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Steroid-resistance in Diamond Blackfan anemia associates with p57<sup>Kip2</sup> dysregulation in erythroid progenitors

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Abstract:

Despite the effective clinical use of steroids for the treatment of Diamond Blackfan anemia (DBA), the mechanistic bases via which glucocorticoids regulate human erythropoiesis remain poorly understood. Here, we report that the sensitivity of erythroid differentiation to dexamethasone is dependent on the developmental origin of human CD34+ progenitor cells, specifically increasing the expansion of CD34+ progenitors from peripheral blood (PB) but not cord blood (CB). Dexamethasone treatment of erythroid-differentiated PB, but not CB, CD34+ progenitors resulted in the expansion of a newly defined CD34+CD36+CD71hi/CD105med immature colony-forming unit-erythroid (CFU-E) population. Furthermore, proteomics analyses revealed the induction of distinct proteins in dexamethasone-treated PB and CB erythroid progenitors. Dexamethasone treatment of PB progenitors resulted in the specific upregulation of p57Kip2, a Cip/Kip cyclin-dependent kinase inhibitor, and we identified this induction as critical; shRNA-mediated downregulation of p57Kip2, but not the related p27Kip1, significantly attenuated the impact of dexamethasone on erythroid differentiation and inhibited the expansion of the immature CFU-E subset. Notably, in the context of DBA, we found that steroid resistance was associated with a dysregulated p57Kip2 expression. Altogether, these data identify a unique glucocorticoid-responsive human erythroid progenitor and provide new insights into glucocorticoid-based therapeutic strategies for the treatment of patients with DBA.
Introduction:

Diamond Blackfan anemia (DBA) is an inherited bone marrow failure syndrome with an incidence of 5-10 cases/million live births, characterized by red cell aplasia, a range of physical anomalies, developmental bone defects and cancer predisposition (1, 2). More than 70% of the patients diagnosed with DBA have defects in ribosome biogenesis due to mutations in genes encoding ribosomal proteins. In addition, mutations in the GATA1 transcription factor, a key regulator of erythroid development, and TSR2, a pre-rRNA processing protein, have recently been identified in a few families with DBA (3, 4). The genetic landscape of DBA is heterogeneous but genotype/phenotype correlations have been noted in association with mutations in the RPL5 and RPL11 ribosomal proteins in patients (5).

The standard of care for patients with DBA after the first year of age is glucocorticoids. Notably, a majority of treated patients have an increase in red cell production and exhibit a reduced dependency on blood transfusion (6-8). However, the therapeutic dose is extremely variable between patients and many patients become refractory to treatment over time. Once patients are glucocorticoid-refractory they become dependent on chronic red blood cell transfusions unless they enter remission or undergo a curative hematopoietic stem cell transplant (7). The actions of glucocorticoids have been well studied in many disease contexts. These molecules interact with the glucocorticoid receptor (GR), resulting in the nuclear translocation of the resulting complex which binds DNA at glucocorticoid response elements (GREs) and ultimately activates gene transcription (9). However, the specific mechanisms of action of glucocorticoids in the erythroid system in both healthy individuals and patients with DBA still remain to be fully elucidated. Several studies have demonstrated that glucocorticoids act at the erythroid progenitor level but the precise stages of erythroid differentiation at which they exert their effects have not been identified (10-13). This is in part due to the considerable heterogeneity of erythroid progenitor populations and the different markers and model systems that are used for
studying glucocorticoid effects on erythropoiesis. Indeed, early burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) progenitors are often characterized based on their functional ability to form erythroid colonies in colony forming assays (CFAs) and immunophenotypic evaluations (14-18) are still progressing.

A further difficulty in evaluating the impact of glucocorticoids on BFU-E and CFU-E progenitors is due to important differences in murine versus human erythroid differentiation. In mice, the vast majority of research has focused on fetal liver progenitors, with a detailed characterization of early and late populations of fetal liver BFU-ES (19-21). While several studies performed on murine fetal liver cells have suggested that dexamethasone acts at the BFU-E stage (11, 13, 19), a more recent study has reported that dexamethasone enhances the maintenance of proliferative murine CFU-E by upregulating p57Kip2, a member of the Cip/Kip cyclin-dependent kinase inhibitor protein family (10). Under these conditions, the proliferative CFU-E population is maintained and there is a delayed differentiation to the less proliferative proerythroblast (Pro-EB) stage. However, it is not clear as to whether these data in fetal murine erythroid progenitors translate to humans, as regards both the potential ex vivo heterogeneity of BFU-E and CFU-E populations and more importantly, the diversity of erythroid progenitor subpopulations in human bone marrow. These limitations have made it challenging to elucidate the mechanisms via which glucocorticoids act at different stages of erythroid progenitor development, both under physiological and pathological conditions.

In the present study, we identified significant differences in the potential of dexamethasone to impact on physiological as well as disordered human erythropoiesis. In the context of physiological differentiation, we found that although it affected terminal erythropoiesis independently of the source, dexamethasone impacted the expansion of CD34+ hematopoietic stem and progenitor cells (HSPCs) isolated from adult peripheral blood (PB) but not from cord blood (CB). Dexamethasone did not affect BFU-E, but using a new set of cell surface markers, we identified a unique transitional CD34+CD36+CD71hiCD105med CFU-E subset generated from
PB progenitors as dexamethasone-responsive. Mass spectrometry-based quantitative proteomics analyses revealed substantial differences in the effects of dexamethasone on PB and CB progenitors with an upregulation of Nuclear Receptor Subfamily 4 Group A Member 1 (NR4A1), a negative cell cycle regulator (22), in the former. Furthermore, we found that the p57<sup>Kip2</sup> Cip/Kip cyclin-dependent kinase inhibitor was specifically upregulated by dexamethasone in PB-derived CFU-E and that its downregulation significantly attenuated the effects of this glucocorticoid. Even more notably, p57<sup>Kip2</sup> was not upregulated by dexamethasone in CFU-E isolated from steroid-resistant patients with DBA. These findings open new avenues for the development of specific therapeutic strategies for these patients.
Results:

**Dexamethasone increases the proliferation of adult human CFU-Es**

It has previously been shown that dexamethasone increases the proliferation of erythroid progenitors derived from both healthy and DBA HSPCs (23-25). However, the differentiation stage at which dexamethasone exerts its effects is still unclear. Using a serum-free expansion media that allows for the effective erythroid differentiation of HSPCs under steady state conditions (without dexamethasone), we studied the effects of dexamethasone on human erythropoiesis (18, 26). Notably, we found that dexamethasone (100nM) increased the expansion of CD34+ cells isolated from adult PB by 7-fold as compared to control conditions (**Figure 1A**). Surprisingly though, we did not detect a dexamethasone-mediated increase in the expansion of CB-derived CD34+ cells. In fact, total numbers of precursors differentiating from CB CD34+ cells were approximately 4-fold lower in the presence as compared to the absence of dexamethasone (**Figure 1A**), strongly suggesting marked developmental differences in the responsiveness of human PB and CB progenitors to this glucocorticoid.

To further elucidate the erythroid differentiation stage at which dexamethasone acts, we used our recently developed experimental strategy for the characterization of BFU-E and CFU-E erythroid progenitors, based on the surface expression levels of GPA, IL3R, CD34, and CD36. The absolute numbers of BFU-E and CFU-E generated from $10^6$ PB and CB CD34+ cells were enumerated on the basis of cell surface marker expression in the absence and presence of dexamethasone (**Figure 1B**). Starting at day 7, the number of BFU-E (defined as GPA- IL3R- CD34+ CD36-) cells decreased in cultures starting with PB as well as CB CD34+ progenitors, both in the absence and presence of dexamethasone and the extent of this decrease was enhanced by dexamethasone. In marked contrast, the absolute number of CFU-E (defined as GPA- IL3R- CD34- CD36+) increased over time and this increase was more pronounced in dexamethasone-treated adult PB CD34+ cells. Notably, dexamethasone decreased the appearance of terminally
differentiated GPA+ cells in both PB- and CB-derived cultures. These data indicate that erythroblasts from both sources were responsive to this glucocorticoid, resulting in a delayed transition to terminal erythroid differentiation (Supplemental Figure 1A and B). However, cells derived from PB are more affected, possibly due to additional effects both at the progenitor levels and at this transitional stage. This decrease in terminal erythroid differentiation was also detected as a function of α4-integrin/Band 3 profiles as well as reduced hemoglobinization (Supplemental Figure 1A).

To further validate that the CFU-E is the population primarily responding to dexamethasone, we sorted CFU-E derived from peripheral and cord blood cultures of CD34+ cells and studied their potential for expansion in serum-free expansion media. As shown in Figure 1C, the expansion of CFU-E purified from adult CD34+ cells increased by 5-fold in the presence of dexamethasone. Once again, dexamethasone had little to no effect on the proliferation of purified CFU-E derived from cord blood, supporting the idea that the response to dexamethasone was linked to the specific developmental stage of erythroid progenitors. We performed the same experiments using increasing concentrations of dexamethasone on sorted populations of erythroid progenitors derived from adult PB cultures and observed that the effect on proliferation was maximal at 100nM (Supplemental Figure 2).

Functional assays using methylcellulose cultures with erythropoietin (Epo) only, a culture condition which supports the growth of CFU-E but not BFU-E (requiring both stem cell factor (SCF) and Epo (18)), revealed that treatment with dexamethasone markedly increased the colony size of PB-derived CFU-E but had very little or no effect on CB-derived CFU-E colony size (Figure 1D, Supplemental Figure 3), in spite of the fact that in the absence of dexamethasone the colony size of purified CB-derived CFU-E was larger than that generated by PB-derived CFU-E. Taken together, these data imply that only human CFU-E derived from adult PB respond to dexamethasone by increasing proliferation.
Previous studies have shown that it takes at least two weeks in vivo for the normal bone marrow to produce reticulocytes from the BFU-E stage and about 7 to 10 days from the CFU-E stage (27-29). We hypothesized that if CFU-E is the progenitor population that responds to glucocorticoids in vivo, then patients treated with steroids should present with reticulocytosis in less than two weeks. We followed patients with DBA over a one-month period before and after treatment with prednisone. We observed that in each patient the reticulocyte count increased within seven to eleven days after initiation of the treatment strongly suggesting that in vivo, the CFU-E is indeed the population responsive to glucocorticoids (Figure 1E). We further noticed a decline in the reticulocyte response in a similar time frame, in association with a decrease in the dose of prednisone administered to the patient. Our finding that patients with DBA exhibit a reticulocytosis within less than 2 weeks of starting prednisone treatment strongly suggests the presence of a mature steroid-responsive progenitor, such as a CFU-E.

**Dexamethasone targets a subpopulation of adult-derived CFU-Es**

We previously described that based on surface expression of CD34 and CD36 we can obtain highly enriched population of BFU-E (GPA⁺ IL3R⁻ CD34⁺ CD36⁻) and CFU-E (GPA⁻ IL3R⁺ CD34⁻ CD36⁺) (18). More recently we reported a transitional progenitor population defined as GPA⁻ IL3R⁻ CD34⁺ CD36⁺ which is more predominant during differentiation of adult PB than that of CB (30). Notably, the kinetics of progression through these differentiation states were also altered with dexamethasone treatment (Figure 2A).

Having identified two phenotypically different CFU-E populations (CD34⁺ CD36⁻ and CD34⁻ CD36⁺) we sought to determine if we can identify additional cell surface markers that would provide further insights into the heterogeneity of these CFU-E cell populations. We focused on CD71, the transferrin receptor, and CD105, endoglin, which both demonstrated large differences in their RNA expression during erythroid differentiation at the progenitor stages (Supplemental Figure 4A).
Based on the expression patterns of CD71 and CD105 on CD34+ CD36+ cells we identified a continuum of cells with two distinct populations – CD71\textsuperscript{hi} CD105\textsuperscript{med} and CD71\textsuperscript{hi} CD105\textsuperscript{hi} (Figure 2B). We then sorted these different cell populations and performed colony forming assays in the presence of either Epo alone, to produce colonies with the traditional definition of CFU-E, or in complete medium with SCF, Epo, IL3, IL6, G-CSF and GM-CSF. Interestingly, while both sorted cell populations from CD34+ CD36+ generated colonies in the presence of Epo only (Figure 2C, left panel), only CD71\textsuperscript{hi} CD105\textsuperscript{med} showed marked responsiveness to SCF in complete medium, while SCF had little or no effect on CD71\textsuperscript{hi} CD105\textsuperscript{hi} cells (Figure 2C, right panel). CD71\textsuperscript{hi} CD105\textsuperscript{hi} cells from CD34+ CD36+ populations responded similarly to CD34− CD36+ populations with a minimal response to SCF. Based on these findings we propose that CD71\textsuperscript{hi} CD105\textsuperscript{med} be termed as immature CFU-E and CD71\textsuperscript{hi} CD105\textsuperscript{hi} as mature CFU-E. Importantly, immature CFU-E cells functionally responded to dexamethasone by increasing their colony size in methylcellulose culture system (Figure 2D). In marked contrast, mature CFU-E responded marginally to dexamethasone in the same functional colony-forming assays and this increase was not statistically significant. When treated with dexamethasone, PB-derived CD34+ cells preferentially maintained this immature CFU-E population. Indeed, while both untreated and treated cells expressed comparable levels of CD71 on their surface, ~ 50% of the PB cells treated with dexamethasone were still CD105\textsuperscript{med} in comparison to untreated controls, ~ 30% of which were CD105\textsuperscript{med} at day 4. (Figure 2E-F, left panel). Consequentially, the population of CD105\textsuperscript{hi} cells was decreased in PB cells treated with dexamethasone (Figure 2F, right panel). Taken together, these data demonstrate that in human erythropoiesis, dexamethasone treatment preferentially maintains the immature CFU-E progenitor population for an extended period of time to increase its proliferative capacity.

Proteomic studies highlight previously unidentified erythroid dexamethasone targets
To begin to explore the mechanisms regulating the differential impact of dexamethasone on PB- and CB-derived progenitors, we elected to use a global, comparative proteomics approach. PB- and CB- derived CD34+ cells were cultured in vitro for 5 days, allowing the acquisition of sufficient flow-sorted purified CFU-Es, for proteomics evaluation (~20x10^6). Biological triplicates of PB- and CB-derived CFU-E were treated in the absence or presence of dexamethasone (100nM) for 24 hours. These cells were then processed for proteomic analysis by liquid chromatography-tandem mass spectrometry. Proteomics quantified 10,045 proteins in PB samples and 10,028 proteins in CB samples (Supplemental Table 1). Proteomic evaluation of PB and CB samples were analyzed in two separate TMT-6 plexes. This allows for precise measurements of changes in protein abundance due to drug treatment in the PB-plex and in the CB-plex, but less precise comparisons between PB and CB plexes. Therefore, the observed differences between PB and CB samples were subsequently validated in follow-up experiments.

Importantly, both PB- and CB-derived CFU-E responded to dexamethasone as demonstrated by the upregulation of the gene for Period 1 (PER1; Figure 3A, B), a major component of the mammalian circadian clock which has long been known to be regulated by glucocorticoids (31, 32). Notably though, the majority of dexamethasone-upregulated proteins in PB- and CB-derived CFU-E differed (Figure 3C, D), consistent with the distinct phenotypic responses of these CFU-E. One of the proteins whose abundance was upregulated by dexamethasone in PB-derived CFU-E but not in CB-derived CFU-E was Nuclear Receptor Subfamily 4 Group A Member 1 (NR4A1, Figure 3C, D), a negative cell cycle regulator. NR4A1 is an interesting target due to its role as a cell cycle regulator (22) and a regulator of T cell differentiation (33). NR4A1 also binds to HIF1alpha (34), an important regulator of erythroid differentiation (35). Identification of NR4A1 as target of dexamethasone was confirmed by Western blot (Figure 3E). Interestingly, the transcription profiles of NR4A1 as well as two other cell cycle inhibitors, p27^Kip1 and p57^Kip2, exhibited a substantial decrease between the CD34+ and BFU-E stages (Supplemental Figure 4B). While p57^Kip2 was not detected in the proteomics
screen, it is notable that only p57<sup>Kip2</sup> was expressed at significantly higher levels in PB than CB CD34<sup>+</sup> progenitors (Supplemental Figure 4B). A single sample gene set enrichment analysis (ssGSEA) was also performed to identify the pathways most differentially affected by dexamethasone treatment in both PB and CB samples (Figure 3F). Notably, we observed an increase in proteins involved in the cell cycle and RNA processing as well as a decrease in proteins implicated in oxidative phosphorylation and biosynthetic pathways. Together, these data point to potential differences in the roles of cell cycle inhibitors in CD34<sup>+</sup> progenitors as a function of their developmental origin.

Dexamethasone increases p57<sup>Kip2</sup> expression in human CFU-Es

The data presented above together with an elegant previous study showing that p57<sup>Kip2</sup> regulates steroid responsiveness in murine erythroid progenitors (10), suggested that dexamethasone would act through p57<sup>Kip2</sup> in PB-derived erythroid progenitors. We first evaluated the expression levels of p57<sup>Kip2</sup> protein as a function of erythroid differentiation from PB and CB CD34<sup>+</sup> cells (Figure 4A). While p57<sup>Kip2</sup> was expressed in early erythroid progenitors derived from both PB and CB CD34<sup>+</sup> cells (unsorted day 4), the loss of p57<sup>Kip2</sup> was significantly more rapid in the former; p57<sup>Kip2</sup> levels in PB erythroid progenitors were reduced by 77% by day 7 whereas expression persisted in CB progenitors (Figure 4A, B). Conversely, p27<sup>Kip1</sup>, a related Cip/Kip family member, was expressed at minimal levels during early differentiation but increased dramatically in terminally differentiating erythroblasts (Figure 4A). These distinct expression profiles are in agreement with previous studies showing that p57<sup>Kip2</sup> is associated with the quiescence of stem and progenitor cells while p27<sup>Kip1</sup> plays a role in the cell cycle exit that occurs during terminal erythroid differentiation (36, 37). Notably though, differences in the kinetics of p57<sup>Kip2</sup> downregulation in CB and PB erythroid progenitors have not, thus far, been appreciated.

Based on these findings, it was of interest to assess whether p57<sup>Kip2</sup> levels would be differentially impacted by dexamethasone in purified PB-derived and CB-derived progenitors. To
this end, progenitors derived from both sources were treated with dexamethasone and the expression of \(p57^{Kip2}\) was quantified by Western blot. Total CFU-E were evaluated as the numbers of immature CFU-E were insufficient in the absence of dexamethasone, particularly in CB cultures where the kinetics of differentiation is increased (30). Notably, within CFU-E, \(p57^{Kip2}\) protein levels were increased by 1.8-fold in PB-derived progenitors but remained unchanged in CB-derived progenitors (\(p<0.05\); Figure 4C, D). These data are even more striking in light of the finding that \(p27^{Kip1}\) levels were not significantly altered in progenitors derived from either PB or CB sources (Figure 4C, D). Altogether, these data strongly suggest a role for \(p57^{Kip2}\) in mediating the dexamethasone-induced changes in PB-derived but not CB-derived progenitors.

As \(p57^{Kip2}\) is a regulator of the cell cycle, by inhibiting the transition from G1 to S phase (38), we evaluated whether dexamethasone-mediated changes in \(p57^{Kip2}\) were associated with differences in the cell cycle dynamics of PB erythroid progenitors. The percentages of progenitors in the S phase of the cell cycle increased significantly between the BFU-E and CFU-E stages, from a mean of 35% to 48% (\(p<0.05\), Figure 4E). Notably though, dexamethasone significantly decreased S phase cells in immature CFU-E, but not in the BFU-E or mature CFU-E subsets (Figure 4E, Supplemental Figure 5). In addition, the percentages of cells in the non-S phases of the cell cycle population were increased in immature CFU-E (Supplemental Figure 5B). Thus, in agreement with our data showing that the immature PB-derived CFU-E subset preferentially undergoes a dexamethasone-mediated expansion (Figure 2), only this subset responded to upregulated \(p57^{Kip2}\) levels with a significant decrease in S phase population. Despite this reduction of cells in S phase, immature CFU-E continued to divide, with increases in G2/M, before further differentiating into mature CFU-E and then proerythroblasts, resulting in increased expansion.

\(p57^{Kip2}\) expression is altered in erythroid progenitors from transfusion-dependent patients with DBA
We further hypothesized that the resistance of patients with DBA to glucocorticoids is mediated, at least in part, by p57^Kip2. To test this hypothesis, we compared dexamethasone-induced changes in p57^Kip2 levels in unsorted cells at day 7 of culture in response to dexamethasone in CD34^+ cells from healthy controls and transfusion-dependent patients with DBA. p57^Kip2 levels were evaluated in unsorted progenitors at day 7 of expansion due to the strongly reduced growth of cells from transfusion-dependent patients with DBA (Figure 5A). Importantly, p57^Kip2 levels, increased in healthy controls and steroid-responsive patients with DBA in response to dexamethasone, were not affected in transfusion-dependent patients with DBA (Figure 5B, C). As expected from the data presented in Figure 4C, p27^Kip1 levels were not impacted by dexamethasone but it is notable that they were dysregulated and pointedly higher or lower in samples from patients with DBA, likely due to early differentiation or defective terminal erythroid differentiation respectively in these unsorted cells. Most critically, p57^Kip2 levels in progenitors derived from steroid-responsive patients with DBA were upregulated in response to dexamethasone similar to healthy controls (Figure 5B, C). Notably, the expansion of PB-derived CD34^+ cells from healthy controls and steroid-responsive patients with DBA were similar after 7 days of culture, while cells from transfusion-dependent patients with DBA demonstrated significantly less expansion (Figure 5A). Taken together, these results suggest a critical role for p57^Kip2-associated cell cycle changes in the steroid responsiveness of both physiological and pathological human erythropoiesis.

Dexamethasone responsiveness is mediated by CDK activity

In order to directly assess the role of p57^Kip2 in mediating dexamethasone effects on erythroid progenitors, we downregulated p57^Kip2 levels through a lentiviral-mediated shRNA approach. Following transduction, p57^Kip2 was downregulated by 80% as compared to cells transduced with a control luciferase targeting shRNA construct (Figure 6A). Notably, p57^Kip2 downregulation abrogated the ability of PB-derived CD34^+ cells to respond to dexamethasone,
monitored as a function of their expansion (Figure 6B). Moreover, erythroid differentiation was accelerated, exhibited by 1.5- and 3-fold increases in the level of glycophorin A expression in control and dexamethasone-treated progenitors, respectively (Figure 6C). Finally, this effect on erythroid progenitors was specific to the p57Kip2 cyclin-dependent kinase inhibitor as downregulation of p27Kip1 did not alter their expansion (Supplemental Figure 6A, B). The effect on p27Kip1 was only noticed at later stages as indicated by a delay in terminal differentiation measured by the surface markers α4-integrin and Band3 (Supplemental Figure 6B). Altogether, these data reveal the function of p57Kip2 in regulating the balance between human erythroid progenitor proliferation and differentiation and furthermore, in controlling glucocorticoid responsiveness under both physiological and pathological conditions.

Based on our finding that augmented levels of p57Kip2, a CKI, is critical for dexamethasone responsiveness, we hypothesized that inhibiting cyclin kinases would have the same effect as increasing p57Kip2. Notably, treatment of PB-derived CD34+ cells with olomoucine (1 μM), a small molecule CDK1 and CDK2 inhibition (39), resulted in a significant increase in the ratio of immature CFU-E to mature CFU-E, to levels similar to those induced by dexamethasone (Figure 6D). Together, these data demonstrate the importance of the CDK/CKI balance in the expansion of the immature CFU-E population.

Dexamethasone upregulates p57Kip2 expression in erythroid progenitors derived from human bone marrow

To assess the effects of dexamethasone on populations of cells that are directly targeted by the drug in vivo, we repeated key experiments with erythroid progenitors derived from human bone marrow (BM). In culture with serum-free expansion media, we observed that the expansion of BM-derived CD34+ cells increases significantly in the presence of dexamethasone (Figure 7A). When examining the expression of CD105 in BM-derived progenitors, we observed that
dexamethasone treatment maintained the population of CD105\textsuperscript{med} cells and correspondingly decreased the population of CD105\textsuperscript{hi} cells (Figure 7B). Furthermore, treatment of BM-derived CFU-E with dexamethasone led to an increase in p57\textsuperscript{Kip2} expression that was not observed in CB-derived CFU-E treated with dexamethasone (Figure 7C). Overall, these data indicate that PB- and BM-derived erythroid progenitors respond to dexamethasone in a similar manner and thus the phenotypes we observed may be relevant in the clinical use of dexamethasone in the treatment of red cell disorders.
**Discussion:**

The present study, focused on identifying the role of dexamethasone during physiological and pathological human erythropoiesis, has generated several unique insights. Our finding that dexamethasone markedly enhanced the erythroid proliferation of CD34\(^+\) cells from adult PB but not from CB was unexpected and very surprising. In this context, it is of interest that the transition trajectories from BFU-E to CFU-E differs considerably following erythroid differentiation of CD34\(^+\) cells derived from adult PB and CB (30). Together, these data strongly suggest that dexamethasone has differential effects on transitional erythroid progenitor populations that are regulated by the source of the CD34\(^+\) progenitors. Importantly, these findings are relevant to bone marrow populations as we found that CFU-E from BM-derived CD34\(^+\) cells exhibit dexamethasone-induced responses that are similar to those detected in PB-derived CFU-E (Figure 7).

While neither BFU-E derived from human PB nor CB were responsive to dexamethasone, BFU-Es from murine fetal liver have been shown to be dexamethasone responsive (19). Murine CFU-E have also been shown to be specifically dexamethasone responsive (10) suggesting that dexamethasone-dependent erythroid developmental stages may be species-dependent. Collectively, these previous studies (10, 19) together with the present work point to important differences between murine and human systems and moreover, demonstrate the importance of developmental origin of hematopoietic stem and progenitor cells in regulating their responsiveness to glucocorticoids during erythropoiesis. These developmental differences as well as steroid resistance in DBA may be mediated by epigenetic regulators (40-43) and further investigations of the mechanisms may offer additional insights into the heterogeneity of progenitor cell populations. Finally, our data show the importance of the p57\(^{\text{Kip2}}\) cell cycle inhibitor in mediating dexamethasone effects during human erythroid differentiation and reveal the critical nature of the p57\(^{\text{Kip2}}\) axis in the dexamethasone responsiveness of patients with DBA.
The marked heterogeneity of human erythroid progenitors has long been recognized (44, 45). Indeed, it has been known for decades that erythroid progenitors give rise to colonies of different sizes and morphology in the methylcellulose culture system which led to the concept of large, intermediate and small BFU-E colonies with the small BFU-E forming cells further differentiate into CFU-E (46). The recent progress of single cell technologies has enabled stringent analyses of these varied populations, in terms of both steroid sensitivity and cell fate decisions (12, 47). While we previously found that human BFU-E and CFU-E can be immunophenotypically defined on the basis of IL3R, GPA, CD34 and CD36 cell surface markers, this characterization did not allow us to fully resolve the heterogeneity of human erythroid progenitors. In the present study, we found that the CD71 and CD105 markers allow for further immunophenotyping, discriminating sub-populations of erythroid BFU-E and CFU-E. Using this method, we identified a new transitional human progenitor CFU-E population, resulting in the designation of immature and mature CFU-Es.

Using this optimized immuno-phenotyping assay, we identified the immature CFU-E population as the steroid-responsive erythroid progenitor subset, as assessed by both proliferation and a decrease in the percentages of cells in the S phase of the cell cycle. This cell cycle phenotype was due to a dexamethasone-mediated increase in p57\textsuperscript{kip2} and under these conditions, differentiation was delayed resulting in a paradoxical expansion of this progenitor subset. We propose that this occurs through the maintenance of the immature CFU-E population, generating larger numbers of mature CFU-E.

The critical role of p57\textsuperscript{kip2} in CFU-E was shown by the shRNA knockdown of p57\textsuperscript{kip2} which resulted in an “opposite” phenotype; erythroid differentiation was accelerated, and this was associated with a decreased level of proliferation. Together with recent work that identifies a role for cell cycle status in cell fate decisions (48), the ensemble of these data demonstrates a clear link between cell cycle progression and differentiation of erythroid progenitors.
Importantly, erythroid progenitors from transfusion-dependent patients with DBA harbor altered levels of p57Kip2 and expression was not sensitive to dexamethasone treatment. Indeed, the absence of changes in p57Kip2 levels in transfusion-dependent patients is a proof of concept for the importance of this cell cycle regulator in the response to steroids. Notably though, there is substantial variability in the phenotype of progenitors from transfusion-dependent DBA patients that is likely mediated by other mechanisms. In the future, it will be important to determine whether steroid-responsiveness in DBA patients is regulated at the level of a specific subset(s) of erythroid progenitors and furthermore, to assess the role of p57Kip2 levels in these subsets.

Our proteomics data revealed previously unidentified dexamethasone targets that may offer additional insights into how dexamethasone influences the cell cycle and increases erythroid proliferation. Of the identified targets, many have been shown to be involved in cell cycle regulation including PER1 and NR4A1, indicating that these proteins may function to negatively regulate cell cycle regulation in association with p57Kip2 (31, 49). In this regard, it is of interest that NR4A1 mediates TGF-β signaling which is also aberrant in DBA and thus, could be involved in corticosteroid resistance mechanisms (50). Our finding that dexamethasone globally regulates RNA processing and biosynthetic pathways may also open additional avenues for investigation on the effects of dexamethasone on erythroid progenitors.

The present findings have direct relevance for the clinical use of corticosteroids for the treatment of hypo-proliferative anemias, most notably DBA. While corticosteroid use stably increases red cell mass and induces hematopoietic remission or marked improvement in many patients with DBA, other patients are not responsive or cannot continue glucocorticoid treatment because of adverse side effects. We anticipate that our new insights into the mechanism of action of glucocorticoids on human erythropoiesis, and specifically on the immature CFU-E subset, will promote the development of new targeted drugs and treatment strategies, inducing a sustained effect on erythroid progenitors in more patients with fewer side effects. In summary, we have identified a new transitional CFU-E subset, between late BFU-E and mature CFU-E stages, which
is responsive to dexamethasone. These findings contribute to our understanding of erythroid progenitor biology and the development of new treatment strategies for erythroid disorders.
Materials and Methods:

Human studies

CD34\(^+\) cells were obtained from deidentified control adult peripheral blood leukoreduction filters, deidentified cord blood units, deidentified bone marrow samples or phlebotomized patients with DBA after informed consent was obtained in writing and prior inclusion in the study. In order to limit sample variability, blood from multiple control peripheral blood or cord blood donors were pooled.

Patients with DBA were defined as transfusion-dependent or steroid-responsive based on their clinical need for chronic red blood cells transfusion or successful management with corticosteroids, respectively.

The three patients with DBA presented in Figure 1E were patients followed after diagnosis and initial treatment with prednisone. The dose of prednisone was decreased after initial response.

Isolation and culture of CD34\(^+\) cells

Mononuclear cells from peripheral blood, cord blood, or bone marrow were separated using Lymphoprep (Stem Cell Technologies) and CD34\(^+\) cells were purified with anti-CD34 microbeads and MACS Columns (Miltenyi Biotec) using manufacturer protocol. CD34\(^+\) cells were cultured at a density of \(10^5\) cells/mL in a serum free expansion media as previously described (26), with a base media of StemSpan SFEM (Stem Cell Technologies) initially supplemented with 100 ng/mL stem cell factor (SCF), 10 ng/mL interleukin 3 (IL3), 0.5 U/mL erythropoietin (Epo), 4 μL/mL lipid mixture 1 (Sigma Aldrich), 2 mM L-glutamine and 200 μg/mL transferrin. Beginning at day 7, the dose of Epo was increased to 3 U/mL and at day 11 the dose of transferrin was
increased to 1 mg/mL. Dexamethasone (Sigma) was added to cultures at 100 nM as indicated as this dose was found to be optimal (Supplemental Figure 2). Olomoucine (Sigma) was added at 1 μM as indicated.

Flow cytometry and cell sorting

Erythroid progenitors were examined using surface markers as previously described (18). Erythroid progenitors were analyzed at day 4 of differentiation where 10⁵ cells were stained with an antibody cocktail containing anti-IL3R PE-Cy7, anti-glycophorin A (GPA) PE, anti-CD34 FITC, anti-CD36 APC, anti-CD71 Alexa Fluor 700, and anti-CD105 Brilliant Violet 421 (BD Biosciences, 560826, 555570, 555821, 550956, 560566, and 563920 respectively) for 15 minutes at room temperature. BFU-E were defined as GPA⁻ IL3R⁻ CD34⁺ CD36⁻, CFU-E as GPA⁻ IL3R⁻ CD34⁻ CD36⁻, and transitional progenitors as GPA⁻ IL3R⁻ CD34⁺ CD36⁺. Dead cells were excluded from further analysis with 7-aminoactinomycin D (BD Biosciences) staining. Analysis was performed using a BD Fortessa flow cytometer with FCS Express 6 and FlowJo 10. BFU-E, CFU-E and transitional progenitors were sorted for downstream experiments using a BD FACSAlra at a low pressure with a 100 μm nozzle. Fluorescence minus one, and isotype controls were used to define the different populations of progenitor cells and resolve the continuum. Functional assays using sorted cells in colony forming assays were used to further confirm identity and purity of these different populations.

Colony forming assays

Sorted erythroid progenitors were seeded to methylcellulose media H4230 (Stem Cell Technologies) supplemented with Epo alone at 0.5 U/mL or complete methylcellulose media
H4435 (Stem Cell Technologies) at 200 cells/mL. BFU-E and CFU-E colonies were counted and measured at day 14 and day 7 of culture respectively. Area of colonies was determined by modeling each colony as an ellipse and measuring its major axis $a$ and minor axis $b$ to calculate area by the formula $A = \pi ab/4$. Dexamethasone was added to cultures as indicated at 100 nM.

**Cell cycle staining**

For live cell cycle staining for examining cell cycle profile, erythroid progenitors were incubated with 5 mg/mL Hoechst 33342 for 3 hours at 37°C prior to staining with antibody cocktail for surface markers.

**Western blot**

Cells were lysed in RIPA Lysis and Extraction buffer (Thermo Fisher Scientific) with 1:100 protease inhibitor cocktail (Sigma Aldrich) on ice for 10 minutes and then centrifuged at max speed for 10 minutes. Supernatants were mixed at 1:1 (vol/vol) with 2X Laemmli Sample Buffer (Biorad) with 0.1M dithiothreitol (DTT) and boiled for 5 minutes. Samples were then separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 1.5 hours at 150V and transferred to nitrocellulose membranes for 1 hour at 95V. Membranes were then blocked with 4% (wt/vol) milk powder and 1% (wt/vol) bovine serum albumin (BSA) in 0.1% Tween 20 (vol/vol) phosphate buffered saline (PBST) for 3 hours. Membranes were then incubated with the listed primary antibodies overnight at 4°C, p57$^{kip2}$ (BD Biosciences, 556346), p27$^{kip1}$ (BD Biosciences, 610241), NR4A1 (BD Biosciences, 554088), GAPDH (Millipore, CB1001) and α-globin (Santa Cruz Biotechnologies, sc-514378). Membranes were washed 5 time for 5 minutes with PBST and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Biorad) for 2 hours at room temperature. Membranes were imaged with Pierce ECL Western
Blotting Substrate (Thermo Fisher Scientific) using a ChemiDoc MP Imaging System (Biorad). Western blot images are representative of multiple experiments and were quantified with ImageJ (NIH).

**Lentiviral transduction**

\(p57^{Kip2}\) and \(p27^{Kip1}\) knockdown experiments were each carried out with 2 lentiviral shRNA knockdown constructs targeting CDKN1C and CDKN1B respectively (Clone ID: NM_000076.2-1451s21c1 and NM_000076.2-1216s21c1 for CDKN1C and NM_004064.3-841s21c1 and NM_004064.3-643s21c1 for CDKN1B, Sigma Aldrich). shRNA knockdown constructs targeting luciferase were used as controls. After 2 days of culture in serum free expansion media, erythroid progenitors were placed in 10% FBS IMDM with 3 U/mL heparin and 8 μg/mL polybrene. Lentiviral particles were then added at a multiplicity of infection (MOI) of 30 and spinoculated for 2 hours at 3000 rpm. Cells were then incubated overnight and placed in serum free expansion media. After 24 hour recovery, lentiviral transduction was positively selected with 1 μg/mL puromycin which was maintained until day 11 of culture.

**Proteomic profiling**

*In-Solution Digestion*

CD34+ cell pellets were lysed for 30 min at 4 °C in 8M urea, 50 mM Tris-HCl pH 8.0, 75 mM NaCl, 1 mM EDTA, 2 μg/μl aprotinin (Sigma-Aldrich), 10 μg/μl leupeptin (Roche), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). Lysates were cleared via centrifugation at 20,000 rcf, and protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce). Remaining lysis buffer was used to equalize sample concentration to the lowest measured concentration before proceeding. Protein reduction was performed with 5 mM
dithiothreitol (DTT) for 1 h at room temperature, followed by alkylation with 10 mM iodoacetamide for 45 min at room temperature in the dark. Sample volumes were then adjusted with 50 mM Tris-HCl pH 8.0 to reduce urea concentration to 2 M preceding enzymatic digestion. Proteins were digested first with endoproteinase LysC (Wako Laboratories) for 2 h at 25 °C, then overnight with sequencing-grade trypsin (Promega) at 25 °C, both at enzyme-to-substrate ratios of 1:50. Following digestion, samples were acidified to a concentration of 1% with neat formic acid, and insoluble peptides and urea was removed via centrifugation at 20,000 rcf. Remaining soluble peptides were desalted using a 100 mg reverse phase tC18 SepPak cartridge (Waters). Cartridges were conditioned with 1 ml 100% MeCN and 1 ml 50% MeCN/0.1% FA, then equilibrated with 4X 1 ml 0.1% TFA. Samples were loaded onto the cartridge and washed 3X with 1 ml 0.1% TFA and 1X with 1 ml 1% FA, then eluted with 2X 600 µl 50% MeCN/0.1% FA. Peptide concentration of desalted samples was again estimated with a BCA assay such that the proper amount for TMT labeling could be removed, dried in a vacuum centrifuge, and stored at -80 °C.

**TMT labeling of peptides**

Samples were divided into two groups—peripheral blood- and cord blood-derived cells—and each set was separately labeled with TMT 6-plex isobaric mass tagging reagents (Thermo Fisher Scientific) as previously described (51). Each 6-plex contained triplicate samples of dexamethasone- and DMSO-treated cells. Digested peptides were resuspended in 50 mM HEPES, pH 8.5 at a concentration of 2.5 mg/ml. Dried TMT reagent was reconstituted at 20 µg/µl in 100% anhydrous MeCN and added to samples at a 1:1 TMT to peptide mass ratio (100 µg for peripheral blood samples and 70 µg for cord blood samples due to limiting material amount). Labeling was performed for 1 hour at 25 °C with shaking. The TMT reaction was quenched with 5% hydroxylamine to a final concentration of 0.2%, shaking for 15 min at 25 °C. TMT-labeled
samples within each plex were then combined, dried to completion via vacuum centrifugation, reconstituted in 1 ml 0.1% FA and desalted with a 100 mg SepPak cartridge as described above.

Basic Reverse Phase (bRP) Fractionation

TMT-labeled peptides were fractionated via offline basic reverse-phase (bRP) chromatography as previously described (52). Chromatography was performed with a Zorbax 300 Extend-C18 column (4.6 x 250 mm, 3.5 µm, Agilent) on an Agilent 1100 high pressure liquid chromatography (HPLC) system. Samples were reconstituted in 900 µl of bRP solvent A (5 mM ammonium formate, pH 10.0 in 2% vol/vol MeCN) and injected with this solvent at a flow rate of 1 ml/min. Peptides were separated at the same flow rate with a 96 min gradient, beginning with an initial increase to 16% bRP solvent B (5 mM ammonium formate, pH 10.0 in 90% vol/vol MeCN) followed by a linear 60 min gradient to 40% and stepwise ramping to 44% and finally 60% bRP solvent B. A total of 96 fractions were collected in a row-wise snaking pattern into a Whatman 2 ml 96-well plate (GE Healthcare), which were then concatenated non-sequentially into a final 24 fractions for proteomic analysis. Fractions were dried via vacuum centrifugation.

Liquid chromatography and mass spectrometry

Dried fractions were reconstituted in 3% MeCN/0.1% FA to a peptide concentration of 1 µg/µl and analyzed via coupled nanoflow liquid chromatography and tandem mass spectrometry (LC-MS/MS) using a Proxeon Easy-nLC 1000 (Thermo Fisher Scientific) and a Q-Exactive Plus series mass spectrometer (Thermo Fisher Scientific). A sample load of 1 µg for each fraction was separated on a capillary column (360 µm outer diameter x 75 µm inner diameter) containing an integrated emitter tip and heated to 50 °C and packed to a length of approximately 30 cm with ReproSil-Pur C18-AQ 1.9 µm beads (Dr. Maisch GmbH)). Chromatography was performed with
a 110 min gradient consisting of solvent A (3% MeCN/0.1% FA) and solvent B (90% MeCN/0.1% FA). The gradient profile, described as min:% solvent B, was 0:2, 1:6, 85:30, 94:60, 95:90, 100:90, 101:50, 110:50, with the first six steps being performed at a flow rate of 200 nl/min and the last two at a flow rate of 500 nl/min. Ion acquisition on the Q-Exactive Plus was performed in data-dependent mode, acquiring HCD-MS/MS scans at a resolution of 17,500 on the top 12 most abundant precursor ions in each full MS scan (70,000 resolution). The automatic gain control (AGC) target was set to $3 \times 10^6$ ions for MS1 and $5 \times 10^4$ for MS2 and the maximum ion time was set to 120 ms for MS2. The collision energy was set to 30, peptide matching was set to preferred, isotope exclusion was enabled, and dynamic exclusion time was set to 20 s.

The original mass spectra and the protein sequence database used for searches have been deposited in the public proteomics repository MassIVE (http://massive.ucsd.edu) and are accessible at ftp://massive.ucsd.edu/MSV000084614/.

Data Analysis

Data was analyzed using Spectrum Mill, version 6.01.202 (Agilent Technologies). In extracting spectra from the .raw format for MS/MS searching, spectra from the same precursor, or within a retention time window of +/- 60 s and m/z range of +/- 1.4 were merged. Spectra were filtered to include only those with a precursor mass range of 750 to 6000 Da and a sequence tag length greater than 0. MS/MS searching was performed against a human UniProt database. Digestion enzyme conditions were set to “Trypsin allow P” for the search, allowing up to 4 missed cleavages within a matched peptide. Fixed modifications were carboxymethylation of cysteine and TMT6 on the N-terminus and internal lysine. Variable modifications were oxidized methionine and acetylation of the protein N-terminus. Matching criteria included a 30% minimum matched peak intensity and a precursor and product mass tolerance of +/- 20 ppm. Peptide-level matches were validated if found to be below the 1.0% false discovery rate (FDR) threshold and within a precursor charge range of 2-6. A second round of validation was then performed for protein-level
matches, requiring a minimum protein score of 0. Protein-centric information, including experimental ratios, was then summarized in a table, which was quality filtered for non-human contaminants, keratins, and any proteins not identified by at least two fully quantified peptides with two ratio counts.

**Statistical analysis**

All statistical evaluations between the different experimental groups were performed using GraphPad Prism 8 (unpaired 2-tailed Student’s t-test, two-way ANOVA with Tukey’s post-hoc test, and Kruskal-Wallis test with Dunn’s post-hoc analysis with corrections for multiple comparisons). A p<0.05 was considered as statistically significant. Raw data corresponding to the additional independent experiments are presented in **Supplemental Figure 7**.

For proteomics analyses, peripheral and cord blood plexes were analyzed separately, each utilizing biologically paired samples to compare dexamethasone treatment to a DMSO control. Data was median normalized and subjected to a one-sample moderated T-test using an internal R-Shiny package based in the limma R library. Correction for multiple testing was performed using the Benjamini-Hochberg false discovery rate method. Single sample gene set enrichment (ssGSEA) was performed as previously described (53) using an R script available at [https://github.com/broadinstitute/ssGSEA2.0](https://github.com/broadinstitute/ssGSEA2.0). ssGSEA was performed separately on PB and CB plexes, using the log2-transformed ratios of dexamethasone/control as input. Parameters were set as follows: sample.norm.type = "rank", weight = 0.75, statistic = "area.under.RES", output.score.type = "NES", nperm = 1e3, min.overlap = 10, correl.type = "z.score", par = T, spare.cores = 1. Heatmap in **Figure 3F** was generated using the average of triplicate enrichment scores, ranked to create a list of top/bottom 10, eliminating redundant rows. It should be noted that the TMT plex design used limits direct quantitative comparisons between PB and CB
samples, as the stochastic sampling of spectra on the mass spectrometer can lead to technical differences in the proteins identified in different plexes that are not necessarily driven by biological causes. Therefore, the observed differences between PB and CB samples were subsequently validated in follow-up experiments.

**Study approval**

All human studies have been approved by the Institutional Review Board (Northwell Health and Stanford University).
Author Contributions:

R.J.A. and H.Y. designed and performed most of the experiments, analyzed data and wrote the manuscript. N.W., J.H., B.M.D., J.P. performed experiments. M.E.O., N.D.U., S.A.C., designed, performed, and analyzed the proteomics studies and edited the manuscript. A.V., J.M.L., L.D.C., analyzed data related to patients with Diamond Blackfan anemia. C.H., S.K., N.T. designed and analyzed data and edited the manuscript. N.M., A.N. and L.B. designed the project, analyzed data, and wrote the manuscript.
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References:


Figure Legends:

Figure 1: Dexamethasone enhances the proliferation of human CFU-Es derived from peripheral blood CD34+ cells.

(A) Fold change in total expansion of PB (red) and CB (black, n = 5) derived CD34+ cells upon erythroid differentiation in the presence or absence of dexamethasone for 14 days (horizontal lines present means ± SE). (B) Total numbers of BFU-E and CFU-E generated from PB and CB cultures in the absence or presence of dexamethasone are presented (data are representative of 1 of 3 independent experiments). (C) Fold change in purified CFU-Es derived from PB (n = 6) and CB (n = 4) in the presence of dexamethasone relative to untreated controls after 14 days of expansion (horizontal lines present means ± SE). (D) Average size (area) of colonies generated from purified CFU-Es derived from untreated PB and CB after 5 days of culture in the absence (-, open circles) or presence (+, closed circles) of dexamethasone (n = 3, horizontal lines present means ± SE). (E) The percentages of reticulocytes (Retic) in 3 patients with DBA following treatment with prednisone, beginning at day 0, are presented. *P < 0.05, ns, non-significant, by 2-tailed Student’s t-test (A, C and D).

Figure 2: Dexamethasone specifically targets a transitional subpopulation of human CFU-Es derived from peripheral blood CD34+ cells.

(A) PB derived CD34+ cells differentiated in the presence or absence of dexamethasone were evaluated as a function of their CD36 and CD34 expression profiles. Representative plots at day 4 of differentiation are presented (1 of 3 independent experiments). (B) Gating strategy to define mature and immature transitional CFU-E erythroid progenitor populations based on the CD71/CD105 profiles of the CD34+CD36+ subset is shown (data are representative of 1 of 3 independent experiments). (C) Representative images of colonies formed by immature and mature CFU-E as defined in (B), in the presence of EPO alone or EPO/SCF/IL-3/IL6/G-CSF/GM-
CSF (n=3). (D) Colony size (area) generated by immature (red) and mature (black) CFU-E, defined as in (B), formed in the presence of Epo alone in the absence (open circles) or presence (closed circles) of dexamethasone (n = 3, horizontal lines present mean ± SE). (E) Representative histograms showing CD71 and CD105 expression in PB-derived CD34⁺ cells differentiated in the absence (black) or presence (red) of dexamethasone (unsorted day 4, data are representative of 1 of 3 independent experiments). (F) Quantification of CD105_med (left) and CD105_hi (right) cells following differentiation of PB-derived CD34⁺ cells in the absence (Ctrl, open circles) or presence (black circles) of dexamethasone (day 4, n=5, horizontal lines present mean ± SE). (*P < 0.05, ns, non-significant, by a 2-tailed Student’s t test (D and F).

**Figure 3: Proteomic studies highlight NR4A1 as a previously unidentified Dexamethasone target in erythroid-differentiated PB progenitors.**

(A) Ranked average log fold change (LogFC) plots of differences in protein expression induced by Dex in erythroid-differentiated PB progenitors. (B) Ranked average LogFC plots of differences in protein expression induced by Dex in erythroid-differentiated CB progenitors. (C) Top 20 upregulated proteins induced by Dex in erythroid-differentiated PB progenitors based on the LogFC. (D) Top 20 upregulated proteins induced by Dex in erythroid-differentiated CB progenitors based on the LogFC. (E) NR4A1 expression levels in purified PB- and CB-derived unsorted progenitors were evaluated by Western blot at day 4 of expansion. Expression of NR4A1 relative to GAPDH is quantified below each lane (data are representative of 1 of 3 independent experiments). (F) Single sample gene set enrichment analysis of proteins differentially regulated by Dex. The top 10 upregulated/downregulated pathways between all samples are listed and redundant pathways were eliminated.

**Figure 4: Dexamethasone increases p57Kip2 expression in Epo-induced PB CD34⁺ cells.**
(A) Expression of p57\textsuperscript{Kip2} and p27\textsuperscript{Kip1} was evaluated in CD34\textsuperscript{+} progenitors during EPO-induced erythroid differentiation by Western blot. Erythroid differentiation was controlled by evaluating α-globin expression (data are representative of 5 independent experiments). (B) Quantification of the differences in p57\textsuperscript{Kip2} protein levels in PB and CB cultures between days 4 and 7 of erythroid differentiation (n=5, values at day 4 are arbitrarily set at “1”). (C) Expression of p57\textsuperscript{Kip2}, p27\textsuperscript{Kip1} and GAPDH in PB-derived and CB-derived progenitors was evaluated in sorted CFU-Es following 4 days of EPO-induced differentiation in the absence (-) or presence (+) of dexamethasone. Representative western blots (1 of 3 independent experiments) are shown. (D) Quantification of the fold changes in p57\textsuperscript{Kip2} (left panel) and p27\textsuperscript{Kip1} (right panel) expression in purified PB-derived (n=7, red circles) and CB-derived (n=6, black circles) CFU-Es in the presence of dexamethasone relative to control conditions (arbitrarily set as “1”; horizontal lines present means ± SE). (E) Quantification of the percentages of PB-derived BFU-E, immature CFU-E and mature CFU-E that are in S phase in the absence (open circles) and presence (closed circles) of dexamethasone. S phase was quantified by Hoechst 33342 staining (n=5, horizontal lines present means ± SE). *P < 0.05, ns-non-significant, by 2-tailed Student’s t-test (D and E).

Figure 5: Aberrant steroid-mediated induction of p57\textsuperscript{Kip2} in erythroid progenitors from transfusion-dependent patients with DBA.

(A) The expansion of CD34\textsuperscript{+} cells derived from healthy controls (black, n=8), transfusion-dependent (TD) patients with DBA (blue, n=5), and steroid-responsive (SR) patients with DBA (red, n=3) is presented following a 7 day stimulation (horizontal lines present means ± SE). (B) Expression of p57\textsuperscript{Kip2} and p27\textsuperscript{Kip1} in erythroid progenitors from healthy controls (Ctrl) and patients with DBA, either transfusion-dependent or steroid-responsive, were evaluated by Western blot at day 7 of differentiation. Expression of p57\textsuperscript{Kip2} relative to GAPDH is quantified below each lane with control levels in the healthy donor arbitrarily set at “1".
(C) Quantification of the fold change in dexamethasone-induced p57Kip2 expression following expansion of CD34+ cells from healthy controls (Ctrl, n=8) as compared to transfusion-dependent patients with DBA (TD, n=5) and steroid-responsive patients with DBA (SR, n=3, horizontal lines present means ± SE). *P < 0.05, ns, non-significant, by Kruskal-Wallis test with Dunn’s post-hoc analysis with corrections for multiple comparisons (A and C).

Figure 6: Downregulation of CDKN1C (p57Kip2) in CD34+ progenitors attenuates the impact of dexamethasone, accelerating erythroid differentiation while olomoucine, a CDK inhibitor, mimics the effect of dexamethasone.

(A) CD34+ progenitors were transduced with a lentiviral vector harboring an shRNA targeting luciferase (shluc) or p57Kip2 (shp57). Expression of p57Kip2 was evaluated by Western blot relative to GAPDH levels at day 7 following transduction (data from 1 of 3 independent experiments are shown). (B) Expansion of control (shluc) and p57Kip2–downregulated (shp57) PB CD34+ cells was evaluated in the absence (open circles) or presence (closed circles) of dexamethasone after 14 days of culture (n = 5; horizontal lines present means ± SE; *P< 0.05, ns, non-significant, by 2-tailed Student’s t-test). (C) Representative histograms of GPA expression in control (shluc) and p57Kip2–downregulated (shp57) progenitors at day 14 of differentiation. Mean fluorescence intensities are indicated (data from 1 of 3 independent experiments are shown). (D) Quantification of the ratio of immature CFU-E to mature CFU-E following expansion of PB-derived CD34+ cells in the absence (black circles) or presence of dexamethasone (100nM, red circles) or olomoucine (1µM, blue circles) at D4 of culture. n=5, horizontal lines present mean ± SE; *P< 0.05 by Kruskal-Wallis test with Dunn’s post-hoc analysis with corrections for multiple comparisons.

Figure 7: Dexamethasone increases p57Kip2 levels in EPO-induced BM-derived progenitors, expanding the immature CD105med CFU-E subset.

(A) BM CD34+ progenitors were differentiated in the absence (-, open circles) or presence (+,
closed circles) of dexamethasone and total cell numbers at day 14 are presented (n=5 independent experiments, horizontal lines present means ± SE). *P< 0.05, by 2-tailed Student’s t-test. (B) Representative histograms of CD105 expression in BM-derived CD34+ cells differentiated in the absence (-, black) or presence (+, red) of dexamethasone at day 4 are shown and the percentages of CD105med cells are indicated. (C) Expression of p57Kip2 and GAPDH in purified BM-derived and CB-derived mature CFU-Es, generated in the absence or presence of dexamethasone, were evaluated by western blot (1 of 3 independent blots are shown).

Supplemental Figure Legends:

Supplemental Figure 1: Dexamethasone significantly delays Glycophorin A upregulation in terminal erythroblasts in both PB- and CB-derived progenitors.

(A) Representative histograms and cell pellets of PB- and CB-derived progenitors differentiated in the absence (-) or presence (+) of Dex (unsorted day 11). GPA expression as well as Band3 and α4-integrin expression of GPA+ cells are shown. (B) Quantification of the percentages of GPA-positive cells following erythroid differentiation of PB- (n=4) and CB-derived (n=4) progenitors in the absence (solid circles) or presence (open circles) of Dex (horizontal lines present means ± SE; *,#P < 0.05, **,#P < 0.01, ns-non-significant, by two-way ANOVA with Tukey’s post-hoc analysis with corrections for multiple comparisons to assess differences between PB-derived cells (*) and CB-derived cells (#) in the presence or absence of Dex).

Supplemental Figure 2: Alterations in BFU-E and CFU-E cells as a function of Dexamethasone treatment.

The percentages of BFU-E and CFU-E generated from PB-derived CD34+ progenitors following expansion in the absence (Ctrl) or presence of Dex (100nm and 1μm) are presented.

Supplemental Figure 3: Measurement of CFU-E colony area.
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Figure 5: Aberrant steroid-mediated induction of p57<sup>Kip2</sup> in erythroid progenitors from transfusion-dependent patients with DBA.

(A) The expansion of CD34<sup>+</sup> cells derived from healthy controls (black, n=8), transfusion-dependent (TD) patients with DBA (blue, n=5), and steroid-responsive (SR) patients with DBA (red, n=3) is presented following a 7 day stimulation (horizontal lines present means ± SE). (B) Expression of p57<sup>Kip2</sup> and p27<sup>Kip1</sup> in erythroid progenitors from healthy controls (Ctrl) and patients with DBA, either transfusion-dependent or steroid-responsive, were evaluated by Western blot at day 7 of differentiation. Expression of p57<sup>Kip2</sup> relative to GAPDH is quantified below each lane with control levels in the healthy donor arbitrarily set at “1”.

(C) Quantification of the fold change in dexamethasone-induced p57<sup>Kip2</sup> expression following expansion of CD34<sup>+</sup> cells from healthy controls (Ctrl, n=8) as compared to transfusion-dependent patients with DBA (TD, n=5) and steroid-responsive patients with DBA (SR, n=3, horizontal lines present means ± SE). *P < 0.05, ns, non-significant, by Kruskal-Wallis test with Dunn’s post-hoc analysis with corrections for multiple comparisons (A and C).
Figure 6: Downregulation of CDKN1C (p57\textsuperscript{Kip2}) in CD34\textsuperscript{+} progenitors attenuates the impact of dexamethasone, accelerating erythroid differentiation while olomoucine, a CDK inhibitor, mimics the effect of dexamethasone.

(A) CD34\textsuperscript{+} progenitors were transduced with a lentiviral vector harboring an shRNA targeting luciferase (shluc) or p57\textsuperscript{Kip2} (shp57). Expression of p57\textsuperscript{Kip2} was evaluated by Western blot relative to GAPDH levels at day 7 following transduction (data from 1 of 3 independent experiments are shown). (B) Expansion of control (shluc) and p57\textsuperscript{Kip2}–downregulated (shp57) PB CD34\textsuperscript{+} cells was evaluated in the absence (open circles) or presence (closed circles) of dexamethasone after 14 days of culture (n = 5; horizontal lines present means ± SE; *P < 0.05, ns, non-significant, by 2-tailed Student’s t-test). (C) Representative histograms of GPA expression in control (shluc) and p57\textsuperscript{Kip2}–downregulated (shp57) progenitors at day 14 of differentiation. Mean fluorescence intensities are indicated (data from 1 of 3 independent experiments are shown). (D) Quantification of the ratio of immature CFU-E to mature CFU-E following expansion of PB-derived CD34+ cells in the absence (black circles) or presence of dexamethasone (100nM, red circles) or olomoucine (1\textmu M, blue circles) at D4 of culture. n=5, horizontal lines present mean ± SE; *P < 0.05 by Kruskal-Wallis test with Dunn’s post-hoc analysis with corrections for multiple comparisons.
Figure 7: Dexamethasone increases p57Kip2 levels in EPO-induced BM-derived progenitors, expanding the immature CD105^med CFU-E subset.  

(A) BM CD34^+ progenitors were differentiated in the absence (-, open circles) or presence (+, closed circles) of dexamethasone and total cell numbers at day 14 are presented (n=5 independent experiments, horizontal lines present means ± SE). *P< 0.05, by 2-tailed Student’s t-test (B) Representative histograms of CD105 expression in BM-derived CD34^+ cells differentiated in the absence (-, black) or presence (+, red) of dexamethasone at day 4 are shown and the percentages of CD105^med cells are indicated. (C) Expression of p57Kip2 and GAPDH in purified BM-derived and CB-derived mature CFU-Es, generated in the absence or presence of dexamethasone, were evaluated by western blot (1 of 3 independent blots are shown).