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5

Nitric Oxide / Cyclic Nucleotide Regulation of Globin Genes in Erythropoiesis

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1. Introduction

1.1. Hemoglobin synthesis

The human hematopoiesis initiates in the second to third embryonic weeks with formation of mesoderm-derived blood islands in the extraembryonic mesoderm of the developing secondary yolk sac (Migliaccio et al., 1986). Erythropoiesis involves proliferation and differentiation of committed erythroid progenitors to mature red blood cells (erythrocytes). Although globins represents <0.1% of proteins at the proerythroblast level, it reaches 95% of all proteins at the level of reticulocytes (Nienhuis & Benz, 1977). Physiologically, the expression of the globin genes is generally regulated in such a way that at any point in development the output of the beta (β)-globin-like chains equals the alpha (α)-globin chains (Lodish & Jacobsen, 1972). The α-globin gene cluster contains three genes and several pseudogenes arranged from the telomere toward the centromere in the following order: 5'-ζ2-ψζ1-ψα2-ψα1-α2-α1-θ1-3' (Higgs et al., 1998). The human β-globin locus consists of five functional β-like globin genes, that are arranged in the order of their expression during development (5′-ε-^{G}γ-^{G}γ-δ-β-3′), and an upstream regulatory element, the locus control region (LCR), that is physically composed of five DNase I-hypersensitive sites (HSs) (Grosveld et al., 1987). The most widely studied changes during red cell ontogeny are the shifts or "switches" in globin types. Embryonic erythroblasts are characterized by the synthesis of the unique hemoglobins Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\gamma_2$). The zeta (ζ)- and epsilon (ε)-globin chains are embryonic α-like and β-like chains, respectively. Thus, a switch from ζ- to α- and ε- to gamma (γ)-globin gene production begins during the embryonic phase of erythropoiesis but is not complete until fetal erythropoiesis is well established. During the transition from yolk sac to fetal liver erythropoiesis (5–8 weeks), erythroid precursors within the fetal liver co-express embryonic (ζ- or ε-) and fetal

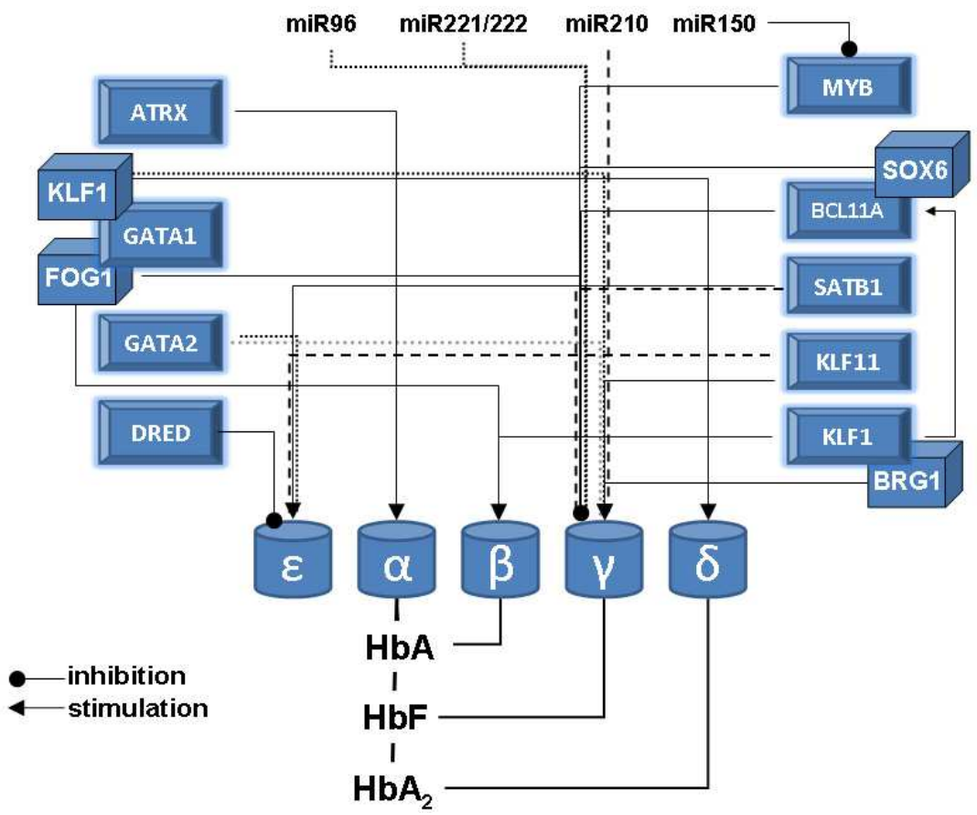
(α- or γ-) globins both in vivo and in vitro (Peschle et al., 1984). The predominant type of hemoglobin synthesized during fetal liver erythropoiesis is fetal hemoglobin (HbF, $\alpha_2\gamma_2$). HbF is formed by γ-globin chain A or G according to the amino acid at the 136 position in the γ chain, i.e., alanine or glycine. The proportion of $^{G}\gamma$ to $^{A}\gamma$ is constant throughout fetal development: about 75% of $^{G}\gamma$ and 25% of $^{A}\gamma$. However, in adult red cells, the small amount of HbF is composed mainly of $^{A}\gamma$ (60% *vs.* 40% of $^{G}\gamma$) (Jensen et al., 1982). α- and β-globin chains combine as a tetramer of two α- and two β-globin polypeptides along with a heme moiety to form the adult hemoglobin molecule (HbA, $\alpha_2\beta_2$). HbA, detectable at the earliest stages of fetal liver erythropoiesis, is also synthesized as a minor component throughout this period, but HbA_2 ($\alpha_2\delta_2$), minor hemoglobin in the adult, is undetectable in these early stages. From about the 30th gestational week onward, β-globin synthesis steadily increases, so that by term 50%–55% of hemoglobin synthesized is HbA. By 4-5 weeks of postnatal age, 75% of the hemoglobin is HbA, this percentage increasing to 95% by 4 months as the fetal-to-adult hemoglobin switch is completed. HbF levels in circulating red cells are in a plateau for the first 2–3 weeks (as a result of the decline in total erythropoiesis that follows birth), but HbF gradually declines and normal levels (<1%) are achieved by 200 days after birth.

1.2 The GATA-1/2 and Krüppel-like factors role in hemoglobin switching

The regulation of globin gene switching is a very complex process requiring the coordination of different signaling pathways and molecular reactions. Many transcription factors controlling globin gene expression have been identified and characterized. These factors form a complex network of protein-protein and protein-DNA interactions with each other, globin gene promoters, LCR HSs, and other *cis*-acting intergenic regions (Stamatoyannopoulos, 2005). The GATA family of proteins (GATA1–6) comprises zinc finger transcription factors that both activate and repress target genes containing a consensus GATA binding motif (Orkin, 1992). Binding sites with this motif are present in many positions in the α- and β-globin loci, as well as many other erythroid-expressed genes. The founding member of this family, GATA1, was discovered as a β-globin locus-binding protein (Pruzina et al., 1991). GATA1 is essential for erythroid cell maturation in vivo (Pevny et al., 1989). In addition, GATA1 homodimerizes and interacts with other transcription factors, such as SP-1 and erythroid Krüppel-like factor (EKLF/KLF1), further contributing to the complex network of GATA factor interactions (Gregori et al., 1996). GATA2 is primarily expressed in primitive erythropoiesis, but later in development GATA1 expression predominates (Leonard et al., 1993). Downregulation of GATA2 is important for progression of erythroid cell differentiation (Persons et al., 1999). The protein Friend of GATA1 (FOG) is co-expressed with GATA1 during embryonic development in erythroid cells (Tsang et al., 1997). GATA1 binds a region upstream of both the HBG1- and HBG2-promoter, necessary for HbF silencing, in a FOG1 dependent manner leading to recruitment of the suppressive NuRD-complex (Harju-Baker et al., 2008). GATA-1, together with FOG-1, functions as an anchor in the formation of chromatin looping, and is required for physical interactions between the β LCR and β globin promoter (Vakoc et al., 2005).

The Krüppel-like factors are a family of C2H2 zinc finger DNA binding proteins that are important in controlling developmental programs. KLF1 gene is an erythroid cell-specific zinc finger transcription factor, containing a DNA-binding domain located at its C-terminus, composed of three 'Krüppel-like' C2H2 zinc finger motifs, and a proline-rich transactivation domain at its N-terminus (Miller & Bieker, 1993). KLF1 preferentially activates the β-globin

gene promoter by binding with high affinity to the CACCC element (Bieker & Southwood, 1995). KLF1 is essential for adult β-globin gene transcription and binds to the βLCR and β-globin promoters, required for direct interactions between the βLCR and the β-globin gene in humans (Donze et al., 1995, Scheme 1). Patients with hereditary persistence of HbF, with elevated levels of HbF, have mutations in the gene encoding erythroid transcription factor KLF1 (Borg et al., 2010). Recent findings demonstrate that KLF1, and the co-activator BRG1, are designated by short-chain fatty acid derivatives to activate the fetal globin genes. The SWI/SNF complex chromatin-modifying core ATPase BRG1 is required for γ-globin induction by short-chain fatty acid derivatives, and is actively recruited to the γ-globin promoter in the KLF1-dependent manner (Perrine et al., 2009). KLF1-GATA1 fusion proteins activated δ-, γ-, and β-globin promoters, and significantly up-regulated delta (δ)- and γ-globin RNA transcript and protein expression in human erythroleukemic cells (Zhu et al., 2011). DRED (direct repeat erythroid-definitive) was identified as a repressor of the ε-globin gene, it appears to prevent binding of KLF1 to the ε-globin gene promoter and silences ε-globin expression during definitive erythropoiesis (Tanimoto et al., 2000). KLF2 also regulates the expression of the human embryonic ε-globin gene but not the adult β-globin gene (Basu et al., 2005). Another erythroid-specific transcription factor, called fetal Krüppel-like factor (KLF11), activates γ- and ε-globin genes in human erythroleukemic cells (Asano et al., 1999, Scheme 1). KLF11 also activates γ-globin transcription via the CACCC element in the promoter (Asano et al., 2000). The protein encoded by this gene is a zinc finger transcription factor that binds to SP1-like sequences in ε- and γ-globin gene promoters.



Sheme 1. Overview of globin genes control by examined transcription factors.

1.3 Other factors that participate in hemoglobin switching

A nuclear protein, special AT-rich binding protein 1 (SATB1), regulates genes through targeting chromatin remodeling and its overexpression increases ε-globin and decreases γ-globin gene expression (Wen et al., 2005, Scheme 1). Global changes to chromatin, including acetylation, phosphorylation, and methylation play roles in LCR activation. Histone acetylation occurs during chromatin remodeling and hyperacetylation is associated with transcriptional activation of a locus (Pazin & Kadonaga, 1997). Similar to acetylation, phosphorylation of histone H3 disrupts DNA-nucleosome interaction and increases transcription factor accessibility to DNA. SATB1 overexpression increased ε-globin and decreased γ-globin gene expression accompanied by histone hyperacetylation and hypomethylation in chromatin from the ε-globin promoter and HS2, and histone hypoacetylation and hypermethylation associated with the γ-globin promoter (Wen et al., 2005). Mitogen activated protein kinase (MAPK) pathways, as well as the stress activated p38 pathway, activate histone H3 phosphorylation (Cheung et al., 2000). Studies on p38 knockout mice established a role for the p38 stress pathway in the switch from primitive to definitive erythropoiesis (Tamura et al., 2000). Mutations in the transcription factor alpha thalassemia/mental retardation syndrome X-linked (ATRX), nearly always downregulate α-globin expression, provide potentially important insight into the trans-regulation of globin gene expression (Gibbons et al., 1995). Alpha hemoglobin stabilizing protein (AHSP) is an erythroid-specific protein that forms a stable complex with free alpha-hemoglobin (Kihm et al., 2002). It has been found that AHSP expression was highly dependent on the larger subunit of nuclear factor erythroid-derived 2 (NFE2) (Guo-wei et al., 2010). The transcription factor NFE2 activation of globin production was stimulated by cAMP-dependent protein kinase (PKA) in erythroid cells (Casteel et al., 1998).

BCL11A gene (encoding the transcription factor B-cell lymphoma/leukemia 11A) maintains silencing of γ-globin expression in adult erythroid cells and functions as a direct transcriptional regulator of the fetal to adult hemoglobin switch in humans. BCL11A protein levels vary in erythroid progenitors over the course of human ontogeny. BCL11A is expressed as short variant proteins in primitive erythroid progenitors that largely express γ-globin and as full-length forms at the adult stage with silenced γ-globin genes (Sankaran et al., 2008). In erythroid progenitors, BCL11A physically interacts with the NuRD chromatin remodelling complex, and the erythroid transcription factors, GATA1 and FOG1. In addition, KLF1, as a key activator of BCL11A, controls globin gene switching by directly activating β-globin and indirectly repressing γ-globin gene expression (Zhou et al., 2010). BCL11A binds the upstream LCR, ε-globin, and the intergenic regions between γ-globin and δ-globin genes. BCL11A and SOX6 co-occupy the human β-globin cluster along with GATA1, and cooperate in silencing γ-globin transcription in adult human erythroid progenitors (Xu et al., 2010, Scheme 1). SOX6 has also been suggested to enhance definitive erythropoiesis in mouse by stimulating erythroid cell survival, proliferation and terminal maturation (Dumitriu et al., 2006). A broad genome expression profile studies led to the identification of common genetic polymorphisms in the locus of the β-globin gene, a region between the HBS1-like gene HBS1L and the oncogene MYB, as well as within the gene BCL11A (Galarneau et al., 2010). HBS1L-MYB intergenic polymorphism on chromosome 6q23 is associated with elevated HbF levels. MYB and HBS1L expression was significantly

reduced in erythroid cultures of individuals with high HbF levels, whereas overexpression of MYB in human erythroleukemic cells inhibited γ-globin expression supporting role of MYB in HbF regulation (Jiang et al., 2006). The human erythroid precursor cells from individuals with higher HbF and higher F cell levels have lower MYB expression associated with lower erythrocyte count but higher erythrocyte volume (Jiang et al., 2006).

MicroRNAs (miRNAs or miRs) are small, 19 to 25 nucleotide long, non-coding RNAs, which target mRNAs in a sequence-specific manner, inducing translational repression or decay. Increased miRNA-210 levels elevated γ-globin levels in hereditary persistence of HbF (Bianchi et al., 2009), while the let-7 family has been associated with hemoglobin switching (Noh et al., 2009). Recently, two miRNAs, miR-221 and miR-222, have been identified to regulate HbF expression in erythropoietic cells via the kit receptor (Gabbianelli et al., 2010). miRNA-150 repression of MYB in hematopoietic progenitor cells, of human bone marrow origin, supported MYB's importance in erythroid and megakaryocytic differentiation (Lu et al., 2008). It has been reported that miRNA-96, miRNA-146a, let-7a, miR-888 and miR-330a-3p are significantly more abundant in reticulocytes obtained from adults than from umbilical cord blood and therefore are potential inhibitors of γ-globin expression. The miR-96 has been identified as a direct inhibitor of γ-globin expression (Azzouzi et al., 2011, Scheme 1). These findings demonstrate that miRNAs contribute to HbF regulation by the post-transcriptional inhibition of γ-globin expression during adult erythropoiesis.

2. Microarray analysis of globin related genes during ontogenesis

2.1 Introduction

Several groups have examined the gene expression profile of human $CD34^+$ hematopoietic progenitor cells from bone marrow (BM), peripheral blood (PB) and cord blood (CB) using microarray technology (He et al., 2005; Ng et al., 2004; Steidl et al., 2002). The modulation of gene expression during ontogeny, in fetal liver (FL)- and CB-derived $CD34^+CD38^-$ hematopoietic progenitor cells, appears to overlap broadly with early response genes of growth factor stimulated adult (BM) hematopoietic progenitor cells (Oh et al., 2000). Recent studies have begun to define a general gene expression profiling for human erythroid cells from different origins - adult BM and PB (Goh et al., 2007; Gubin et al., 1999; Fujishima et al., 2004). In general, it has been hypothesized that globin gene switching may be mediated by proteins expressed during different stages of ontogeny.

Following the same intention, we have performed serial gene expression profiling in human differentiating erythroid cells by oligonucleotide microarrays. The several expressed genes (GATA1, ALAS2, EPOR, globins, etc.) linked to known erythroid differentiation confirms the validity of our approach in establishing the appropriate in vitro cell culture conditions. To study the mechanism of globin gene switching, we have performed gene expression profiling of erythroid progenitor cells derived from hematopoietic tissues during ontogeny, using a large Gene Array to gain insight into the associated molecular pathways. Gene expression patterns of $CD71^+$ erythroid progenitor cells, differentiated from human FL, CB, BM, PB and granulocyte colony-stimulating factor (G-CSF) mobilized PB (mPB), were compared to establish the expression patterns of representative genes.

2.2 Material and methods

2.2.1 Liquid erythroid cell cultures

$CD34^+$ hematopoietic progenitor cells were purified by positive immunomagnetic selection using the MACS cell isolation system (Miltenyi Biotec, Auburn, CA). Fresh BM, PB and G-CSF stimulated mPB $CD34^+$ cells were collected (AllCells LLC, Berkeley, CA) and proceeded with selection. CB $CD34^+$ cells (AllCells LLC) and FL $CD34^+$ cells (Cambrex Bio Science, Inc., Walkersville, MD) were collected and frozen. For analysis, $CD34^+$ cells were resuspended in medium with erythropoietin (EPO), as already described (Cokic et al., 2003). After 6 days of EPO treatment, $5x10^5$ cells were washed and incubated for 20 minutes in the presence of the monoclonal antibody anti-CD71 Tricolor for cell staining (Beckman-Coulter, Miami, FL). Cells were then washed, fixed and acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed with Flowjo software (Tree Star, San Carlos, CA). After 6 days of erythropoietin treatment and incubation at 37°C (5% CO_2, 95% humidity), we used the RNeasy 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 was assessed using an 8453 UV/Visible Spectrophotometer (Hewlett-Packard GmbH, Waldbronn, Germany) and Agilent 2100 Bioanalyzer Software (Agilent Technologies, Waldbronn, Germany).

2.2.2 Microarray studies

In microarray studies, the numbers of total genes overexpressed in erythroid cells of CB, BM and PB origin were determined from three independent samples as biological repeats. FL and mPB-derived samples were analyzed in independent duplicate samples at day 6 of erythroid liquid culture. High quality oligonucleotide glass arrays were produced containing a total of 16,659 seventy-mer oligonucleotides (Operon Inc. Valencia, CA). The array includes probes for 2121 hypothetical proteins and 18-expressed sequence tags (ESTs) and spans approximately 50% of the human genome (Operon Inc.). The arrays were produced in house by spotting oligonucleotides on poly-L-lysine coated glass slides by Gene Machines robotics (Omnigrid, San Carlos, CA).

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) served as a universal reference control in the competitive hybridization. Labeled cDNA probes were produced as described (Risinger et al., 2003). cDNA was purified by the MinElute column (Qiagen). Binding buffer PB was added to the coupled cDNA, and the mixture applied to the MinElute column and centrifuged. After discharging the flow-through, washing buffer PE was added to the column, and centrifuged. Then the columns were placed into a fresh eppendorf tube and elution buffer added to the membrane, incubated and centrifuged and probe collected. The probe was dried in speed-vac. Finally, 5 µl of 2X coupling buffer and 5 µl Cy3 and Cy5 dye (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) were mixed into the control (HuURNA) and experimental cDNAs (huES cell-derived) respectively and incubated at room temperature in dark for 90 minutes. After incubation, the volume was raised to 60 µl by DEPC water and then cDNA was purified by the MinElute column and eluted with 13 µl elution buffer by centrifugation. For hybridization, 36 µl hybridization mixture was pre-heated at 100°C for 2 minutes and

cooled for 1 minute. Total volume of probe was added on the array and covered with cover slip. Slides are placed in hybridization chamber and 20µl water was added to the slide, and incubated overnight at 65^{o}C. Slides were then washed for 2 minutes each in 2X SSC, 1X SSC and 0.1X SSC and spin-dried. Microarray slides were scanned in both Cy3 (532nm) and Cy5 (635nm) channels using Axon GenePix 4000B scanner (Axon Instruments, Inc., Foster City, CA) with a 10-micron resolution. Scanned microarray images were exported as TIFF files to GenePix Pro 3.0 software for image analysis. The average of the total Cy3 and Cy5 signal gave a ratio that was used to normalize the signals. Each microarray experiment was globally normalized to make the median value of the log2-ratio equal to zero. The Loess normalization process corrects for dye bias, photo multiplier tube voltage imbalance, and variations between channels in the amounts of the labeled cDNA probes hybridized. The data files representing the differentially expressed genes were then created. For advanced data analysis, gpr and jpeg files were imported into microarray database, and normalized by software tools provided by NIH Center for Information Technology (http://nciarray.nci.nih.gov/). Spots with confidence interval of 99 (≥ 2 fold) with at least 150-fluorescence intensity for both channel and 30 µm spot size were considered as good quality spots for analysis. Paired t test was used in microarray analysis.

2.3 Results

2.3.1 Predominantly elevated genes expression in ontogenic tissues

In the presence of EPO and other cytokines, CD34^{+} cells were differentiated in vitro into erythroid progenitor cells. 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 same culture conditions (Cokic et al., 2003, 2008). The transferrin receptor (CD71) is present on early erythroid cells but is lost as reticulocytes differentiate into mature erythrocytes (Cokic et al., 2003). At day 6 of erythroid cell culture, the erythroid progenitor cells were sorted as 100% CD71^{+}, a well-known early marker of erythroid differentiation. In microarray studies, the quantities of tissue specific overexpressed genes were determined from two to four independent samples (biological repeats). During microarray analysis genes are upregulated or downregulated versus HuURNA, what we used as a control alongside each sample. We observed largely upregulated genes in all tissues (Table 1).

Besides common highly expressed genes in erythroid progenitor cells: CD71, Rh-associated glycoprotein (RHAG), SERPINE1 mRNA binding protein 1 (SERBP1); the highly expressed genes in erythroid cells of FL origin are ribonuclease, RNase A family 2 and 3 (RNASE2/3) and serpin peptidase inhibitor, clade B (SERPINB1, Table 1).

The highly expressed genes in erythroid cells of CB origin are hemoglobin gamma A (HBG1), NFE2 and eosinophil peroxidase (EPX). The greatly expressed genes in erythroid cells of BM origin are latexin (LXN), coproporphyrinogen oxidase (CPOX) and carbonic anhydrase II (CA2). The highly expressed genes in erythroid cells of mPB origin are KLF1, aminolevulinate, delta-, synthase 2 (ALAS2) and THO complex 2 (THOC2). The genes with reduced expressed in erythroid cells of PB origin are proteoglycan 2 (PRG2), Charcot-Leyden crystal protein (CLC), EPX and v-myb myeloblastosis viral oncogene homolog

Gene Name	Description	Fold induction vs. HuURNA (±SD)				
		FL	CB	BM	mPB	PB
HBG1	hemoglobin, gamma A	4.2±0.7	4.5±0.9	4.1±1	4.3±0.2	4.7±0.6
CLC	Charcot-Leyden crystal protein	4.3±0.4	4.3±0.7	4.6±0.6	4.8±0.4	**2.9±0.6**
PRG2	proteoglycan 2, bone marrow	5±0.3	4.2±0.9	4.7±0.5	4.1±0.7	**2.9±0.4**
RNASE2	ribonuclease, RNase A family, 2	**4.3±0.7**	3.7±0.9	3.1±0.6	3.5±0.7	**1.4±0.8**
HBD	hemoglobin, delta	**2±1.3**	3.5±0.3	4.3±0.4	4.2±0.4	3.2±0.5
EPX	eosinophil peroxidase	**4±0.2**	**3.4±0.8**	2.9±0.7	2.6±1.1	**0.9±0.5**
SRGN	serglycin	4.3±1	**2.8±1**	4.4±1.3	4.6±0.4	**2.1±0.5**
TFRC	transferrin receptor (p90, CD71)	3.1±1	3.3±1.2	4.4±0.3	4.3±0.03	3±0.9
HBE1	hemoglobin, epsilon 1	3±1.2	2.6±1.5	**4.4±1.7**	**4.6±0.01**	3.4±1.3
RHAG	Rh-associated glycoprotein	2.7±1.3	3.2±1.2	2.1±0.9	3.1±0.7	2.1±0.5
MPO	myeloperoxidase	3.8±0.2	3.2±0.6	3.4±0.1		
MYB	v-myb myeloblastosis viral oncogene homolog	3±0.3	3.1±0.7	2.6±0.4	3.5±0.4	**1.7±1**
RNASE3	ribonuclease, RNase A family, 3	**3.6±1.2**	2.7±1.5	2±1	3.1±0.7	**0.6±0.2**
LXN	latexin	1.5±0.4	1.9±0.7	**4.7±1**	**4.1±0.5**	2.7±0.2
CD36	CD36 molecule (thrombospondin rec)	**1.6±1.1**	2.6±0.5	**4±1**	3.2±0.5	2.1±0.8
CPOX	coproporphyrinogen oxidase	2±0.8	2.7±0.6	**3.5±0.7**	2.9±0.4	1.8±0.6
CA2	carbonic anhydrase II	**0.15**	1±0.3	**3.3±0.6**	1.8±0.2	1.7±0.5
CYTL1	cytokine-like 1	1.4±0.8	1.7±0.4	2.8±0.4	**3.7±0.3**	2.4±0.3
KLF1	Kruppel-like factor 1 (erythroid)	**1±0.4**	1.7±0.5	2.5±0.9	**3.6±0.3**	2.3±0.2
ALAS2	aminolevulinate, delta-, synthase 2	1.5±0.4	1.4±0.3	2.5±0.9	**3.5±0.7**	2.7±0.3
CPA3	carboxypeptidase A3 (mast cell)	1	0.9±0.3	1.9±0.7	**3.2±0.4**	1.2±0.5
THOC2	THO complex 2	0.6±0.4	0.9±0.5	1.4±0.7	**3.1±0.2**	1.6±0.5
GATA1	globin transcription factor 1	**0.9**	2.1±0.4	2.7±0.6	2.5±0.3	2.3±0.3
NFE2	nuclear factor (erythroid-derived 2)	1.4±0.4	1.9±0.5	2.6		1.6±0.5
SERPINB1	serpin peptidase inhibitor, clade B	**2.5±0.2**	1.8±0.3	1.7±0.4	0.9±0.3	0.7±0.3
SERBP1	SERPINE1 mRNA binding protein 1	2.1±0.4	2.4±0.5	1.9±0.7	1.9±0.5	1.6±0.6

Table 1. Largely up-regulated genes vs. HuURNA among different tissues: FL, CB, BM, mPB, PB. Bolded black values represent increased genes expression, whereas bolded gray values represent decreased genes expression in comparison to other tissues.

(MYB). Presence of certain gene in the least two samples (66% filtering), in one group of tissues, reduced largely the total gene expression in all tissues. Using the range of microarray analysis and filtering reduction, the erythroid cells of FL tissue origin expressed 1772 genes, CB-derived erythroid cells expressed 3846 genes, BM derived erythroid cells expressed 1827 genes, mPB derived erythroid cells expressed 4008 genes, and PB derived erythroid cells expressed 1320 genes. The observed gene expression is more than doubled in CB and mPB tissues in comparison to other tissues.

2.3.2 A comparison in genes expression of erythroid cells during subsequent stages of development

Using Venn diagrams we compared total gene expression, determined by microarray analysis, among all examined ontogenic tissues. By 66% filtering, we analyzed only genes

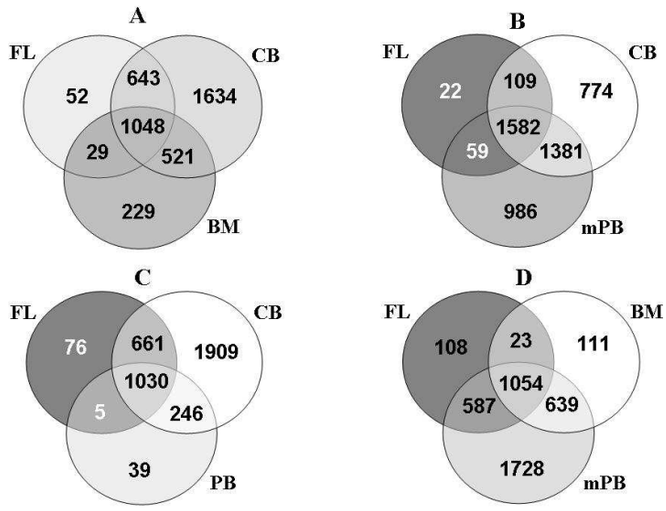


Fig. 1. **Venn diagram of genes expression in erythroid progenitor cells between FL and other developmental tissues.** A: Comparison among FL, CB and BM tissues; B: Comparison among FL, CB and mPB tissues; C: Comparison among FL, CB and PB tissues; D: Comparison among FL, BM and mPB tissues.

present in at least two donor samples per tissue. We compared gene expression of FL-derived erythroid cell with other ontogenic derived tissues in Figure 1. Shared genes expression was more prominent between CB and FL/mPB derived erythroid cells, than between FL and BM/PB tissues (Figure 1A-C). Moreover, the FL- and CB-derived erythroid cells have the more common genes with mPB-derived erythroid cells than with BM and PB tissues (Figures 1D, 2C). The genes related to FL tissue shared the similar expression with BM- and PB-derived cells (Figure 2A). In addition, the genes expression in mPB-derived erythroid cells contains the majority of genes expressed in FL, BM and PB tissues (Figure 2B,

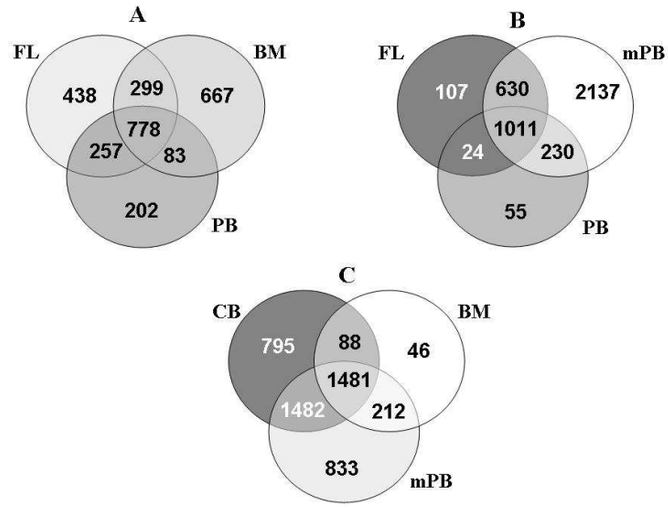


Fig. 2. **Venn diagram of genes expression in erythroid progenitor cells among ontogenic tissues.** A: Comparison among FL, BM and PB tissues; B: Comparison among FL, mPB and PB tissues; C: Comparison among CB, BM and mPB tissues.

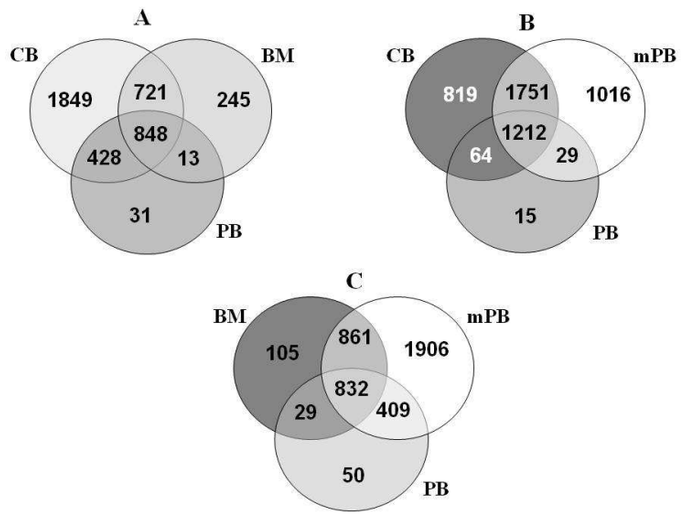


Fig. 3. **Venn diagram of genes expression in erythroid progenitor cells between PB and other ontogenic tissues.** A: Comparison among CB, BM and PB tissues; B: Comparison among CB, mPB and PB tissues; C: Comparison among BM, mPB and PB tissues.

3C), but not in CB tissue (Figures 2C, 3B). Also, mPB-derived erythroid cells shared more genes with BM tissue than with PB tissue (Figure 3C). The genes expression in CB-derived erythroid cells overwhelmed the gene expression in BM tissues and almost completely in PB tissue (Figure 3A).

Comparison in genes expression between FL- and CB-derived erythroid cells revealed couple statistically significant genes ($p<0.01$, Table 2): POLE3 and BRP44. Negative values in Tables represent downregulated genes expression in ontogenic tissues in contrast to HuURNA, whereas positive values represent upregulated genes.

Gene	Description	p value	FL Mean±SD	CB Mean±SD	Tissue presence
POLE3	polymerase epsilon 3	0.0022	0.4±0.22	0.8±0.4	FL, CB, mPB, PB
SNRPD3	small nuclear ribonucleoprotein D3	0.0036	0.8±0.02	1.6±0.4	FL, CB, mPB
ZFP36	zinc finger protein 36	0.0072	-1.6±0.07	-2.3±0.6	FL, CB, mPB
BRP44	brain protein 44	0.0063	-0.14±0.09	0.5±0.1	All tissues

Table 2. Comparison of statistically significant ($p<0.01$) genes between FL and CB tissues.

Brain protein 44 (BRP44) gene expressions was highly significantly increased in CB-derived erythroid cells vs. FL-derived cells (Table 2), but was reduced in comparison to PB-derived cells (Table 8). BRP44 gene was stable expression in all examined ontogenic tissues, whereas POLE 3 gene expression was absent in BM tissue (Table 2). Comparison in genes expression between FL- and BM-derived erythroid cells exposed more statistically significant genes (Table 3): SBNO2, WDR1 and CTAG2 present in all tissues.

WD repeat domain 1 (WDR1) gene expressions was significantly increased in BM- vs. PB-derived erythroid cells (Table 10), but was reduced in comparison to FL-derived cells (Table

3). Evaluation in genes expression between FL- and mPB-derived erythroid cells exposed also statistically significant genes (Table 4): ME2 present just in FL and mPB tissues, highly upregulated EEF1B2 expressed in all tissues. IFI30 was downregulated in mPB-derived erythroid cells, but was upregulated in FL-derived cells (Table 4).

Gene	Description	p value	FL Mean±SD	BM Mean±SD	Tissue presence
SPRR2B	small proline-rich protein 2B	0.0001	-0.8±0.03	-0.9±0.5	FL, CB, BM,mPB
SRI	sorcin	0.0014	0.9±0.04	1.1±0.2	All tissues
KPNA2	karyopherin alpha 2	0.002	0.5±0.005	1 ±0.2	All tissues
SDHC	succinate dehydrogenase complex, subunit C	0.0021	0.1±0.05	0.2±0.2	All tissues
NCOA4	nuclear receptor coactivator 4	0.0048	2±0.3	1.8±0.4	All tissues
SBNO2	strawberry notch homolog 2	0.005	-1.5±0.02	-1.1±0.6	All tissues
TTC3	tetratricopeptide repeat domain 3	0.0057	0.3±0.08	0.6±0.1	FL, CB, BM,mPB
PTTG1	pituitary tumor-transforming1	0.0078	0.7±0.05	1±0.2	All tissues
WDR1	WD repeat domain 1	0.0079	0.5±0.002	0.3±0.1	All tissues
MINK1	Misshapen-like kinase 1	0.0089	-1.2±0.1	-0.9±0.5	All tissues
CTAG2	cancer/testis antigen 2	0.0094	-0.1±0.25	-0.2±0.54	All tissues

Table 3. Comparison of statistically significant ($p<0.01$) genes between FL and BM tissues.

Gene	Description	p value	FL Mean±SD	mPB Mean±SD	Tissue presence
ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	0.0068	1.19±0.15	0.9±0.14	FL, mPB
INSIG1	insulin induced gene 1	0.0073	-0.08±0.4	-0.01±0.4	FL, mPB
EEF1B2	eukaryotic translation elongation factor 1 beta 2	0.0009	1.77±0.12	2.3±0.12	All tissues
ZNF224	zinc finger protein 224	0.0015	-0.2±0.06	0.9±0.06	FL, CB, BM, mPB
CSDE1	cold shock domain containing E1, RNA-binding	0.004	0.56±0.08	0.6± 0.02	All tissues
IFI30	Interferon, gamma-inducible protein 30	0.0059	0.06±0.05	-2±0.07	FL, CB, BM, mPB
PSMD11	proteasome 26S, non-ATPase,11	0.0091	0.67±0.17	0.18±0.16	All tissues
PGAM1	phosphoglycerate mutase 1	0.0093	0.87±0.15	0.06±0.16	FL, CB, mPB, PB
PTPRC	protein tyrosine phosphatase, receptor type, C	0.0093	1.42±0.75	1.5±0.75	FL, CB, BM, mPB

Table 4. Comparison of statistically significant ($p<0.01$) genes between FL and mPB tissues.

Comparison between FL- and PB-derived erythroid cells revealed just two significant genes: TOP1 and CAT (Table 5). TOP1 and CAT genes have higher levels in FL tissue than in PB.

Gene	Description	p value	FL Mean±SD	PB Mean±SD	Tissue presence
TOP1	topoisomerase I	0.0017	1.3±0.34	0.9±0.7	All tissues
CAT	catalase	0.0042	1.8±0.18	1.3±0.26	FL, CB, mPB, PB

Table 5. Comparison of statistically significant ($p<0.01$) genes between FL and PB tissues.

Evaluation in genes expression between CB- and BM-derived erythroid cells exposed several genes: WAPAL, GRB2, GOLIM4, etc. (Table 6). CTDSP1 has the elevated expression in CB tissue, while TPST2 has the higher expression in BM tissue (Table 6).

Gene	Description	p value	CB Mean±SD	BM Mean±SD	Tissue presence
SCAMP2	secretory carrier membrane protein 2	0.0022	0.4±0.22	0.8±0.4	CB, BM, PB
ABCF2	ATP-binding cassette, sub-family F	0.0075	-0.2±0.17	0.22±0.08	CB, BM, PB
ZNF16	zinc finger protein 16	0.0093	0.2±0.2	0.6±0.18	CB, BM, PB
GRIPAP1	GRIP1 associated protein 1	0.0014	-0.6±0.45	-0.06±0.45	All tissues
WAPAL	wings apart-like homolog	0.0023	1.3±0.1	1.7±0.1	All tissues
TPST2	tyrosylprotein sulfotransferase 2	0.0029	0.8±0.26	1.6±0.21	All tissues
TPSB2	tryptase beta 2	0.0035	-0.7±0.43	1±0.33	All tissues
CCNB2	cyclin B2	0.0037	1.3±0.21	1.7±0.19	All tissues
RPS13	ribosomal protein S13	0.0041	1.9±0.23	1.5±0.21	All tissues
GRB2	growth factor receptor-bound protein 2	0.0067	-0.06±0.4	0.4±0.38	All tissues
GSK3A	glycogen synthase kinase 3α	0.0071	-1±0.04	-0.8±0.06	All tissues
CTDSP1	carboxy-terminal domain, A polypeptide small phosphatas 1	0.0077	0.4±0.23	-0.05±0.01	All tissues
ZNF43	zinc finger protein 43	0.0086	0.9±0.11	1.2±0.09	All tissues
GOLIM4	golgi integral membrane protein 4	0.0094	0.4±0.04	0.85±0.07	All tissues

Table 6. Comparison of statistically significant ($p<0.01$) genes between CB and BM tissues.

Zinc finger protein 43 (ZNF43) has also significantly increased gene expressions in BM-derived erythroid cells compared to CB- and PB-derived erythroid cells (Tables 6, 10). Golgi integral membrane protein 4 (GOLIM4) gene has also significantly increased expressions in BM-derived erythroid cells compared to CB- and mPB-derived erythroid cells (Tables 6, 9). Assessment in genes expression between CB- and mPB-derived erythroid cells uncovered several genes: ARF4, PHIP, ACIN1, etc. (Table 7). ARF4 has highly upregulated gene expression in CB-derived cells compared to mPB tissue.

Measurement in genes expression profile between CB and PB tissues revealed the downregulated genes PPFIA4 and WIPI2, as well as upregulated genes FBL and BRP44 (Table 8). WIPI2 was less downregulated in PB tissue than in CB tissue.

Determination of statistical significance between BM and mPB tissues showed the prevalent quantity of genes: MYCL2, ADIPOR2 and POP7 present in CB, BM and mPB tissues; NFATC3, YY1, GCA present in all tissues except PB; YWHAZ, TACC3, UBE2D3 present in BM, mPB and PB tissues (Table 9).

Gene	Description	P value	CB Mean±SD	mPB Mean±SD	Tissue presence
GRN	granulin	0.0007	-1.1±0.35	-1.5±0.16	All tissues
PHIP	pleckstrin homology domain interacting protein	0.0009	0.5±0.38	1.4±0.29	All tissues
ARF4	ADP-ribosylation factor 4	0.0037	0.4±0.3	0.04±0.15	FL, CB, BM, mPB
UBE2V1	ubiquitin-conjugating enzyme E2 variant 1	0.0044	0.7±0.06	0.4±0.04	All tissues
ACIN1	apoptotic chromatin condensation inducer 1	0.0058	0.6±0.11	0.3±0.05	All tissues
XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6	0.0067	1.2±0.13	0.8±0.11	All tissues
F2R	coagulation factor II (thrombin) receptor	0.0072	-0.2±0.22	-0.9±0.21	FL, CB, BM, mPB
SNRPA	small nuclear ribonucleoprotein polypeptide A	0.008	1.6±0.37	1.1±0.02	FL, CB, mPB
LGALS1	lectin, galactoside-binding, soluble, 1	0.0093	-1.3±0.48	-0.5±0.57	FL, CB, mPB

Table 7. Comparison of statistically significant ($p<0.01$) genes between CB and mPB tissues.

Gene	Description	p value	CB Mean±SD	PB Mean±SD	Tissue presence
PPFIA4	protein tyrosine phosphatase, f polypeptide, interacting protein α 4	0.0016	-1.3±0.37	-0.06±0.44	All tissues
WIPI2	WD repeat domain, phosphoinositide interacting 2	0.0043	-0.3±0.08	-0.02±0.14	All tissues
FBL	fibrillarin	0.0056	1.5±0.29	0.9±0.36	All tissues
BRP44	brain protein 44	0.0059	0.5±0.13	1±0.14	All tissues

Table 8. Comparison of statistically significant ($p<0.01$) genes between CB and PB tissues.

Gene	Description	p value	BM Mean±SD	mPB Mean±SD	Tissue presence
MYCL2	v-myc avian myelocytomatosis viral oncogene homolog 2	0.0006	-0.7±0.26	-1.4±0.26	CB, BM, mPB
SKAP2	src kinase associated phosphoprotein 2	0.0012	-1.1±0.3	-1.6±0.31	CB, BM, mPB
MPHOSPH9	Mphase phosphoprotein mpp9	0.0012	-0.7±0.18	-1.3±0.19	CB, BM, mPB
SLC25A39	solute carrier family 25, member 39	0.0013	1.5±0.13	0.9±0.07	CB, BM, mPB

Gene	Description	p value	BM Mean±SD	mPB Mean±SD	Tissue presence
UBE2NL	ubiquitin-conjugating enzyme E2N-like	0.0015	0.9±0.17	0.6±0.17	CB, BM, mPB
POP7	Processing of precursor 7, ribonuclease P/MRP subunit	0.0027	-0.4±0.21	0.28±0.12	CB, BM, mPB
ANKH	ankylosis, progressive homolog	0.0031	-0.5±0.22	-1.2±0.21	CB, BM, mPB
ADIPOR2	adiponectin receptor 2	0.0045	0.6±0.32	0.6±0.01	CB, BM, mPB
ZBTB43	zinc finger and BTB domain containing 43	0.0041	-0.9±0.22	-1.4±0.21	CB, BM, mPB
RBM8A	RNA binding motif protein 8A	0.0048	-0.9±0.45	-0.3±0.13	CB, BM, mPB
ZYG11B	zyg-11 homolog B	0.0062	-0.2±0.28	-0.9±0.23	CB, BM, mPB
APBB2	amyloid beta precursor protein-binding, family B, member 2	0.0074	-0.8±0.16	-1.1±0.17	CB, BM, mPB
CEP97	centrosomal protein 97kDa	0.0082	-0.6±0.09	-1.4±0.1	CB, BM, mPB
KLRD1	killer cell lectin-like receptor subfamily D	0.0011	-0.4±0.16	-1.3±0.16	FL, CB, BM, mPB
TOMM34	translocase of outer mitochondrial membrane 34	0.0035	-0.5±0.21	-0.6±0.15	FL, CB, BM, mPB
NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	0.0045	1.3±0.19	0.9±0.23	FL, CB, BM, mPB
YY1	YY1 transcription factor	0.0047	1.4±0.18	0.8±0.22	FL, CB, BM, mPB
SSB	Sjogren syndrome antigen B	0.0050	2.7±0.72	2.3±0.003	FL, CB, BM, mPB
UBC	ubiquitin C	0.0069	-1±0.53	-1.3±0.08	FL, CB, BM, mPB
UBA1	ubiquitin-like modifier activating enzyme 1	0.0083	-0.4±0.6	-0.6±0.17	FL, CB, BM, mPB
LRRC59	leucine rich repeat containing59	0.0086	1.9±0.25	1.5±0.03	FL, CB, BM, mPB
GCA	grancalcin, EF-hand calcium binding protein	0.0098	1.2±0.21	0.4±0.17	FL, CB, BM, mPB
TACC3	transforming, acidic coiled-coil containing protein 3	0.0011	0.02±0.01	-0.3±0.03	BM, mPB, PB
LMNB1	lamin B1	0.0014	1±0.1	-0.1±0.04	BM, mPB, PB
SREBF2	sterol regulatory element binding	0.0019	-0.6±0.05	-2.1±0.18	BM, mPB, PB
UBE2D3	ubiquitin-conjugating enzyme E2D 3	0.0032	1.02±0.06	0.5±0.001	BM, mPB, PB
YWHAZ	tyrosine 3-mono oxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	0.0042	1.4±0.08	0.7±0.04	BM, mPB, PB

Gene	Description	p value	BM Mean±SD	mPB Mean±SD	Tissue presence
GOLIM4	golgi integral membrane protein 4	0.0044	0.8±0.02	0.29±0.05	BM, mPB, PB
GLE1	GLE1 RNA export mediator homolog	0.0049	-0.4±0.02	0.29±0.12	BM, mPB, PB
CORO1C	coronin actin binding protein 1C	0.0049	1.4±0.1	0.6±0.08	BM, mPB, PB
ARHGDIB	Rho GDPdissociation inhibitorβ	0.0075	1.4±0.02	1.2±0.03	BM, mPB, PB
TMEM187	transmembrane protein 187	0.0082	-0.4±0.08	-0.9±0.03	BM, mPB, PB
TIMM23	translocase of inner mitochon-drial membrane 23 homolog	0.0088	0.9±0.1	0.3±0.05	BM, mPB, PB
PSMB5	proteasome subunit, β type, 5	0.0092	0.3±0.02	0.02±0.02	BM, mPB, PB

Table 9. Comparison of statistically significant ($p<0.01$) genes between BM and mPB tissues.

YY1 transcription factor has significantly increased gene expressions in BM-derived erythroid cells compared to PB- and mPB-derived erythroid cells (Tables 9, 10). Measurement of statistical significance between BM and PB tissues revealed the following significant genes: NUCKS1 and KDM3B prevalent in BM tissue, ATF5 and ATP5L prevalent in BM tissue (Table 10).

Gene	Description	p value	BM Mean±SD	PB Mean±SD	Tissue presence
NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	0.0092	1.1±0.62	0.7±0.12	CB, BM,PB
KDM3B	lysine (K)-specific demethylase 3B	0.0002	0.8±0.01	0.15±0.03	BM, mPB, PB
WDR1	WD repeat domain 1	0.0018	0.3±0.1	-0.2±0.03	BM, mPB, PB
YY1	YY1 transcription factor	0.0018	1.4±0.2	0.25±0.14	BM, mPB, PB
FEN1	flap structure-specific endonuclease 1	0.004	2.7±0.1	1.13±0.2	BM, mPB, PB
ATF5	activating transcription factor 5	0.0057	-0.8±0.1	-0.2±0.14	BM, mPB, PB
ZNF43	zinc finger protein 43	0.0058	1.2±0.08	0.5±0.15	BM, mPB, PB
ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	0.0071	0.3±0.08	0.78±0.09	BM, mPB, PB
HLA-C	MHC class I human leukocyte antigen	0.0046	-2.7±0.7	-2.1±0.88	CB,BM,PB

Table 10. Comparison in statistically significant ($p<0.01$) gene between BM and PB tissues.

Flap structure-specific endonuclease 1 (FEN1) gene has significantly decreased expressions in PB-derived erythroid cells compared to BM- and mPB-derived erythroid cells (Tables 10, 11). Similarity in genes expression between mPB and PB tissues was limited on four

significant genes: TXNIP, EIF3E, FEN1 and FECH (Table 11). TXNIP was present and downregulated just in mPB and PB tissues, whereas EIF3E and FEN1 was highly upregulated in all tissues and predominantly in mPB-derived erythroid cells. FECH has more prominent expression in PB- than in mPB-derived cells.

Gene	Description	p value	mPB Mean±SD	PB Mean±SD	Tissue presence
TXNIP	Brain-expressed HHCPA78 homolog VDUP1	0.0063	-1.3±0.05	-0.14±0.18	mPB, PB
EIF3E	eukaryotic translation initiation factor 3, subunit E	0.0003	2.5±0.14	1.2±0.24	All tissues
FEN1	flap structure-specific endonuclease 1	0.0028	2±0.21	1.13±0.21	All tissues
FECH	ferrochelatase	0.0074	1.6±0.7	2.5±0.59	FL, CB, mPB, PB

Table 11. Comparison in statistically significant ($p<0.01$) gene between mPB and PB tissues.

2.3.3 Signaling pathways related to globin genes expression

It has been already reported that γ globin genes expression is regulated by nitric oxide (NO) and p38 MAPK signaling pathways (Cokic et al., 2003; Ramakrishnan & Pace, 2011). We examined the genes related to those pathways in erythroid progenitor cells during ontogeny in succeeding tissues (Figure 4). Protein kinase, cAMP-dependent, regulatory, type II, beta (PRKAR2B) has the highest expression in NO signaling pathways linked genes throughout the ontogeny reaching the top in PB-derived erythroid cells. Calmodulin 2 (CALM2) gene demonstrates decline in expression during ontogeny, as well as protein phosphatase 3 beta isoform (PPP3CB, Figure 4A). Downregulation in gene expression during ontogeny was shown for protein kinase, cAMP-dependent, regulatory, type I, alpha (PRKAR1A) and calmodulin 3 (CALM3). Regarding p38 MAPK signaling pathway, transforming growth factor, beta 1 (TGFB1) gene expression was predominant in FL- and BM-derived erythroid cells, while heat shock 27kDa protein 1 (HSPB1) was decreased in BM-derived erythroid cells (Figure 4B). Linked to p38 MAPK, v-myc myelocytomatosis viral oncogene homolog (MYC) has the most upregulated gene expression throughout the ontogeny (Figure 4B).

2.3.4 Discussion

To recognize sets of genes that reveal the essential mechanisms in hematopoiesis, as potential novel therapeutic targets, several groups have performed individual gene expression profiling in erythroid cells from certain tissues during ontogenesis. We extended those studies, of gene expression pattern of ontogenic tissues, to compare gene expression from fetal to adult hematopoiesis as a more reflective and comprehensive overview of erythropoiesis. Gene expression in normal human erythroid progenitor cells has been described and generally static expression analysis was performed on cultured human erythroid progenitor $CD71^+$ cells derived from $CD34^+$ cells in the presence of EPO and cocktail of cytokines. We presented the number of total genes overexpressed in evaluated tissues, the most dominant in CB and mPB tissues. Also, the highly expressed genes are SERPINE1, PRG2, CLC, HBG1, NFE2 and EPX. General genes expression was more present between CB and FL/mPB derived erythroid cells, than between FL and adult tissues.

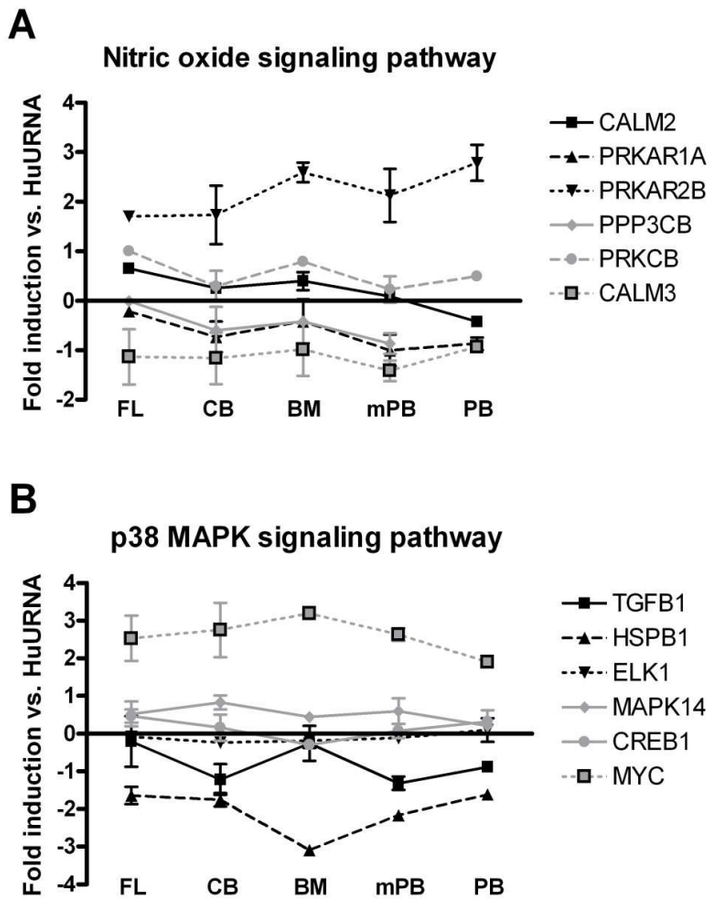


Fig. 4. **Gene expression in signaling pathways related to globins stimulation determined by microarray analysis.** A: Induction of Nitric oxide signaling pathway related genes in erythroid progenitor cells during human ontogeny. B: Induction of p38 MAPK signaling pathway related genes in erythroid progenitor cells during human ontogeny.

Comparison between certain tissues revealed the statistically significant genes: TOP1, CAT, IFI30 in FL tissue, ARF4, CTDSP1 in CB tissue, WDR1, ATF5, YWHAZ in BM tissue, EIF3E and FEN1 in mPB tissue, FECH in PB tissue. PRKAR2B has the highest expression in NO signaling pathways, while MYC has the most upregulated gene expression in p38 MAPK signaling pathway in erythroid progenitor cells throughout ontogeny.

3. Nitric oxide interaction with signaling pathways related to erythropoiesis

It has been found that proliferation of erythropoietic cells is more related to activation of JAK-STAT and MAPK p42/44 signaling pathways, whereas the survival of erythropoietic cells correlated better with activation of PI-3K-AKT, JAK-STAT and MAPK p42/44 pathways (Ratajczak et al., 2001). During erythroid maturation, the p38 MAPK regulates γ-globin transcription through its downstream effector cAMP response element binding protein 1 (CREB1) which binds the Gγ-globin 3′,5′-cyclic adenosine monophosphate (cAMP) response element (Ramakrishnan & Pace, 2011). NO is a diffusible free radical that plays a

role as a chemical messenger involved in vasodilator, neurotransmitter, and anti-platelet aggregation. NO is produced and released from three different isoforms of NO synthase (NOS) that convert the l-arginine and molecular oxygen to citrulline and NO in cells: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) (Cokic & Schechter, 2008). NO readily diffuses across cell membranes into neighboring cells, or may produce effects distant from its site of production transported by vehicles such as low-molecular weight S-nitrosotiols, S-nitrosylated proteins including hemoglobin and albumin, and nitrosyl-metal complexes which liberate NO spontaneously or after cleavage by ectoenzymes (Bogdan, 2001). A significance of NO in erythroid differentiation has been founded on demonstration that NO donors inhibit growth of erythroid primary cells and colony cultures (Maciejewski et al., 1995). Besides observation that NO inhibited erythroid differentiation induced by butyric acid, antitumour drugs aclarubicin and doxorubicin, but not by hemin (Chénais et al., 1999), additional study demonstrated inhibitory effect of NO in the hemin-induced erythroid differentiation (Kucukkaya et al., 2006). NO decreased colony-forming unit-erythrocytes (CFU-E) and CFU-granulocyte macrophage (CFU-GM) formation derived from human bone marrow mononuclear cells. Moreover, NO increased CFU-GM and decreased CFU-E formation derived from $CD34^+$ hematopoietic progenitor cells (Shami & Weinberg, 1996). Although NO increased intracellular levels of guanosine 3',5'-cyclic monophosphate (cGMP) in bone marrow cells, addition of a membrane permeable cGMP analogue did not reproduce previously mentioned effects of NO in bone marrow derived colonies (Shami & Weinberg, 1996). We have previously shown that HbF stimulation is dependent on NO/cGMP signaling pathway in erythroid progenitor cells (Cokic et al., 2003). NO-releasing agents and cGMP analogues inhibit murine erythroleukemia cell differentiation and suppress erythroid-specific gene expression such as beta-globin and delta-aminolevulinate synthetase (Suhasini et al., 1995). Serum nitrate and nitrite (NOx) concentrations correlated inversely with hemoglobin levels (Choi et al., 2002).

EPO increased the level of phosphorylated eNOS and stimulated NO production and cGMP activity during hypoxia (Beleslin-Cokic et al., 2004; Su et al., 2006). Phospho-eNOS and eNOS were significantly induced by hypoxia (Beleslin-Čokić et al., 2011). NO participates in stability control of hypoxia inducible factor (HIF)-1α and induces HIF-1α accumulation and HIF-1-DNA binding (Kovacević-Filipović et al., 2007). Hypoxia and EPO increased erythropoietin receptor (EPOR) gene expression and protein level (Beleslin-Čokić et al., 2011). The physiologically low oxygenation of bone marrow is a regulator of hematopoiesis maintenance, and physiological levels of O_2 should be considered as an important environmental factor that significantly influences cytokine activity (Brüne & Zhou, 2007; Krstic et al., 2009a). The proportion of γ–globin mRNA (the γ/(γ+β) mRNA ratio) increased with reduced oxygen, reaching a maximum value at 5% O_2 of 1.5 to 4-fold higher than at 20% O_2, and then decreased as the O_2 dropped to 2%. In parallel, the proportion of HbF (the HbF/(HbF+HbA) ratio) also peaked at 5% O_2. Reported increase in the HbF was generally lower than that of the γ-globin mRNA, suggesting that although globin mRNA accumulation is primarily under transcriptional regulation, additional post-transcriptional processing such as globin chain stability contribute to the amount of produced hemoglobins (Rogers et al., 2008).

4. Nitric oxide influence on hematopoietic microenvironment in bone marrow

Various growth factors, cytokines, and chemokines are secreted by human hematopoietic progenitor cells, myeloblasts, erythroblasts, and megakaryoblasts to regulate normal

hematopoiesis in an autocrine/paracrine manner. Furthermore, each stromal cell in the bone marrow may provide the preferable microenvironment for a rapid expansion of the lineage-restricted progenitor cells (Kameoka et al., 1995; Majka et al., 2001). We showed that the human endothelial cells and macrophages contain NOS activity, representing the potential pool for NO production. The erythroid progenitor cell co-cultures with either macrophages or endothelial cells, stimulated by NO-inducers, demonstrated more elevated levels of γ-globin gene expression than in the erythroid cells only (Čokić et al., 2009). This observation suggests that NO could come out of the bone marrow stromal cells and diffuse into the erythroid progenitor cells largely participating in γ-globin gene induction, linked to stromal cells augmented capability for NO production. This supplemented NO production, in the hematopoietic microenvironment, has the potential to enhance HbF synthesis in erythroid progenitor cells, which still have low hemoglobin levels and presumably low scavenging activity. Accumulating evidence emphasized the involvement and important role of NO in the regulation of hematopoiesis (Krstić et al., 2009, 2010). Spleen is also an active hematopoietic organ where response of hematopoietic cells to cytokines depends on the tissue microenvironment (Jovčić et al., 2007). Improper signaling inside bone marrow stromal cells can lead to their failure and inconsistent microenvironmental niche for hematopoietic stem cells. It has been recently shown that basal NO/cGMP/cGMP-dependent protein kinase (PKG) activity is necessary for preserving bone marrow stromal cell survival and promoting cell proliferation and migration (Wong & Fiscus, 2011). Co-culture studies of human macrophages, as well as human bone marrow endothelial cell line, with erythroid progenitor cells resulted in induction of γ-globin mRNA expression in the presence of cytostatic hydroxyurea. NOS-dependent stimulation of NO by lipopolysaccharide and interferon-γ has been observed in human macrophages. In addition, lipopolysaccharide and interferon-γ together increased γ-globin gene expression in human macrophage/erythroid cell co-cultures (Čokić et al., 2009). These observations are in accord to the intimate contact between erythroid and stromal cells, effects and associations in physiological hematopoietic microenvironment. The endothelial cells as well as macrophages, normal components of bone marrow stroma, play an active role in the modulation of human hematopoietic stem cell growth (Ascensao et al., 1984; Davis et al., 1005; Hanspal & Hanspal, 2004). The murine endothelial cell lines also stimulate the proliferation and differentiation of erythroid precursors, where close cell contact is necessary for erythropoiesis (Ohneda & Bautch, 1997). Mice deficient in eNOS, expressed by bone marrow stromal cells, demonstrated a defect in progenitor cell mobilization (Aicher et al., 2003). Hemoglobin synthesis of erythroleukemia cells line was increased after co-culture with endothelial cells and monolayers of bone-marrow-derived macrophages, as well as with cell-free culture media conditioned by blood-monocyte-derived macrophages (Zuhrie et al., 1988). NO has also an important role in bone marrow angiogenesis, together with vascular endothelial growth factor (VEGF), with implications in patients with leukemic malignancies (Antic et al., 2010, 2011). NO-cGMP pathway stimulates the proliferation and osteoblastic differentiation of primary mouse bone marrow-derived mesenchymal stem cells and osteoblasts (Hikiji et al., 1997). NO donors, as well as proteasome inhibitors, inhibited cytokine induced intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression (Cobb et al., 1996; De Caterina et al., 1995). The proteasome inhibition also significantly enhanced endothelial-dependent vasorelaxation of rat aortic rings (Stangl et al., 2004), as well as NO production in endothelial cells (Cokic et al., 2007).

5. Cyclic nucleotides induction of globin genes expression

5.1 NO/cGMP stimulation of γ-globin gene expression

We demonstrated that NO increases γ-globin gene expression in erythroid cells during differentiation. Inhibition of soluble guanylate cyclase (sGC) prevents NO-induced increase in γ-globin gene expression (Cokic et al., 2003). In addition, we have shown that the well known γ-globin gene inducer hydroxyurea stimulated HbF by the NO-dependent activation of sGC in human erythroid progenitor cells (Cokic et al., 2003). It has been shown that both sGC activators and cGMP induce γ-globin gene expression in human erythroleukemic cell line and primary erythroblasts (Ikuta et al., 2001). Therefore, intracellular pathway including sGC and PKG induced expression of the γ-globin gene (Ikuta et al., 2001). Moreover, it has been reported that hydroxyurea increased NOx levels and NOS-dependent γ-globin transcription in erythroleukemic and primary erythroid cells. This γ-globin gene activation demonstrated cGMP-dependence (Lou et al., 2009). We found that during human erythroid differentiation in vitro, eNOS mRNA and protein levels were initially high but then declined steadily, as did the production of NO derivatives, in contrast with steady elevation of hemoglobin levels, a potent scavenger of NO (Cokic et al., 2008). According to our previous results, hydroxyurea dose- and time-dependently induced rapid but transient activation of eNOS in endothelial cells (Cokic et al., 2006). Hydroxyurea stimulated NO production in endothelial cells, both as short and long term effects (Cokic et al., 2006, 2007). Chronic hydroxyurea therapy significantly increased NO, cGMP, and HbF levels in patients with sickle cell anemia (Nahavandi et al., 2002). cGMP levels were found to be significantly higher in red blood cells (RBCs) of sickle cell patients than in RBCs of normal individuals, and were further increased in RBCs of sickle cell patients on hydroxyurea therapy (Conran et al., 2004). NOS activity was also higher in RBCs of sickle cell disease patients on hydroxyurea therapy than in untreated patients (Iyamu et al., 2005). It is in accordance with results that l-arginine alone does not increase serum NOx production in steady-state patients, however it does when given together with hydroxyurea (Morris et al., 2003). Neither l-arginine alone nor l-arginine in combination with NOS inhibitor effected hydroxyurea-mediated induction of HbF synthesis in erythroid progenitors (Haynes et al., 2004). L-arginine did not change the suppression of burst forming unit-erythroid (BFU-E) colony growth and stimulation of HbF synthesis by hydroxyurea in erythroid progenitors (Baliga et al., 2010). Inhibition of NOS attenuated the hydroxyurea and l-arginine effects on BFU-E colony growth and HbF synthesis (Baliga et al., 2010), but did not decrease NOx production in RBCs during incubation with hydroxyurea (Nahavandi et al., 2006).

5.2 Nitric oxide synthase levels in red blood cells

It has been demonstrated that human RBCs contain iNOS and eNOS as well as calmodulin, suggesting that RBCs may synthesize its own NO (Jubelin & Gierman, 1996). This notion was supported by the observation that RBCs have an active eNOS protein (Chen & Mehta, 1998). Addition of l-arginine to RBCs stimulated NO production (measured as plasma nitrite) in a dose-dependent manner (Nahavandi et al., 2006), whereas it did not significantly change NOx levels (Chen & Mehta, 1998). However, it was later reported that RBCs possess iNOS and eNOS, but the proteins are without catalytic activity (Kang et al., 2000). Recent studies, revealed eNOS protein activity in the cytosol and in the internal side of membrane RBCs, serving essential regulatory functions for RBCs deformability and platelet

aggregation (Kleinbongard et al., 2006). In vitro NOx production by RBCs (normal and sickle) is increased by treatment with hydroxyurea, but it's not decreased by NOS inhibition (Nahavandi et al., 2006). In difference to this result, we showed that hydroxyurea increased NO production via induction of eNOS activity in endothelial cells (Cokic et al., 2006). Thus, hydroxyurea may increase the plasma concentration of NO by combining endothelial cell NOS activity and interaction with oxy/deoxy hemoglobin in RBCs. We found previously that hydroxyurea increased cAMP and cGMP levels in human endothelial cells (Cokic et al., 2006), as well as NO levels via activation of eNOS and proteasome inhibition (Cokic et al., 2007). Previous reports indicated that cAMP elevation activated the L-arginine/NO system and induced vasorelaxation in rabbit femoral artery in vivo and human umbilical vein in vitro (Xu et al., 2000). It is known that agents that increase cAMP stimulate eNOS activity in human umbilical vein endothelial cells (Ferro et al., 1999). A recent report revealed that a rapid increase in endothelial NO production by bradykinin is mediated exclusively by PKA signaling pathway (Bae et al., 2003). PKA signaling acts by increasing phosphorylation of Ser1177 and dephosphorylation of Thr495 to activate eNOS (Michell et al., 2001). Shear stress stimulates phosphorylation of bovine eNOS at the corresponding serine in a PKA-dependent, but PKB/Akt-independent, manner, whereas NO production is regulated by the mechanisms dependent on both PKA and PKB/Akt (Boo et al., 2002).

5.3 A role of cAMP-dependent pathway in γ-globin gene induction

During erythroid differentiation adenylate cyclase and cAMP phosphodiesterase activity, as well as cellular cAMP concentrations, decline in a synchronized manner (Setchenska et al., 1981). The cAMP-dependent pathway plays a negative role in a γ-globin gene expression in K562 erythroleukemic cell line, in contrast to a cGMP positive role (Inoue et al., 2004). It has been also found that, upon activation of the cAMP pathway, expression of the γ-globin gene is induced in adult erythroblasts (Kuroyanagi et al., 2006). The subsequent study found that the cAMP-dependent pathway efficiently induced γ-globin expression in adult erythroblasts of beta-thalassaemia (Bailey et al., 2007). In patients with beta-thalassaemia intermedia, cAMP levels were elevated in both RBCs and nucleated erythroblasts but no consistent elevation was found with cGMP levels. The transcription factor cAMP response element binding protein (CREB) was phosphorylated in nucleated erythroblasts and its phosphorylation levels correlated with γ-globin gene expression of the patients (Bailey et al., 2007). According to previous study, guanylate cyclase inhibition minimally reduced HbF induction, whereas adenylate cyclase inhibition markedly decreased HbF induction by hydroxyurea in $CD34^+$-derived erythroid cells. Activation of the adenylate cyclase modestly induced HbF production, while hydroxyurea failed to significantly stimulate adenylate cyclase activity on days 7 to 10 of erythroid cells liquid culture (Keefer et al., 2006). However, in our cultures we found that in early erythroid progenitor cell cultures (day 4), hydroxyurea stimulated cAMP production (Cokic et al., 2008). It has been also postulated that cJun activates the ^{G}γ-globin promoter via an upstream cAMP response element in a way equivalent to CREB1 (Kodeboyina et al., 2010).

5.4 Cyclic nucleotides interaction with phosphodiesterases

It has been shown that nitrite increased blood flow in the human circulation as well as vasodilatation of rat aortic rings. Formation of both NO gas and NO-modified hemoglobin

resulted from the nitrite reductase activity of deoxyhemoglobin and deoxygenated erythrocytes levels (Cosby et al., 2003). Studies of nitrite activation of sGC demonstrated that nitrite alone activated sGC in solution (Jeffers et al., 2005). In our performed in vitro studies nitrite failed to induce cGMP, in purified form of sGC in solution, what confirmed a major role of NO molecule in hydroxyurea interaction with sGC (Cokic et al., 2008). It has been also demonstrated that eNOS is rapidly activated and phosphorylated on both Ser1177 and Thr495 in the presence of cGMP-dependent protein kinase II and the catalytic subunit of PKA in endothelial cells. These processes are more prominent in the presence of Ca^{2+}/calmodulin (Butt et al., 2000). The transient rises of cGMP levels induced by bradykinin and endothelin-1, which caused release of Ca^{2+} from internal stores, were similarly enhanced by activation of adenylate cyclase and increased cAMP levels. The cAMP seems to enhance NO formation, which depends on Ca^{2+} release from internal stores (Reiser, 1992). An elevated cGMP level attenuated the store-operated Ca^{2+} entry in vascular endothelial cells (Kwan et al., 2000). The cGMP-mediated [Ca^{2+}]i-reducing mechanisms may operate as a negative reaction to protect endothelial cells from the damaging effect of excessive [Ca^{2+}]i. The main targets of cGMP are phosphodiesterases (PDEs), resulting in interference with the cAMP-signaling pathway (Vaandrager & de Jonge, 1996). cAMP hydrolyzing PDE isozymes in endothelial cells are represented by PDE2 and PDE4 as cGMP-stimulated and cGMP-insensitive PDE, respectively. In endothelial cells, PDE4 inhibition may up-regulate basal production of NO, being supported by PDE2 inhibition (Lugnier et al., 1999). cGMP-inhibited PDE3 was expressed in K562 erythroleukemic cells at a high level (Inoue et al., 2004), while PDE3/4 inhibitor treatment reduced asymmetrical dimethylarginine, an endogenous NOS inhibitor, and elevated NO/cGMP levels (Pullamsetti et al., 2011). In addition, PDE9A gene expression is increased in $CD34^+$-derived erythroid cells and K562 erythroleukemic cells. Inhibition of PDE9A enzyme significantly increased production of the γ-globin gene in K562 cells (Almeida et al., 2008).

6. Nitric oxide-related therapy in hemoglobinopathies

NO inhibits HbS polymer formation and has anti-sickling properties. NO may disrupt HbS polymers by abolishing the excess positive charge of HbS, resulting in increased oxygen affinity in patients with sickle cell disease (Ikuta et al., 2011). In sickle cell disease, HbS polymerization and intravascular sickling lead to reperfusion injury, hemolysis, decreased NO bioavailability and oxidative stress. Increased expression of HbF decreased intravascular sickling, accompanied by decreased hemolysis, oxidative stress and increased NO metabolites (NOx) levels (Dasgupta et al., 2010). Nitrite can react similarly with adult oxy- and deoxy-hemoglobin (HbA), resulting in oxidative denitrosylation of nitrosyl-hemoglobin and rapid dissociation of NO. RBCs containing oxy-HbF (F-cells) had accelerated oxidative denitrosylation. So, induction of HbF present in sickle cell disease may enhance vasodilatation in addition to direct inhibition of polymerization of deoxy sickle hemoglobin (Salhany, 2008). The role of NO in erythrocyte function, sickle cell anemia, malaria, and damage to banked blood has been already reviewed, as well as the use of NO targeted therapies for erythrocyte disease (Maley et al., 2010). Pain from vaso-occlusive crisis is the major cause of hospitalization in patients with sickle cell disease, where beneficial therapeutic effects of inhaled NO have been demonstrated (Head et al., 2010). Decreased exhaled nitric oxide levels (FE_{NO}) have been described in patients with sickle cell disease, together with deficiency in plasma arginine. Additional study shows that sickle cell

disease patients, with and without a history of acute chest syndrome, have similar FE_{NO} at baseline when compared with healthy controls (Sullivan et al., 2010).

The protecting effects of exogenous NO on murine cerebral malaria are associated with decreased brain vascular expression of inflammatory markers, ICAM-1 and P-selectin, resulting in attenuated endothelial damage and facilitating blood flow (Zanini et al., 2011). Previous reports demonstrated reduced NO levels in severe malaria related to impaired production of NO, reduced mononuclear cell iNOS expression and NOS substrate arginine (Anstey et al., 1996; Lopansri et al., 2003). Responsible factors for low NO levels in malaria include scavenging of NO by free hemoglobin and superoxide anion, and reduced levels of nitrate, a NO precursor molecule (Lopansri et al., 2003). Endothelial activation plays a central role in the pathogenesis of severe malaria with angiopoietin-2 as a key regulator. NO is a major inhibitor of angiopoietin-2 release from endothelium and has been shown to decrease endothelial inflammation and reduce the adhesion of parasitized RBCs. Low-flow inhaled NO is an attractive new candidate for the adjunctive treatment of severe malaria (Hawkes et al., 2011). Exhaled NO was also lower in severe malaria in comparison to moderately severe falciparum malaria and controls. Intravenous administration of l-arginine increased exhaled NO in moderately severe malaria (Yeo et al., 2007).

7. Conclusion: Nitric oxide and soluble guanylate cyclase

Besides direct stimulation of sGC in erythroid cells, NO is produced by stromal cells of bone marrow hematopoietic microenvironment. NOS enzymes in stromal cells are activated via PKA, supported by intracellular Ca^{2+} elevation. NO has been released into the intercellular space and then passed through the plasma membrane of erythroid cells, where it binds directly to ferrous-deoxy heme of sGC, activating the enzyme. Activation of NO-sGC increases conversion of GTP to cGMP, resulting in elevation of cGMP and subsequent activation of cGMP-dependent protein kinases (PKG) and cGMP-hydrolyzing PDEs. Activation of sGC and PKG increases expression of the γ-globin gene in erythroid cells. NO reduces cAMP levels in erythroid cells, whereas cAMP appears to enhance NO formation. The NO-mediated cAMP-reducing mechanisms may operate as a negative feedback in control of cAMP levels. In addition, cGMP enhances cAMP level and cAMP-signalling pathway by competing for the PDEs active site that has modest cyclic nucleotides selectivity (e.g., PDE3 isozymes). By this way, cGMP inhibits the activity of cAMP-specific PDE3, which results in the increase in intracellular cAMP levels and thereby leads to the activation of PKA. Activation of the cAMP-dependent pathway also induces expression of the γ-globin gene in erythroblasts. The phosphorylation levels of CREB correlated with elevated γ-globin gene expression. Moreover, inhibition of PDE9A enzyme significantly increases production of the γ-globin gene. Therefore, it appears that activation of the linked cGMP- and cAMP-signalling pathways regulates γ-globin expression.

Presented results contribute to the understanding of the significance of NO participation in γ-globin induction. These results should support future studies, with the emphasis focused on the hematopoietic microenvironment, in search of therapy of sickle cell disease. In addition to the possibility of NOS presence and activity in mature RBCs, our data show strong eNOS protein levels and function in more primitive human erythroid progenitor and precursor cells, where control of gene expression occurs. While mechanisms involved in globin gene expression have been recognized at different levels within the regulatory

hierarchy, relations between molecular pathways are only emerging. Our presented microarray results demonstrated the broad gene expression profile and related pathways linked to stimulation of globin genes during ontogeny. The presented genes and signaling pathways, involved in the mechanism of globin genes activation, might be targets for therapeutic agents that upregulate γ-globin gene expression and HbF levels in hemoglobinopathies. This ontogenic overview linked to specific genes and transcriptional programs in normal erythropoiesis may contribute to further understanding of erythroid progenitor cell development.

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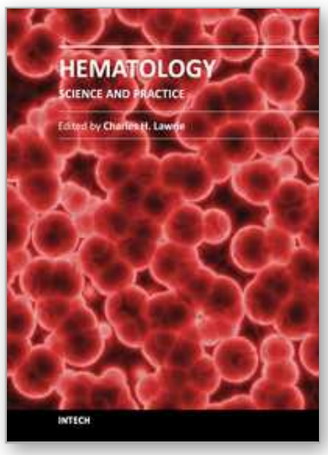

Hematology - Science and Practice

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Hematology encompasses the physiology and pathology of blood and of the blood-forming organs. In common with other areas of medicine, the pace of change in hematology has been breathtaking over recent years. There are now many treatment options available to the modern hematologist and, happily, a greatly improved outlook for the vast majority of patients with blood disorders and malignancies. Improvements in the clinic reflect, and in many respects are driven by, advances in our scientific understanding of hematological processes under both normal and disease conditions. Hematology - Science and Practice consists of a selection of essays which aim to inform both specialist and non-specialist readers about some of the latest advances in hematology, in both laboratory and clinic.

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