erythrocytes (Figure 7). Overall hemoglobin values in these populations (differentiated for 6 days) reached ~40% (12.9±1.1pg; n=5) of the level in circulating human erythrocytes, an underestimate due to the fraction of non-enucleated cells (Figure 7A, cells with condensed, acentric nuclei, smaller than original blasts). Improved synchrony of maturation by T3 was also evident from the much narrower size distribution at each time point (Figure 7E, compare to 6D). No major differences in expression of c-Kit, GPA and CD71 were detected by cytofluorometry in cells differentiating in the presence or absence of T3.

Like for serum, this T3 effect raised the question, whether the hormone would shift the balance between proliferation and differentiation, as previously observed in chicken erythroblasts\textsuperscript{49}. Indeed, addition of T3 under proliferation conditions increased the proportion of spontaneous differentiation, as judged from reduced proliferation rates, gradual decrease of cell size and detectable amounts of hemoglobin synthesized (Supplementary Figure 4').
Discussion

The expansion of hematopoietic progenitors from umbilical cord blood or peripheral blood ex vivo is of great interest, both for basic research and the exploitation of clinical potential. Here we demonstrate—at least for the erythroid compartment—that steroid hormones play a larger than anticipated role in regulating the balance between sustained proliferation and terminal differentiation. Previously used procedures failed to yield long-term proliferating but immature erythroid progenitors, due to massive spontaneous differentiation\textsuperscript{15,33,34}. The use of serum-containing media limited the expansion of erythroid progenitors even when Epo, SCF plus glucocorticoid were provided\textsuperscript{14}. Using serum-free media together with Epo, SCF, glucocorticoid and androgen, we could expand human erythroid progenitors from umbilical cord blood for about 7 weeks. This yielded mass populations with an in vitro life-span of approximately 30 generations, almost absent spontaneous differentiation and essentially no hemoglobinization, largely facilitating future biochemical and molecular characterization. Optimization of differentiation conditions allowed induction of synchronous terminal maturation, resulting in enucleated cells virtually indistinguishable from erythrocytes of peripheral blood.

The role of glucocorticoids in enhancing proliferation and blocking differentiation of erythroid progenitors becomes increasingly recognized in vitro and in vivo. Glucocorticoids regulate multiple physiological- and developmental processes by binding to and modulating the transcriptional activity of their cognate nuclear receptor (GR)\textsuperscript{50,51,52}. In both chicken and mouse erythroblasts, ligand-activated GR cooperates with activated Epo receptor (EpoR) and c-Kit, the stem cell factor (SCF) receptor to retain erythroid cells in an immature state and thus to induce long-term proliferation\textsuperscript{12,42,53,54}. A similar effect of glucocorticoids was described in human erythroblasts\textsuperscript{14}, but their expansion was limited to 15-22 cell divisions, probably due to the use of serum-containing media. Importantly, GR is also required for stress erythropoiesis following hypoxia or blood loss in the mouse\textsuperscript{10}.

In our hands, the serum-free medium (StemSpan™) employed here was critical for generating long-term cultures. Other serum-free media proved less efficient, the specific type of human serum albumin contained in this medium being one likely candidate. The inclusion of even small amounts of serum (human, calf) strongly increased the rate of spontaneous differentiation\textsuperscript{34,55}. Given our results on the differentiation-promoting effects of thyroid hormone and numerous reports on
similar effects of TGFβ56-58, it may well be that the absence of these serum components from the synthetic StemSpan™-medium was critical for achieving long term proliferation of erythroid progenitors. Comparing to other current procedures, it should also be noted that our conditions did not require positive pre-selection for immature, pluri-potent progenitors (e.g. CD34+/c-Kit–)55,59, negative selection (depletion) against lymphoid or granulocytic cells nor highly complex mixtures of multiple cytokines60. Importantly, the use of serum-free medium yielded immature erythroid progenitors with an in vitro life-span approaching the “Hayflick Limit” of human fibroblasts (40-50 generations). Routinely, a (calculated) 10⁸-fold expansion was obtained, in some experiments even a 10¹⁰-fold increase in cell numbers (about 35 generations). Thus, the procedure described here enables the production of large numbers of homogenous primary human cells for detailed molecular characterization of mechanisms underlying progenitor renewal versus differentiation, including expression profiling.

The clear-cut effect of glucocorticoids, together with the renewal-enhancing role of estrogens on chicken erythroblasts42,43, prompted us to evaluate the effects of additional (steroid) hormones on expansion/maturation of human erythroid progenitors. While estradiol or progesterone were ineffective, androgen clearly promoted proliferation of cord blood erythroid cells from female but not from male donors. This gender-specific effect of androgens could be completely blocked by the androgen-antagonist cyproterone acetate, demonstrating specificity.

The recent literature on androgen and hematopoietic cells is scarce. While androgen ablation reduced the number of peripheral B-cells in male mice61, the hormone or an anabolic derivative thereof increased the number of red cells and CFU-Es in rats and mice, respectively62,63. There is some biochemical evidence for the expression of androgen receptor (AR) in erythroblasts from human bone marrow cultures64 and megakaryocytes65. Given the major role of GR in these cells, one might speculate about a cross-talk between AR and GR, similarly to the cross-talk described between estrogen receptor and progesterone receptor66 or TGFalpha receptor (cErbB)41. Similar to inhibition of the PR-ER cross-talk with estrogen antagonists, liver GR activity could be inhibited with cyproterone acetate67. At present, we cannot explain the gender-specificity of proliferation enhancement by androgen. No differences in GR potency/expression levels between males and females have been reported. Also, promiscuity for the corresponding hormone
response elements is unlikely, since despite sequence similarities they are clearly distinguished by GR versus AR\textsuperscript{68}. Moreover, so far we failed to detect AR protein or mRNA in human erythroblasts. Thus, androgen might exert its effects via binding to other hormone-receptor family members\textsuperscript{69} or non-classical pathways in the absence of AR via androgen binding proteins. The latter can stimulate second messenger cascades like the Src/MAPK and cAMP/PKA pathways, or might increase intracellular calcium levels\textsuperscript{70}, e.g. via G-protein coupled receptors.

Clinically, effects of androgen administration on red cell numbers as evidenced by hematocrit levels are well documented e.g. for hypogonadal men\textsuperscript{71}, patients treated for prostate hyperplasia\textsuperscript{72}, male hemodialysis patients\textsuperscript{73} as well as in athletes upon doping with androgens\textsuperscript{74}. In addition, females receiving dihydro-testosterone therapy also show higher hematocrits\textsuperscript{75,76}, which was already noted decades ago in breast cancer patients receiving androgen for therapeutic reasons\textsuperscript{77,78}. To our knowledge, however, there were so far no data on female-restricted proliferation-promoting effects of androgen in erythroid cells \textit{in vitro}, which should facilitate molecular evaluation of the pathways involved.

Also the direct, differentiation promoting effect of thyroid hormones (in particular 3,5,3'-tri-iod-L-thyronin, T3) on human erythroid progenitors merits further mechanistic exploration. Although thyroids are required for normal development, differentiation and metabolism of many cell types\textsuperscript{79,80}, little is known about their role in normal erythropoiesis. In mice lacking TRalpha, TRbeta or both, standard hematopoiesis appears unaffected\textsuperscript{81-83}. Primary chicken erythroblasts over-expressing TRalpha variants (gag-cErbA) in the absence of T3 underwent sustained proliferation in co-operation with liganded c-Kit\textsuperscript{13}. In contrast, T3 accelerated differentiation of normal avian erythroid progenitors and even promoted differentiation under proliferation conditions\textsuperscript{49,84}. Thus, in this avian model, TRalpha appeared as a ligand-operated “switch”, modulating the balance between renewal and differentiation\textsuperscript{13}. So far we could not reproduce these results in the mouse (HB, unpublished). It was thus interesting to find clear evidence for a function of T3 in human erythroid progenitor maturation, operating efficiently at the endogenous TR without requiring over-expression.

Our data, show profound effects of steroids and thyroids on renewal, proliferation and differentiation of long-term proliferating, immature human erythroid progenitors, leading to several additional considerations. On the medical side, it may
be worthwhile to (re)evaluate and eventually adjust the hormone status in some forms of (mild) anemia, e.g. in post-menopausal women, which although not life-threatening are reducing quality of life. The protocol for cell expansion reported here can also be applied to cells from peripheral blood: In preliminary tests, we not only expanded erythroid progenitors but also cells of other hematopoietic lineages in factor combinations used for multipotent progenitors (C. Leberbauer, D. Genz; in preparation). With respect to molecular mechanisms, our cells are ideal for expression profiling approaches, aimed at identifying target genes specifically modulated in their expression by the hormones used in this study.

Taken together, the advances in long-term expansion of hematopoietic (erythroid) cells described here can facilitate extensive biochemical analyses of cells from patients with hereditary hematopoietic disorders (e.g. thalassemias), which were technically demanding so far due to lack of material. Our system will also be ideal to address novel pathways modulating the balance between renewal and erythroid maturation in vitro.
Acknowledgments

We thank G. Fritsch, D. Prinz (Children Cancer Research Institute, Vienna) and P. Steinlein (IMP) for their initial introduction to flow cytometry of human cells as well as T. Sauer (IMB) for excellent technical assistance. This work was supported by the “Fonds zur Förderung der wissenschaftlichen Forschung” (FWF), Austria (grants to EWM and HB) and the Herzfelder-Family Foundation (EWM).

SUPPLEMENTAL MATERIAL IS AVAILABLE ONLINE AT THE TIME OF FINAL PUBLICATION ONLY.
References


Table 1A. Conditions for optimal long-term proliferation of human cord blood-derived erythroblasts in serum-free StemSpan™-medium

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>erythropoietin</td>
<td>2 U/mL</td>
</tr>
<tr>
<td>dexamethasone (synthetic glucocorticoid)</td>
<td>1 µM</td>
</tr>
<tr>
<td>IGF-1</td>
<td>40 ng/mL</td>
</tr>
<tr>
<td>SCF (stem cell factor)</td>
<td>100 ng/mL</td>
</tr>
<tr>
<td>cholesterol-rich lipid mix</td>
<td>40 µg/mL</td>
</tr>
<tr>
<td>dihydro-testosterone*</td>
<td>1 µM</td>
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</tbody>
</table>

* female erythroblasts only

Factors tested for proliferation of human erythroblasts without any effect

<table>
<thead>
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<th>factor</th>
<th>concentration</th>
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</thead>
<tbody>
<tr>
<td>IL-3</td>
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</tr>
<tr>
<td>TGF-β</td>
<td>5 ng/mL</td>
</tr>
<tr>
<td>heparin-binding EGF</td>
<td>20 ng/mL</td>
</tr>
<tr>
<td>testosterone*</td>
<td>1 µM</td>
</tr>
<tr>
<td>progesterone</td>
<td>1 µM</td>
</tr>
<tr>
<td>estrogen</td>
<td>1 µM</td>
</tr>
<tr>
<td>cyproteron-acetate (androgen antagonist)</td>
<td>1 µM</td>
</tr>
<tr>
<td>fibronectine coated plates</td>
<td>20 ng/mL</td>
</tr>
<tr>
<td>LY (PI-3-K inhibitor)</td>
<td>1 µM</td>
</tr>
</tbody>
</table>

* male erythroblasts only
**Table 1B.** Standard conditions allowing efficient terminal differentiation of human erythroblasts in serum-free StemSpan™ medium

<table>
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<th>factor</th>
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<td>erythropoietin</td>
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<td>insulin</td>
<td>4x10^-4 IE/mL</td>
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<tr>
<td>human transferrin (iron saturated)</td>
<td>1 mg/mL</td>
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<tr>
<td>ZK-112993 (glucocorticoid-antagonist)</td>
<td>3 µM</td>
</tr>
<tr>
<td>T3 (thyroid hormone)</td>
<td>1 µM</td>
</tr>
<tr>
<td>human serum (commercial; Sigma)</td>
<td>3%</td>
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</table>

Factors tested in differentiation of human erythroblasts without any effect

<table>
<thead>
<tr>
<th>factor</th>
<th>concentration (range) tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholesterol-rich lipid mix (Sigma)</td>
<td>20-100 µg/mL</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1-25 pg</td>
</tr>
<tr>
<td>activin</td>
<td>1-25 pg</td>
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<tr>
<td>fetal chicken serum</td>
<td>10%</td>
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<td>chicken serum</td>
<td>5-10%</td>
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<tr>
<td>human anemic serum</td>
<td>2-10%</td>
</tr>
<tr>
<td>human cord blood serum</td>
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<td>dihydro-testosterone</td>
<td>1 µM</td>
</tr>
<tr>
<td>progesterone</td>
<td>1 µM</td>
</tr>
<tr>
<td>estrogen</td>
<td>1 µM</td>
</tr>
<tr>
<td>LY (PI-3-K inhibitor)</td>
<td>1 µM</td>
</tr>
<tr>
<td>fibronectine coated plates</td>
<td>20 µg/mL</td>
</tr>
<tr>
<td>cyproteron-acetate (androgen antagonist)</td>
<td>1 µM</td>
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</table>
Figure 1. Outgrowth of erythroid progenitors from umbilical cord blood. Left panels: Changes in cell size distribution during proliferation of erythroblast cultures for 45 days in optimized medium (see Table 1A). Cell volume changes were monitored with an electronic cell analyzer. Right panels: At the times indicated, cells were cytocentrifuged onto glass slides and stained with cytological dyes. During the first few days, multiple cell types like leukocytes, macrophages and only few erythroblasts are present, the proportion of which increases upon culture in specific erythroid factors (Epo, Dex, mSCF and IGF1). At day 10, the majority of cells in the rapidly proliferating cultures (mitoses indicated by arrows) show an erythroblast morphology.
Figure 2. Long term expansion of human erythroid progenitors (see Table 1A). (A) Cell size- and proliferation kinetics were determined in the presence (from day 0; = standard medium composition for all other experiments if not stated otherwise) or absence of cholesterol-rich lipids by daily measurements in an electronic cell counter. For clarity, values are shown for every third day only; for full data set, see Supplementary Figure 1’. Cumulative cell numbers were calculated as described in Material and Methods; error bars: S.D. of mean, n=5. Cultures without lipid were terminated at day 30 due to excessive cell death (cross symbol). Insets (right): size distribution of cultures grown +/− lipids; a narrow size range corresponds to healthy cultures. (B) DNA content of cells (+ lipid) was monitored by flow cytometry after DAPI staining and the percentage of cells in particular cell cycle phases determined as described in Materials and Methods.
Figure 3. Human erythroid progenitors require Epo, SCF plus Dex for long-term expansion. (A) After pre-cultivation for 18 days (in the absence of lipids), cumulative cell numbers (see Figure 2) were determined from cultures kept for 8 more days in either the absence of Epo (full squares), or SCF (open circles), or Dex plus glucocorticoid antagonist ZK (full circles), or without any factors (open triangles) by daily counting in an electronic cell counter. Control (open squares), cultures grown in the presence of all factors. Cross symbol, termination of cultures due to excessive cell death. (B) Erythroblasts from cultures in (A) were analyzed for the percentage of apoptotic cells by TUNEL staining and flow cytometry (see Material and Methods); symbols as in (A).
Figure 4. Progression of cell surface marker expression in developing erythroblast cultures. Aliquots of proliferating cells were harvested at the times indicated, stained with combinations of fluorescently labeled antibodies against markers characteristic for different hematopoietic lineages and stages of development, and subjected to flow cytometry. (A) Histograms showing the changes in CD117 (c-Kit) and CD71 (TfR) expression over time. Light gray histograms, background fluorescence. (B) Quantitation (as percentage of positive cells) of erythroid (left panel) versus selected non-erythroid marker expression (right panel) during prolonged in vitro cultivation (see also Supplementary Table 1'). To eliminate non-specific staining of myeloid cells by the glycoporphin antibody, the fraction of cells expressing the erythroid-specific protein glycoporphin A (GPA+) is expressed as percentage of TfR positive cells. (C) Quantitation of pluripotent (CD34-positive; gated to be CD45<sup>dim</sup>) hematopoietic progenitors over time. The fraction of CD34+ cells within the myeloid (CD33+; left panel) and lymphoid compartment (CD38+; middle panel) is shown. Right panel, total numbers of CD34 positive cells for comparison, to depict the overall abundance of immature haematopoietic progenitors during the early phases of erythroblast culture.
Figure 5. Proliferation of male versus female human erythroblasts upon addition of androgen. Cell numbers from cultures of male versus female cord blood donors (pre-grown for 8 days in the absence of sex steroids) were counted daily in an electronic cell counter and cumulative cell numbers determined (see Figure 2 and Material and Methods). For clarity, values are shown for every third day only; for full data set, see Supplementary Figure 2'. Cells were cultivated either under standard proliferation conditions (control; diamonds), or in the presence of 1µM testosterone (filled squares) or testosterone plus the androgen antagonist cyproterone-acetate (1µM; open circles); error bars: S.D. of mean, n=3.
Figure 6. Terminal differentiation of human erythroid progenitors. Proliferation kinetics (A) and volume decrease (B) during differentiation in the presence or absence of 3% human serum (HS) was monitored daily by an electronic cell counter. (C) Quantitation of hemoglobin accumulation (for photometric assay, see Materials and Methods); 500 A.U. correspond to 11.0 pg of hemoglobin). Error bars in (A), (B) and (C) represent the S.D. of mean, n=5. (D) Kinetics of changes in cell size distribution during differentiation of human erythroblasts without serum (upper panel) and with HS (lower panel). (E) Cells before (top) and after differentiation for 6 days in the absence (middle panel) or presence of HS (bottom panel) were cytocentrifuged onto slides and stained with neutral benzidine (to detect hemoglobin; brownish stain) plus histological dyes (see Material and Methods). (F) Expression of erythroid cell surface markers in proliferating and differentiating erythroblasts (left panel, labeled “control”: differentiation without serum; right panel, differentiation in the presence of 3% HS). Proliferating cells or erythroblasts induced to differentiate for 6 days were washed with PBS/1%FCS, stained with fluorescently labeled antibodies against c-Kit (CD117), transferrin receptor (CD71) and glycophorin A (GPA) and subjected to FACS analysis. Orange graphs, FACS profiles of self-renewing erythroblasts; red graphs, cell surface antigen expression in differentiating cells; light gray histograms: background fluorescence.
Figure 7. Improved differentiation of human erythroblasts in the presence of thyroid hormone. Human erythroblasts were induced to differentiate either in the presence or absence of T3 (1µM). (A) Cells were analyzed by cytocentrifugation plus histochemical staining (see Figure 6). Enucleated cells observed at day 6 of differentiation in the presence of T3 are indicated by arrows. Proliferation rates (B) and volume decrease (C) during differentiation were determined daily (see Figure 6) using an electronic cell counter. (D) Quantitation of hemoglobin accumulation (see Material and Methods; 500 A.U. correspond to 11.0 pg of hemoglobin). Error bars in (B), (C), and (D) represent the S.D. of mean, n=5. (E) Changes in cell size distribution during differentiation plus T3 (compare to Figure 6D).
Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors

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