Globin gene expression in correlation with G protein-related genes during erythroid differentiation

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Abstract

Background: The guanine nucleotide binding protein (G protein)-coupled receptors (GPCRs) regulate cell growth, proliferation and differentiation. G proteins are also implicated in erythroid differentiation, and some of them are expressed principally in hematopoietic cells. GPCRs-linked NO/cGMP and p38 MAPK signaling pathways already demonstrated potency for globin gene stimulation. By analyzing erythroid progenitors, derived from hematopoietic cells through in vitro ontogeny, our study intends to determine early markers and signaling pathways of globin gene regulation and their relation to GPCR expression.

Results: Human hematopoietic CD34+ progenitors are isolated from fetal liver (FL), cord blood (CB), adult bone marrow (BM), peripheral blood (PB) and G-CSF stimulated mobilized PB (mPB), and then differentiated in vitro into erythroid progenitors. We find that growth capacity is most abundant in FL- and CB-derived erythroid cells. The erythroid progenitor cells are sorted as 100% CD71+, but we did not find statistical significance in the variations of CD34, CD36 and GlyA antigens and that confirms similarity in maturation of studied ontogenic periods. During ontogeny, beta-globin gene expression reaches maximum levels in cells of adult blood origin (176 fmol/μg), while gamma-globin gene expression is consistently up-regulated in CB-derived cells (60 fmol/μg). During gamma-globin induction by hydroxycarbamide, we identify stimulated GPCRs (PTGDR, PTGER1) and GPCRs-coupled genes known to be activated via the cAMP/PKA (ADIPOQ), MAPK pathway (JUN) and NO/cGMP (PRPF18) signaling pathways. During ontogeny, GPR45 and ARRD1 genes have the most prominent expression in FL-derived erythroid progenitor cells, GNL3 and GRP65 genes in CB-derived cells (high gamma-globin gene expression), GPR110 and GNG10 in BM-derived cells, GPR89C and GPR172A in PB-derived cells, and GPR44 and GNAQ genes in mPB-derived cells (high beta-globin gene expression).

Conclusions: These results demonstrate the concomitant activity of GPCR-coupled genes and related signaling pathways during erythropoietic stimulation of globin genes. In accordance with previous reports, the stimulation of GPCRs supports the postulated connection between cAMP/PKA and NO/cGMP pathways in activation of γ-globin expression, via JUN and p38 MAPK signaling.

Keywords: G protein, G protein-coupled receptors, Erythroid progenitors, Ontogeny, Globins

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Background

The guanine nucleotide binding protein (G protein)-coupled receptor (GPCRs) family represents the largest group of cell surface receptors that regulate cell growth, proliferation, and differentiation [1]. The silencing of *Gpr48*, as GPCR is highly expressed in the fetal liver (FL) and premature erythroblast, has no effects on primitive erythropoiesis but significantly reduces definitive erythropoiesis through the cAMP/PKA/CREB pathway [2]. Inactivation of *Gpr48* induces remarkable decreases in the proliferation of definitive erythroid progenitors and erythroblast islands in FL [2]. GPCRs are linked via G proteins to adenyl cyclase, phospholipases, and ionic conductance channels [3]. Thus, the Gs protein is known to couple GPCRs to adenyl cyclase to stimulate formation of the second messenger cAMP. It has been found that, upon activation of the cAMP pathway, expression of the gamma (γ)-globin gene is induced in adult erythroblasts [4]. Once formed, cAMP consecutively stimulates cAMP-dependent protein kinase (PKA). According to our previous results, cytostatic hydroxy-carbamide (hydroxyurea) also induces phosphorylation of endothelial nitric oxide synthase (eNOS) in a PKA-dependent manner [5]. Hydroxy-carbamide, as a γ-globin inducer, increases intracellular cAMP levels as well as cGMP levels in human erythroid progenitor cells [6]. Fetal hemoglobin induction by hydroxy-carbamide is mediated by the nitric oxide (NO)-dependent activation of soluble guanylyl cyclase (sGC) [7].

**Results**

Characterization of erythroid cell cultures

Growth potential of CD34⁺ hematopoietic progenitor cells is determined by counting the viable cells. Growth capacity is found to be most abundant for FL-derived CD34⁺ cells during erythroid differentiation. CB- and mPB-derived CD34⁺ cells have a higher cell growth potential than BM- and PB-derived cells during early erythroid differentiation (Figure 1A). In the presence of EPO and other cytokines, CD34⁺ cells are differentiated in vitro into erythroid progenitor cells, confirmed by flow cytometry analysis using four different markers: CD34, CD36, CD71 and GlyA (Figure 1B,C). The transferrin receptor (CD71) is present at early erythroid cells and disappears as reticulocytes differentiate into mature erythrocytes [7]. At day 6 of erythroid cell culture, the erythroid progenitor cells are sorted as 100% CD71⁺, a well-known early marker of erythroid differentiation. Erythroid progenitor cells from all examined cells, except from FL-derived, retain high levels of CD34 antigen expression (about 50–60%). Moreover, all of them and particularly erythroid cells of CB and BM origin, express CD36 antigen (50–70%) also present at early erythrocyte cells (Figure 1B). We did not find statistical significance in the percentage of CD34, CD36 and GlyA antigen positive cells among erythroid progenitors derived from examined hematopoietic cells (Figure 1B). Besides flow cytometry for analysis of *in vitro* erythroid differentiation, we already reported measurement of hemoglobin content by benzidine staining and high-performance liquid chromatography in erythroid progenitor cells during their *in vitro* differentiation in the same culture conditions [6,7].
Globin genes expression during erythroid differentiation

In erythroid progenitor cells derived from PB, γ- and β-globin gene expression is initially similar, but during erythroid maturation β-globin gene expression becomes elevated in erythroid precursor cells and mature erythroid cells (Figure 2A). We already described erythroid differentiation stages, using erythroid specific cell surface markers [7]. The γ-globin gene expression in erythroid cells, of FL origin, has stable expression during erythroid differentiation (about 20 fmol/μg), whereas erythroid cells of CB demonstrate elevation (about 60 fmol/μg). The γ-globin gene expression of adult hematopoietic progenitor-derived erythroid cells demonstrates reduction during differentiation reaching minimum at day 14 (3–15 fmol/μg), while its expression was at maximum in CB-derived erythroid cells (Figure 2B). The β-globin gene expression is almost absent in erythroid progenitors of FL origin, whereas its expression is slightly increased in erythroid cells of CB origin (35 fmol/μg at day 14) and reaches maximum in cells of adult blood origin: BM (176 fmol/μg) and PB (110 fmol/μg) (Figure 2C).

To compare quantitative PCR results and microarray data of γ-globin gene expression, we combine them to demonstrate their expected similar tendencies during ontogeny (Figure 2D). At day 6 of erythroid culture, the γ-globin gene expression is the most elevated in CB- and PB-derived erythroid progenitors, as determined by quantitative PCR. The lowest level of γ-globin gene expression is in BM-derived erythroid progenitors, as determined by microarray (Figure 2D).

Effect of hydroxycarbamide on G protein-coupled receptor signaling pathways in human erythroid progenitor cells

It has been reported that hydroxycarbamide, a γ-globin inducer, activated p38 mitogen-activated protein kinase (MAPK) and c-jun expression [18]. Since these pathways are also activated by GPCRs, we are interested to determine additional related pathways in PB-derived erythroid progenitor cells in a steady state and after incubation with hydroxycarbamide using GPCRs Signaling Pathway Finder Gene Array at day 6 of erythroid culture (Table 1). In the steady state, we find elevated gene expression of DRD5, S1PR2, PTGDR and PTGER1 in human erythroid progenitor cells (Figure 2A). We also find increase in gene expression activated by the cAMP/PKA pathway.
ADIPOQ, PKC pathway (LHB and ELK4), MAPK pathway (YWHAZ), NO-cGMP pathway (PRPF18) and JAK-STAT pathway (HSPA4, SOCS1 and HSP90AA1). HSP90AA1 has the steady upregulated levels during ontogeny in erythroid cells, as well as HSPA4 and YWHAZ gene expression (not shown). Hydroxycarbamide treatment of erythroid progenitor cells induces the statistically significant expression of following GPCRs: PTGDR (1.9 fold), and PTGER1 genes (1.9 fold, Figure 3B).

Hydroxycarbamide also stimulates gene expression activated by cAMP/PKA pathway (ADIPOQ, 3.1 fold), NO/cGMP pathway (PRPF18, 2.6 fold), MAPK pathway (JUN, 1.6 fold) and JAK-STAT pathway (SOCS1, 2.7 fold, Figure 3B). Hydroxycarbamide increases γ-globin gene expression up to 2.5 fold in erythroid progenitor cells used for GPCRs Gene Array, as measured by real-time quantitative PCR and microarray analysis at day 6 of liquid culture (n=2-4). *P < 0.05 PB vs. FL by qPCR. **P < 0.01 BM vs. PB by microarray. Values are mean ± SEM.

Figure 2 Measurement of globin genes expression during human erythroid differentiation. (A) Expression of γ- and β-globin genes during erythroid differentiation of CD34+ cells derived from normal human PB (n=10). (B) Expression of γ-globin gene during erythroid differentiation of hematopoietic CD34+ progenitors of FL, CB, BM, PB and mPB origin (n=2-4). *P < 0.05 PB vs. FL at day 6, **P < 0.01 CB vs. all other tissues at day 14. (C) Expression of β-globin gene during erythroid differentiation (n=2-4). **P < 0.01 FL-, CB-derived erythroid cells vs. all other cells at day 6, 10 and 14, except *P < 0.05 CB vs. mPB at day 6; *P < 0.05 PB vs. BM at day 14; *P < 0.01 mPB vs. BM. (D) Comparative expression of γ-globin genes of erythroid progenitor cells as measured by real time quantitative PCR and microarray analysis at day 6 of liquid culture (n=2-4). *P < 0.05 PB vs. FL by qPCR, *P < 0.05 BM vs. PB by microarray.

G-protein related genes in erythroid progenitor cells during ontogenesis

The G protein superfamily consists of heterotrimeric complexes of distinct α-, β-, and γ-subunits. Heterotrimeric G proteins are classified according to α subunit into four subfamilies: Gs, Gi, Gq, and G12/13 [3]. To distinguish significant G proteins and GPCRs during erythropoiesis we performed microarray analysis of erythroid progenitor cells in certain stages of human ontogeny. During microarray analysis the genes are upregulated or downregulated versus reference HuURNA, what we use as a control alongside each sample. G-protein related genes: GNAI2 and GNB1 are continuously upregulated vs. GNB2L1 upregulation during ontogeny (Figure 4). GNAI3 gene expression was prominently upregulated in BM- and mPB-derived cells, but downregulated in FL-derived cells. Steady upregulation also demonstrates GNL2 and GNL3 genes. The increased GNG10 and GRK6 gene expression is observed in BM-derived cells (Figure 4), with elevated β-globin gene expression (Figure 2C). The expression of GNAQ gene is
significantly increased only in erythroid progenitors of mPB origin. In addition, GPCRs genes: 
GPR65 gene has decreased gene expression only in BM-, whereas GPR135 only in PB-derived cells. 
GPR45 gene has elevated expression in erythroid cells of FL origin, whereas GPR108 has decreased expression in BM-derived cells, opposite to GPR110 and GPR172A genes. In addition, β-arrestins are involved in termination of GPCRs activation after prolonged agonist binding [9]. 

**Table 1 Human G protein-coupled receptor signaling pathway profile in erythroid progenitor cells, of peripheral blood origin, after treatment with hydroxycarbamide**

| UniGene | Symbol | Description | Gene expression | Gene expression |
|---------|--------|-------------|-----------------|-----------------|
| Hs.2551 | ADRB2  | Beta 2 adrenergic receptor | 0.04±0.04, 0.15±0.1 | 0.107 |
| Hs.2624 | DRD1   | D1 dopamine receptor | 0.12±0.1, 0.34±0.2 | 0.1206 |
| Hs.73893 | DRD2  | D2 dopamine receptor | 0.13±0.02, 0.28±0.01 | 0.0715 |
| Hs.380681 | DRD5 | D5 dopamine receptor | 1.23±0.4, 2.03±1.1 | 0.2115 |
| Hs.458474 | S1PR2 | Sphingosine-1-phosphate receptor 2 | 1.92±0.4, 2.6±1.1 | 0.2614 |
| Hs.306381 | PTGDR | Human DP prostaglandin receptor | 0.39±0.1, 0.76±0.1 | 0.0391 |
| Hs.159360 | PTGER1 | Prostaglandin E receptor 1, EP1 subtype | 1.02±0.2, 1.89±0.5 | 0.0305 |

**PI3 kinase pathway:**

- Hs.525622 | AKT1  | v-akt murine thymoma viral oncogene homolog 1 | 0.04±0.01, 0.07±0.06 | 0.139 |

**cAMP/PKA pathway:**

- Hs.80485 | ADIPOQ | Adiponectin, C1Q and collagen domain containing | 0.28±0.09, 0.87±0.2 | 0.0432 |

**NO/cGMP pathway:**

- Hs.161181 | PRPF18 | PRP18 pre-mRNA processing factor 18 homolog | 0.36±0.28, 0.93±0.6 | 0.0497 |

**PKC pathway (Ca²⁺,MEK, etc.):**

- Hs.154704 | LHB  | Luteinizing hormone beta polypeptide | 0.8±0.1, 1±0.4 | 0.1705 |

**JAK-STAT pathway:**

- Hs.525600 | HSP90AA1 | Heat shock 90 kDa protein 1, alpha | 1.84±0.5, 2.21±1.2 | 0.3635 |

**MAP kinase pathway (p42/p44MAP, p38MAP):**

- Hs.414795 | SERPINE1 | Plasminogen activator inhibitor, type 1 | 0.11±0.1, 0.13±0.06 | 0.3309 |

**Discussion**

To reveal the essential mechanisms in erythropoiesis several studies have performed gene expression profiling in erythroid cells from hematopoietic tissues through ontogeny. The findings in these reports are heterogeneous, reflecting the variation in the experimental systems used. By choosing early erythroid progenitors differentiated from purified CD34+ cells, we extend those studies to evaluate globin gene expression in correlation to GPCR-coupled genes from fetal to adult erythropoiesis. The γ-globin gene expression is most prominent in CB-derived erythroid progenitors, whereas β-globin gene expression is major in adult blood-derived (BM, PB, mPB), low in CB-derived and almost completely absent in FL-derived erythroid progenitors. The GPCR-coupled genes have been studied in erythroid
progenitor cells during stimulation of γ-globin gene production. Hydroxycarbamide stimulates the expression of several genes (ADIPOQ, SOCS1, HSP90AA1, PRPF18) activated by GPCRs via cAMP/PKA, p38 MAPK, and NO/cGMP signaling pathways. A certain number of G-proteins (α, β, γ isoforms) and GPCRs demonstrate variation or stability of gene expression in erythroid progenitor cells during ontogeny. Genes GPR45 and ARRD1C have the most prominent expression in FL-derived, genes GNL3 and GRP65 in CB-derived (with high γ-globin gene expression), GNL2, GPR110, ARRD2 and GNG10 in BM-derived (with β-globin gene expression). GNAQ, GNA13 and GPR44 in mPB-derived, GPR89C and GPR172A in PB-derived erythroid progenitors.

As previously reported, G protein expression and MAPKs are involved in hemin-induced erythroid differentiation, another γ-globin stimulator [19]. Hydroxycarbamide increased phosphorylation of p38 in erythroid differentiation [18]. In accordance with hydroxycarbamide and G protein linked activity, Gα12/13 also induced activation of p38 MAPK (Figure 5) [20]. It has been revealed that the p38 activation resulted in the stimulation of NF-kappaB-specific DNA-protein binding and the subsequent expression of inducible NOS and NO release [21]. Moreover, G protein α12 (GNA12) stimulation of the eNOS protein expression is in accordance with hydroxycarbamide induction of eNOS protein levels (Figure 5) [11,22]. We have previously shown that fetal hemoglobin stimulation, by hydroxycarbamide, is dependent on NO/cGMP signaling pathway in erythroid progenitor cells [7]. In addition, the activated G protein α13 (GNA13) induced the NF-kappaB accompanied by augmented secretion of IL-8 (Figure 5) [23]. Induced expression of IL-8, HSPA4, SERPINE1 and JUN by hydroxycarbamide, achieved in our experiments, is also observed upon hemin-induced erythroid differentiation [24]. It has been reported that hydroxycarbamide increased JUN gene and protein expression in erythroid cells, by increasing the rate of synthesis as well as stabilizing the mRNA [25]. Initial EPO-dependent JunB induction was not sufficient, but the late EPO-independent JunB expression was necessary for differentiation of primary erythroid cells [26]. GNA13 gene expression has been elevated in erythropoiesis throughout ontogeny, while GNA12 gene expression is prominent in erythroid progenitors of adult cells origin (Figure 4).

G-protein α15 (GNA15) is expressed particularly in hematopoietic cells [27]. Beta 2-adrenergic receptor (ADRB2), induced by hydroxycarbamide, can specifically couple to GNA15 upregulated in erythroid progenitors of CB and adult cells origin in our microarray study [28]. It has been reported that calcium-sensing receptor-mediated MAP kinase (ERK1/2) activation requires GNA12 coupling [29]. Inhibition of the ERK pathway lead to increased hemoglobin levels [30]. Furthermore, according to our results GNA12 gene expression is decreased in every examined erythroid progenitors. The activation of p38 MAPK signaling pathway, induced by GNA12/J3, is also involved in butyrate-mediated erythroid differentiation, another γ-globin inducer as well as inhibitor of histone deacetylases [31]. Additionally, the inhibition of histone deacetylases induced a high increase of γ-globin mRNA and activated p38 signaling during fetal hemoglobin stimulation [32].
| Symbol | Gene names | FL | CB | BM | mPB | PB |
|--------|------------|----|----|----|-----|----|
| GNL3   | G protein-like 3 (nucleolar) | ↓↑ |    |    | ↑↑  |    |
| GNL2   | G protein-like 2 (nucleolar)  | ↑  |    |    |    |    |
| GNAQ   | G protein, q polypeptide      |    |    |    | ↑↑  |    |
| GNA12  | G protein, α inhibiting activity polypeptide 2 |    |    |    |    |    |
| GNA13  | G protein, α inhibiting activity polypeptide 3 |    |    | ↑  | ↑  |    |
| GNA11  | G protein, α 11 (Gq class)   |    |    |    |    | ↓  |
| GNA12  | G protein α 12               |    |    |    |    |    |
| GNA13  | G protein, α 13              |    |    |    |    | ↑  |
| GNA15  | G protein, α 15 (Gq class)   |    |    | ↑  | ↑  |    |
| GNB1   | G protein, β polypeptide 1   |    |    |    |    | ↓  |
| GNB2L1 | G protein, β polypeptide 2-like 1 |    |    |    |    |    |
| GNG2   | G protein, γ 2               |    |    | ↑  |    |    |
| GNG10  | G protein, γ 10              |    |    |    |    | ↑  |
| GRK6   | GPR kinase 6                 |    |    |    | ↑  |    |
| GPR4   |                         |    |    |    | ↑  |    |
| GPR44  |                         |    |    |    |    |    |
| GPR45  |                         |    |    |    |    |    |
| GPR65  |                         |    |    |    |    |    |
| GPR75  |                         |    |    |    |    |    |
| GPR88  |                         |    |    |    |    |    |
| GPR9C  |                         |    |    |    |    | ↑  |
| GPR107 |                         |    |    |    |    | ↓  |
| GPR108 |                         |    |    |    |    |    |
| GPR110 |                         |    |    |    | ↑  |    |
| GPR135 |                         |    |    |    |    | ↑  |
| GPR160 |                         |    |    |    |    |    |
| GPR172A |                        |    |    |    | ↑  |    |
| ARRB1  | Arrestin, beta 1            |    |    |    |    |    |
| ARRB2  | Arrestin, beta 2            |    |    |    |    |    |
| ARRD1C | Arrestin domain containing 1| ↑  |    |    |    |    |
| ARRD2C | Arrestin domain containing 2|    |    |    |    |    |

Figure 4 (See legend on next page.)
We show that hydroxycarbamide stimulates GPCRs PTGDR and PTGER1 gene expression. The activity of PTGDR receptor is mediated by $G_\alpha_S$ proteins that stimulate adenylate cyclase resulting in an elevation of intracellular cAMP and Ca$^{2+}$, while PTGER1 mediates activity through $G_\alpha_Q$ proteins that stimulate phosphatidylinositol-calcium second messenger system (Figure 6) [33,34]. It has been reported that expression of the $\gamma$-globin gene is induced upon activation of the cAMP pathway, while adenylate cyclase inhibition markedly decreased fetal hemoglobin induction by hydroxycarbamide in human erythroid cells [4,35]. Hydroxycarbamide increased intracellular cGMP as well as cAMP levels in human erythroid progenitors, while cAMP levels in human erythroid cells during differentiation. In addition, inhibition of sGC prevents NO and hydroxycarbamide stimulation of $\gamma$-globin gene expression [7]. Furthermore, sGC activators or cGMP analogs augmented $\gamma$-globin gene expression in primary human erythroblasts [36]. The NO-cGMP pathway is known to increase $JUN$ mRNA levels [37]. We show that hydroxycarbamide increases GPCR-coupled $JUN$ gene expression, which is in accordance with previous studies related to induction of $JUN$ and $\gamma$-globin gene expression (Figure 6) [25,38]. It has been also postulated that $JUN$ activates the $\gamma$-globin promoter via an upstream cAMP response element (CRE) in a way comparable to transcription factor CRE binding protein 1 ($CREB1$) [39]. $CREB2$, a key transcription factor in erythropoiesis, was down-regulated in $Gpr48^{-/-}$ fetal livers through the cAMP-PKA-CREB pathway, with decreased adult hemoglobin $\alpha$ and $\beta$ chains [2]. There have been previous reports of p38 MAPK increased phosphorylation by both NO and cGMP [40,41]. Hydroxycarbamide also augmented phosphorylation of p38, and demonstrated dependence of p38 activity during stimulation of fetal hemoglobin production [18]. Finally, the $CREB1$ activated $\gamma$-globin expression via p38 MAPK signaling in erythroid progenitors [42,43]. This observation emphasizes the mutual stimulation of GPCRs and NO/cGMP pathway, via $JUN$ and p38 MAPK signaling, in activation of $\gamma$-globin expression (Figure 6).

Figure 5 Principle of GPCR signaling. Acute agonist stimulation of GPCRs leads to activation of multiple signaling pathways, including second messengers cAMP, IP3 and Ca$^{2+}$. GPCRs in the plasma membrane couple to these pathways via G proteins that connect the receptors to enzymes adenyl cyclase, phospholipase C (PLC) or ionic conductance channels such as Ca$^{2+}$ channels. Prolonged stimulation of the receptor leads to recruitment to the cell membrane of $\beta$-adrenergic receptor kinases and $\beta$-arrestin. Detected genes by microarray are presented in colored boxes corresponding to G protein isoforms ($\alpha$, $\beta$, $\gamma$). The detected genes are upregulated (gray arrow) or downregulated (black arrow) vs. HuURNA through ontogeny. PKA, protein kinase A; PKC, protein kinase C.
Conclusions

While mechanisms involved in globin gene expression have been recognized at different levels within the regulatory hierarchy, relations among these molecular pathways are only emerging. We associate our new results with the NO/cGMP pathway described in our previous publications [6,7], and demonstrate the induction of G-proteins and GPCR-coupled genes during γ-globin stimulation and erythropoiesis through ontogeny. These genes and related signaling pathways, involved in the mechanism of globin activation, might be targets for the therapeutic agents to upregulate γ-globin gene expression and fetal hemoglobin levels in hemoglobinopathies. Therefore, further direct studies are required to confirm that modifications in the level of expression of GPCRs lead to significant changes in NO/cGMP and other signaling pathways important for γ-globin gene expression in erythroid cells.

Methods

Liquid erythroid cell cultures

For erythroid progenitor cell cultures, blood was obtained from consenting normal volunteers from the National Institutes of Health, Department of Transfusion Medicine according to the regulations and guidelines of the Office of Human Subjects Research. Adult PB mononuclear cells are isolated fromuffy coats of healthy donors (3 individuals per experiment) using Lymphocyte Separation Medium (BioWhittaker, Walkersville, MD). We wash mononuclear cells twice with Dulbecco's phosphate-buffered saline (PBS, Invitrogen Corporation, Carlsbad, CA), and CD34+ hematopoietic progenitors are purified by positive immunomagnetic selection using the MACS cell isolation system (Miltenyi Biotec, Auburn, CA). Commercial FL- (Cambrex Bio Science, Inc., Walkersville, MD), CB-, BM- and granulocyte-colony stimulating factor (G-CSF) stimulated mobilized PB- (mPB, AllCells LLC, Berkeley, CA) derived CD34+ cells are also collected by positive immunomagnetic selection (Miltenyi Biotec). To stimulate erythroid differentiation, the labeled CD34+ cells of all samples are cultured in the medium that contains 30% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% deionized bovine serum albumin, 10 mmol/L mercaptoethanol, 1 mmol/L dexamethasone, 33 μg/ml holo-transferrin, 10 ng/ml SCF, 1 ng/ml IL-3 and 1 ng/ml GM-CSF (Sigma, St. Louis, MO), and 1 U/ml human recombinant EPO (Amgen Inc, Thousand Oaks, CA) [7]. For microarray analysis, erythroid progenitors are isolated at day 6 of erythroid cell culture at 37°C and 5% CO2 with balanced 95% room air. At different time points during in vitro erythroid differentiation, the viable cell counts are performed with the use of a trypan-blue exclusion technique (BioWhittaker).

Immunofluorescence analysis

After 6 days of erythroid culture, 5×10^5 cells are washed in PBS containing 0.5% FCS and 0.02% sodium azide and incubated for 20 minutes at the ambient temperature in the presence of the appropriate monoclonal antibodies at a twofold saturating concentration. Anti-glycophorin-A (GlyA) FITC, anti-CD34 PE, anti-CD71
Triclor, anti-CD36-APC markers are used for cell staining at day 6 of erythroid culture (Beckman-Coulter, Miami, FL). Erythroid cells are then washed, fixed in PBS containing 4% formaldehyde, and acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA) equipped with lasers emitting at wavelengths 355, 488, 532, 407 and 638 nm using DIVA4.1.2 software. The saturation of un-specific binding sites is achieved by normal mouse serum control present in the staining buffer. Data are analyzed with Flowjo software (Tree Star, San Carlos, CA).

**Isolation of total RNA**

After 6 days of erythropoietin treatment and incubation at 37°C (5% CO₂, 95% humidity), we use the RNaseasy protocol for isolation of total RNA from erythroid progenitor cells (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Concentration and integrity of total RNA is assessed using an 8453 UV/Visible Spectrophotometer (Hewlett-Packard GmbH, Waldbronn, Germany) and Agilent 2100 Bioanalyzer Software (Agilent Technologies, Waldbronn, Germany) comparing the ratio of 28S and 18S RNA peaks to ensure that there is minimal degradation of the RNA sample. One microgram of total RNA is reverse-transcribed with SuperScript II RNase H’ Reverse Transcriptase (Invitrogen Corporation).

**Quantitative PCR**

Quantitative real-time PCR assay of γ- and β-globin mRNA transcripts is carried out with the use of genespecific double fluorescently labeled probes in a 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The specific primers and TAQMAN probes (synthesized by the NIDDK core oligonucleotide facility) are designed using Primer Express software (Applied Biosystems) and prepared on an ABI 394 synthesizer (Applied Biosystems) as previously described [44]. Platinum Quantitative PCR SuperMix-UDG (Invitrogen Corporation) is used for each of the primer pairs containing a final concentration of 200 μM dNTPs, 0.5 μM Rox reference dye (Invitrogen Corporation), 0.2 μM each of TAQMAN probe, forward and reverse primers. Expression levels are determined using the associated SDS software (ABI Prism, Applied Biosystems) and Microsoft Excel (Redmont, WA). Standard curves are constructed using dilutions of an accurately determined plasmid containing the cDNA of interest as template.

**Microarray studies**

In microarray studies, the numbers of total genes overexpressed in erythroid cells of CB, BM and PB origin are determined from three independent samples as biological repeats at day 6 of erythroid liquid culture. On the other hand in case of FL and mPB-derived samples, the numbers of total overexpressed genes are determined in independent duplicate samples at day 6 of erythroid liquid culture. High quality oligonucleotide glass arrays are produced containing a total of 16,659 seventy-mer oligonucleotides chosen from 750 bases of the 3’ end of each ORF (Operon Inc. Valencia, CA). The arrays are produced in house by spotting oligonucleotides on poly-L-lysine coated glass slides by Gene Machines robotics (Omnigrid, San Carlos, CA). We have followed the MIAME (minimum information about a microarray experiment) guidelines for the presentation of our data [45].

i) **Probe preparation**

Total human universal RNA (HuURNA) isolated from a collection of adult human tissues to represent a broad range of expressed genes from both male and female donors (BD Biosciences, Palo Alto, CA) serve as a universal reference control in the competitive hybridization. All 5 blood tissues are hybridized against HuURNA. The correlation coefficients among those biological repeats themselves are consistently ≥ 0.8, which documented the quality of hybridization and consistency of expression among the replicates of all 5 tissues. Labeled cDNA probes are produced as described [46].

ii) **Hybridization**

For hybridization, 36 μl hybridization mixture (cDNA mixture, 10 μg COT-1 DNA, 8–10 μg poly (da), 4 μg l yeast total RNA, 20X SSC and 10% SDS) is pre-heated at 100°C for 2 minutes and cooled for 1 minute. Total volume of probe is added on the array and covered with cover slip. Slides are placed in hybridization chamber and 20 μl water is added to the slide, and incubated overnight at 65°C. Slides are then washed for 2 minutes each in 2X SSC, 1X SSC and 0.1X SSC and spin-dried.

iii) **Data Filtration, normalization, and analysis**

Microarray slides are scanned in both Cy3 (532 nm) and Cy5 (635 nm) channels using Axon GenePix 4000B scanner (Axon Instruments, Inc., Foster City, CA) with a 10-micron resolution. Scanned microarray images are exported as TIFF files to GenePix Pro 3.0 software for image analysis. For advanced data analysis, gpr and jpeg files are imported into microarray database, and normalized by software tools provided by NIH Center for Information Technology (http://nciarray.nci.nih.gov). We gathered a set of 8,719 erythroid cells gene expression data derived from 11 datasets that have been posted on the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database.

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Human G Protein-coupled receptors signaling pathway finder gene array

Erythroid progenitor cells, of PB origin, are treated with hydroxyurea at day 4 after stimulation with EPO and incubated 48 hours at 37°C. At day 6, total RNA is isolated. GPCRs array is completed using the GEArray Q Series Chemiluminescence Detection User system (SABiosciences, Frederick, MD). Briefly, the preheated annealing mix with 2 μg of total RNA is added to labeling mix with biotin-16-dUTP (Roche Applied Science, Indianapolis, IN) and reverse transcriptase (Promega Corporation, Madison, WI). The biotin labeled cDNA probe is denatured before addition of the probe to the hybridization solution with the GEArray Q Series membrane. After hybridization with continuous agitation, and washing, the chemiluminescent detection is performed. We use a digital imaging system (FluorChem Imaging system, Alpha Innotech Corporation, San Leandro, CA) to record the chemiluminescent image of the array. The relative abundance of a particular transcript is estimated by directly comparing its signal intensity to the signal derived from a housekeeping gene cyclophilin A. The list of 110 genes for human GPCRs signaling pathway finder gene array is available from the manufacturer (SABiosciences, Frederick, MD).

Statistical analysis

The one way ANOVA Tukey’s Multiple Comparison tests and paired t test are applied using Prism 4 software (GraphPad Software Inc., San Diego, CA) for measurement of statistical significance in cell growth and antigen levels among blood tissues, as well as for γ- and β-globin expression. For microarray data management and analysis, we use NCI/CIT microArray database (mAdb) system.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

VCIC carried out experimental work described in the paper, participated in designing the study, drafted the manuscript and performed the statistical analysis. RDS carried out the quantitative real-time PCR assays and performed the statistical analysis. AB carried out the immunofluorescence analysis and performed the statistical analysis. CTN participated in designing the study and coordination and helped to draft the manuscript. RKP conceived of the study, and participated in its design and coordination and helped to draft the manuscript. ANS participated in designing the study and the writing of the manuscript. All authors read and approved the final manuscript.

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