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Clonogenicity, gene expression and phenotype during neutrophil versus erythroid differentiation of cytokine-stimulated CD34⁺ human marrow cells *in vitro*

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Summary

With the objective to correlate clonogenicity, gene expression and phenotype during differentiation, human bone marrow CD34⁺ cells were cultured *in vitro* to stimulate erythroid or neutrophil development, and sorted into five subpopulations according to their surface expression of CD15/CD33 and blood group antigen A/CD117 respectively. Sorted cells were cultured in methylcellulose and analysed by real-time reverse transcription polymerase chain reaction for expression of neutrophil and erythroid marker genes. Surface expression of CD15 coincided with restriction to neutrophil/monocyte differentiation and A antigen with restriction to erythroid differentiation. GATA-2 mRNA was down-regulated during both neutrophil and erythroid maturation, whereas GATA-1, SCL, ABO, erythropoietin receptor, Kell, glycophorin A, β -globin and α -haemoglobin stabilizing protein were up-regulated during erythroid differentiation and silenced during neutrophil differentiation. CCAAT/enhancer-binding protein (C/EBP)- α , PU.1, granulocyte colony-stimulating factor receptor, PR3, C/EBP- ϵ and lactoferrin were sequentially expressed during neutrophil differentiation but rapidly down-regulated during the early erythroid stages. Nuclear factor erythroid-derived 2 (NF-E2) and glycophorin C were expressed both during neutrophil and erythroid differentiation. Our data support the notion of early expression of several lineage-associated genes prior to actual lineage commitment, defined by surface expression of CD15 and A antigen as markers for definitive neutrophil/monocyte and erythroid differentiation respectively. Previous findings, primarily from cell lines and mouse models, have been extended to adult human haematopoiesis.

Keywords: clonogenicity, erythroid cell differentiation, immunophenotype, myeloiesis, transcription factors.

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Haematopoietic stem cells develop into progenitor cells committed to one lineage of differentiation through a process governed by transcription factors and stimulated by haematopoietic growth factors to sustain survival and proliferation (Zhu & Emerson, 2002). Gene targeting experiments in mice and overexpression of certain genes in transduced cell lines of human or murine origin have provided important information about the role of many of the genes critical for haematopoietic development and terminal differentiation. Results from a number of such studies have formed our present understanding of haematopoietic differentiation. It is generally

believed that early neutrophil commitment depends on the transcription factors CCAAT/enhancer-binding protein (C/EBP)- α , PU.1, RAR, core-binding factor (CBF) and c-Myb, and terminal neutrophil differentiation on C/EBP- ϵ and PU.1 (Friedman, 2002). Likewise, GATA-1, FOG-1, erythroid Kruppel-like factor (EKLF) and core-binding protein (CBP) are essential for early erythroid and megakaryocytic development (Cantor & Orkin, 2002). However, only selected parts of the developmental schemes have been confirmed in studies of primary human progenitors and their progeny. For instance, single cell reverse transcription polymerase chain

reaction (RT-PCR) has been applied to cells from different stages of erythroid and myeloid colonies to characterize the gene expression of transcription factors stem cell leukaemia (SCL), GATA-1, GATA-2, nuclear factor erythroid-derived 2 (NF-E2), PU.1, acute myeloid leukaemia (AML)1B, and C/EBP α , as well as the cytokine receptors, erythropoietin receptor (EpoR), granulocyte colony-stimulating factor receptor (G-CSFR) and macrophage colony-stimulating factor receptor (M-CSFR) (Cheng *et al*, 1996). The role of C/EBP α (Radomska *et al*, 1998; Reddy *et al*, 2002; Cammenga *et al*, 2003) and C/EBP ϵ (Morosetti *et al*, 1997) in neutrophil differentiation has been investigated in several human cell lines and essentially confirmed in limited studies of primary human progenitors and retrovirally transduced CD34⁺ cells. In a recent investigation, the transcription factor profiles of bone marrow populations enriched for myeloblasts/promyelocytes, myelocytes/metamyelocytes, band and segmented forms, and mature blood neutrophils, respectively, were characterized (Bjerregaard *et al*, 2003). With regard to erythroid development, the time course for expression of blood group antigens has been characterized in a few studies (Bony *et al*, 1999; Southcott *et al*, 1999; Daniels & Green, 2000). Southcott *et al* (1999) used CD34⁺ cells isolated from cord blood and found that the surface expression of Kell glycoprotein appears first. Bony *et al* (1999) presented similar results and added A antigen as an early marker during erythroid differentiation, appearing simultaneously with Kell. Also, gene expression during erythroid differentiation in *in vitro* cultures has been partly characterized (Gubin *et al*, 1999; Ziegler *et al*, 1999; Pope *et al*, 2000; Scicchitano *et al*, 2003). Ziegler *et al* (1999) in an elegant study, used single cell RT-PCR on cord blood CD34⁺/Lin-cells stimulated towards erythroid maturation and analysed the expression of transcription factors GATA-1, GATA-2, NF-E2, EKLF, SCL, PU.1, cytokine receptors EpoR and c-kit, and surface markers glycophorin A (GPA) and CD36 after two to 16 cell divisions.

However, most of the studies on gene expression during neutrophil or erythroid differentiation lack the phenotypic characterization of the cells analysed and it is conceivable that cells harvested at a specific day of culture are not homogeneous in terms of maturation. In addition, they mostly lack information on the clonogenic capacity of the cells studied. We believe that it is important to perform this type of study on phenotypically well-characterized populations and to include an assessment of the clonogenic capacity in parallel with gene expression profiling, to better understand the relationship between lineage commitment, phenotype and gene expression as the determining features of haematopoietic differentiation. Therefore, we have utilized two culture systems sustaining differentiation of normal human CD34⁺ marrow cells into morphologically mature neutrophils or erythroid cells in advanced differentiation, respectively, to study the relationship between clonogenicity, gene expression and phenotype during the early stages of neutrophil and erythroid differentiation. We found that restriction to neutrophil/monocyte commitment

was concomitant with surface expression of CD15 and erythroid commitment was concomitant with surface expression of blood group antigen A. With regard to these two restriction points, the gene expression pattern has been characterized for transcription factors GATA-1, GATA-2, SCL, C/EBP- α , C/EBP- ϵ , PU.1 and NF-E2, as well as erythroid marker proteins ABO transferase, α -haemoglobin stabilizing protein (AHSP), EpoR, GPA, glycophorin C (GPC) and Kell, and neutrophil differentiation markers, such as G-CSFR, and granule components proteinase 3 (PR3) and lactoferrin.

Material and methods

Isolation of CD34⁺ cells from bone marrow

Human adult bone marrow was obtained from healthy volunteers after ethical approval and informed consent. Mononuclear cells (MNCs) were isolated by separation on Lymphoprep (Nycomed Pharma, Oslo, Norway), and CD34⁺ cells enriched by labelling with magnetic beads according to the manufacturer's instructions (CD34 Progenitor Cell Isolation Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity was regularly above 90%, as determined by flow cytometry. Bone marrow MNCs were genotyped as blood group A1O1 (Olsson & Chester, 1995).

Liquid culture

To obtain *in vitro* erythroid differentiation, CD34⁺ cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% fetal calf serum (FCS), 2-mercaptoethanol (100 μ mol/l), hydrocortisone (1 μ mol/l), granulocyte/macrophage colony-stimulating factor (GM-CSF, 1 pg/ml; Leucomax[®], Schering-Plough, Novartis, Kenilworth, NJ, USA), interleukin 3 (IL-3, 10 pg/ml; Stem Cell Technologies, Vancouver, BC, Canada) and Epo (10 U/ml; Eprex[®], Janssen-Cilag, Wycombe, Buckinghamshire, UK) over 21 d, modified after Malik *et al* (1998). To obtain neutrophil differentiation CD34⁺ cells were cultured in IMDM with 20% FCS, G-CSF (50 ng/ml; Neupogen[®], Amgen, Thousand Oaks, CA, USA) and stem cell factor (SCF, 50 ng/ml; Stem Cell Technologies) over 15 d. Cells were plated at 3×10^5 cells/ml in 24-well plates and after day 4 repeatedly diluted in fresh complete medium when reaching 1×10^6 cells/ml. To evaluate cell morphology, cytospin preparations were produced at serial time intervals and stained with May-Grünwald-Giemsa.

Flow cytometric analysis and cell sorting

Cells were labelled with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against surface markers CD13, CD15, CD66, CD71 (DakoCytomation, Glostrup, Denmark), A antigen (BRIC 145) and Kell (BRIC 68) [International Blood Group Reference Laboratory (IBGRL), Bristol, UK]; phycoerythrin (PE)-conjugated antibodies

against CD11b, CD13, CD33 (DakoCytomation), GPA, CD71 and CD117 (BD Biosciences Pharmingen, San Diego, CA, USA), Kell (BRIC 203) and GPC (BRIC 4 and BRIC 10; IBGRL); peridinin chlorophyll – cyanin 5·5 (PerCP-Cy5·5)-conjugated antibodies against CD34 and CD33 (BD Biosciences); allophycocyanin (APC)-conjugated anti-CD33 and anti-CD15 (BD Biosciences). Cells were analysed on a FACSCalibur and sorted on a FACS Aria flow cytometer equipped with an automatic cell deposition unit (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Cells were sorted (single cell mode) directly into 100 µl methylcellulose culture medium in 96-well plates for clonogenic assay, or into PCR-tubes containing lysis buffer for real-time RT-PCR assay. 4',6-Diamidino-2-phenylindole (DAPI, 3 µmol/l; Molecular Probes, Leiden, the Netherlands) was included to discriminate between live and dead cells and excited by the violet laser (405 nm FACS Aria).

In vitro colony assay

Sorted cells (10 cells/well and 12 wells/population) were cultured over 14 d in IMDM-based methylcellulose medium containing 30% FCS, 1% bovine serum albumin (BSA), 100 µm 2-mercaptoethanol, 2 mmol/l L-glutamine, recombinant human (rh)SCF 50 ng/ml, rhGM-CSF 20 ng/ml, rhIL-3 20 ng/ml, rhIL-6 20 ng/ml, and rhG-CSF 20 ng/ml (Methocult GF H4535; Stem Cell Technologies) and Epo 3 U/ml (Eprex®, Janssen-Cilag) to evaluate the clonogenic growth of mixed lineage (CFU-GM), macrophage (CFU-M), granulocyte (CFU-G) colony-forming units and erythroid burst-forming units (BFU-E).

Sample processing and RT

About 500 cells from selected populations were sorted directly into PCR-tubes containing 42 µl of cell lysis buffer containing 0·5% nonidet P-40 (NP-40) in nuclease-free water and 40 U RNase inhibitor (Applied Biosystems, Foster City, CA, USA). Samples were heated for 1 min at 65°C for complete cell lysis, cooled to 22°C for 3 min and kept on ice, a modified method derived from Cheng *et al* (1996) and Chen *et al* (2000). The RT reaction was performed using the Sensiscript RT kit (Qiagen, Hilden, Germany). 1X RT buffer; 0·5 mmol/l each of dATP, dCTP, dGTP and dTTP; 0·5 U RNase inhibitor (Applied Biosystems) per µl reaction volume; 2·5 µmol/l random hexamers (Applied Biosystems) and 0·05 µl of Sensiscript reverse transcriptase per µl reaction volume, were added to the lysate, after which the samples were incubated at 37°C for 1 h followed by 5 min at 95°C.

To obtain cDNA for use in serial dilutions for standard curves, total RNA was isolated, using RNeasy mini kit (Qiagen), from fresh bone marrow MNCs, fresh CD34⁺ cells and cells cultured in neutrophil differentiation culture for 5 d and in erythroid differentiation culture for 5 and 6 d. RNA concentration was measured by spectrophotometry (Eppen-

dorf BioPhotometer, Hamburg, Germany). RT was performed using TaqMan RT Reagents (Applied Biosystems) according to the manufacturer's guidelines, with 1 µg RNA/100 µl reaction, whereupon the different cDNA were pooled together, to be used in standard curves for all targets (see below).

Real-time RT-PCR and data analysis

Approximately 7·5% of the cDNA from 500 sorted cells was used per PCR-reaction (25 µl) with 1X TaqMan Universal PCR Master Mix (with AmpErase UNG) and 1X Assays-on-Demand Target or Endogenous Control Assay (listed in Table I). The gene-specific primers and fluorogenic probe for β-globin (Table I) were designed using PRIMER EXPRESS 1·5A software (PE Applied Biosystems) and a final concentration of 250 nmol/l probe and 900 nmol/l primers were used. All samples were run in triplicates. Data were collected and quantitatively analysed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Standard curves were generated for each gene and the serial dilutions of cDNA enabled the quantification of relative levels of the specific mRNA of interest, by the relative standard curve method described by the manufacturer [Applied Biosystems User Bulletin no. 2: Relative Quantitation of Gene Expression (PN 4303859)]. Target quantities were normalized to 18S ribosomal RNA and calibrated using values from fresh CD34⁺ cells, defined as 1·0. For those targets not expressed in this population, the population with the lowest positive value was used as calibrator. All other quantities were expressed as an *n*-fold difference relative to the calibrator. We considered as positive any sample with at least two detected values within the triplicate. The SD of values obtained from three different targets applied on five identical sorted populations of 500 cells was 0·17, 0·24 and 0·16 respectively.

Results

Suspension cultures

In neutrophil culture, the cells expanded more than 150-fold and in erythroid culture more than 20-fold (Fig 1). On day 14 of neutrophil culture most cells were mature neutrophils, although occasional metamyelocytes, myelocytes and promyelocytes were also present (Fig 2A). In erythroid culture, differentiation was evident from the first 2–3 d of culture when most cells were pronormoblasts, followed sequentially by maturing normoblasts, until the enucleation of pycnotic nuclei, as shown in Fig 2B. Occasional monocyte/macrophages were also present.

Flow cytometric-analysis of differentiation into mature neutrophils and erythrocytes

Differentiation of CD34⁺ cells over time, as represented by surface markers, is shown in Fig 3. CD34-positivity was

Gene	Gene product/protein	Assay ID
<i>ABO</i>	ABO transferase	Hs 00220850_m1
<i>CEBPA</i>	C/EBP α	Hs 00269972_s1
<i>CEBPE</i>	C/EBP ϵ	Hs 00357657_m1
<i>AHSP/ERAF</i>	AHSP/EDRF	Hs 00372339_g1
<i>EPOR</i>	EpoR	Hs 00181092_m1
<i>GATA1</i>	GATA-1	Hs 00231112_m1
<i>GATA2</i>	GATA-2	Hs 00231119_m1
<i>CSF3R</i>	G-CSFR	Hs 00167918_m1
<i>GYP A</i>	GPA (glycophorin A)	Hs 00266777_m1
<i>GYP C</i>	GPC (glycophorin C)	Hs 00242583_m1
<i>KEL</i>	Kell antigen	Hs 00220850_m1
<i>LTF</i>	Lactoferrin	Hs 00158924_m1
<i>NFE2</i>	NF-E2	Hs 00232351_m1
<i>PRTN3</i>	PR3 (proteinase 3)	Hs 00160521_m1
<i>SPI1</i>	PU.1	Hs 00231368_m1
<i>TAL1</i>	SCL/Tal1	Hs 00268434_m1
	18S rRNA	Hs 99999901_s1
		Primer/probe sequence
<i>HBB</i>	β -globin	
	Forward primer	5'-CACCTTTGCCACACTGAGTGA-3'
	Reverse primer	5'-GTGATGGGCCAGCACACA-3'
	Probe	5'-FAM-TGAGAACTTCAGGCTCCT-MGB-3'

Table I. Primers and probes for reverse transcription polymerase chain reaction (RT-PCR).

Genes assayed for expression by real-time RT-PCR. The identification number (Applied Biosystems) for Assays-on-Demand Targets and Endogenous Control Assays are shown in the third column. Primer express-designed primers/probe for β -globin are shown at the bottom.

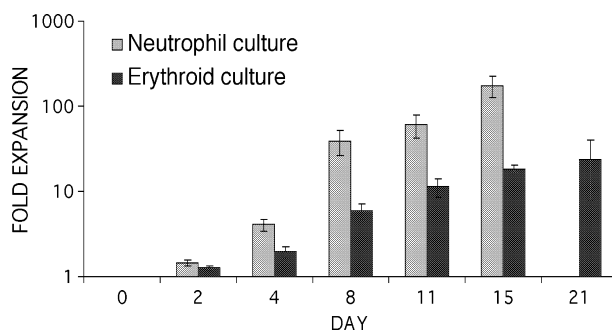


Fig 1. Total cell expansion in liquid culture. CD34⁺ cells were plated at 3×10^5 cells/ml and stimulated with either granulocyte colony-stimulating factor (G-CSF, 50 ng/ml) and stem cell factor (SCF, 50 ng/ml) to sustain neutrophil differentiation, or granulocyte/macrophage colony-stimulating factor (GM-CSF, 1 pg/ml), interleukin (IL)-3 (10 pg/ml) and erythropoietin (Epo, 10 U/ml) to sustain erythroid differentiation. The cultures were diluted and replenished with fresh medium whenever cell numbers reached 10^6 cells/ml. Results are mean values and SD of three marrow samples, with fold expansion represented on a logarithmic scale.

reduced to 40% of the cells after 2 d in both neutrophil and erythroid cultures, and declined further to become negative on days 12 and 15 respectively. The surface expression of CD117 showed a different pattern; CD117 declined rapidly in neutrophil culture whereas it was maintained at decreasing levels over the entire erythroid culture period. This was seen even when SCF was excluded from the neutrophil culture and

was thus not a result of cytokine-mediated receptor down-modulation (data not shown). Neutrophil-expressed markers, such as CD11b, CD13, CD15 and CD66, increased from low initial levels and reached maximum expression (75–100%) from day 7 onwards in neutrophil culture, while they were down-regulated and virtually absent from late erythroid culture. The erythroid markers A antigen and GPA, on the other hand, had low or no expression in neutrophil culture. In erythroid culture the pattern was reversed, with more than 80% GPA⁺ and A⁺ cells after 2 weeks. Notably, the A antigen appeared earlier than the more commonly used erythroid marker GPA, while both blood group markers GPC and Kell glycoprotein, although appearing early, were similarly expressed in the two cultures and thus not erythro-specific.

However, in erythroid culture, most cells expressed higher levels of GPC than in neutrophil culture and the Kell-positive cells were mostly CD33-positive in neutrophil culture, whereas the proportion of Kell⁺/CD33-negative cells increased with time in the erythroid culture (data not shown). The results presented in Fig 3 were obtained using the anti-GPC BRIC 10 (sialic acid-independent) and anti-Kell BRIC 68 antibodies, but subsequent use of the anti-GPC BRIC 4 (sialic acid-dependent; Daniels & Green, 2000) and anti-Kell BRIC 203 antibodies resulted in similar expression patterns (data not shown). CD71-expression differed between erythroid and neutrophil cultures; similar to GPC, the majority were CD71^{hi} cells in erythroid culture, while the neutrophil culture contained mostly CD71^{lo} cells (data not shown). CD33, which

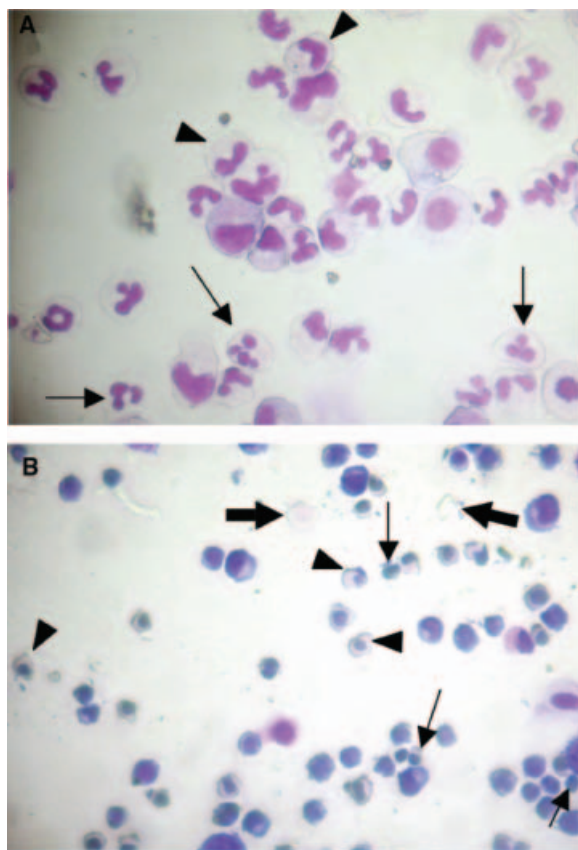


Fig 2. Terminal maturation in liquid culture. Photomicrographs of May-Grunwald-Giemsa stained cells. (A) At day 14, neutrophil culture was dominated by band cells (arrowhead) and mature segmented neutrophils (arrow). (B) At day 13, erythroid culture contained mostly orthochromatic normoblasts with pycnotic nuclei (arrowhead), expelled nuclei (arrow) and reticulocytes (bold arrow), although earlier differentiation stages were also present. Original magnification $\times 400$.

was expressed on about 50% of the cells at the start, increased and reached a plateau in neutrophil culture from day 4 to day 10 before decreasing, while it decreased over the entire erythroid culture period.

Results from three- and four-colour FACS analysis of the evolution of phenotypic patterns over time indicated that, from the same day of culture, certain combinations of surface markers could separate cells according to their degree of maturation, as illustrated in Fig 4. In neutrophil culture, differentiating cells first gained CD33-expression, followed by CD15 and finally down-regulation of CD33. In contrast, in the erythroid culture, erythroid differentiation was represented by CD117-expression, up-regulation of A antigen and ultimately, loss of CD117. Within the CD34⁺ population on day 0, the subfraction of CD15⁺ cells (10–15%) was also CD117⁺ and CD33⁺, and the subfraction of A⁺ cells (5–10%) was CD117⁺ and CD33^{lo} or negative. There were no CD15 and A antigen double-positive cells among the freshly isolated cells or during the culture period (data not shown). These findings were used in subsequent cell sorting experiments, for analysis of

clonogenic capacity and gene expression during erythroid and neutrophil differentiation.

Clonogenic capacity

Early stages of the maturation pathways were further characterized by sorting defined subpopulations of cells into methylcellulose for clonogenic growth, on day 2 and 5 of liquid culture. As shown in Fig 5, CD15⁻/CD33⁻ cells and CD15⁻/CD33⁺ cells produced CFU-GM/M/G and BFU-E. However, with the appearance of CD15 on the surface, the cells lost their erythroid capacity and could only form CFU-GM/M/G. In a similar way, A⁻/CD117⁻ and A⁻/CD117⁺ cells from the erythroid culture gave rise to both CFU-GM/M/G and BFU-E, but expression of A antigen only allowed the formation of erythroid colonies. Notably, the A⁺/CD117⁻ cells gave rise to no colonies at all, indicating that erythroid cells lose their colony-forming ability as CD117 is down-regulated. There was no significant change in cloning capacity between day 0 and day 2 of culture, but it declined rapidly after day 4–5 of culture although a small number of both neutrophil and erythroid progenitors remained after 1 week (data not shown). The purity of two adjacent sorting gates was regularly >90% (e.g. gate numbers 3 and 4 in Fig 5) and >99% for two non-adjacent gates (e.g. gate numbers 2 and 4).

Gene expression in neutrophil and erythroid maturation

The same populations that were cultured in methylcellulose were also sorted directly to PCR tubes for analysis of gene expression by real-time RT-PCR (Fig 6). The gene expression in freshly isolated and sorted CD34⁺ cells was used for comparison with the exception of GPA and lactoferrin, which were undetectable in fresh CD34⁺ cells. Transcription factor GATA-2 mRNA was up-regulated during the early stages of neutrophil differentiation but was rapidly down-regulated, concomitant with the appearance of CD15 surface expression, and in erythroid culture GATA-2 was down-regulated after A antigen surface expression. The erythroid-associated transcription factors SCL and GATA-1 were expressed at low mRNA levels in the early stages of both cultures, but disappeared with neutrophil differentiation, while they markedly increased with erythroid differentiation with the highest expression after the appearance of A antigen (day 5). NF-E2, another erythroid-associated transcription factor, was more highly expressed during early neutrophil differentiation stages than in early erythroid stages, whereas the opposite was true for late neutrophil and erythroid development. The EpoR gene, although predominantly expressed in erythroid cells at increasing levels with differentiation, retained a low expression throughout most neutrophil developmental stages. GPC mRNA was also expressed at all neutrophil stages and increased markedly during erythroid differentiation. The ABO gene expression disappeared from neutrophil culture when CD15 appeared and increased several-fold

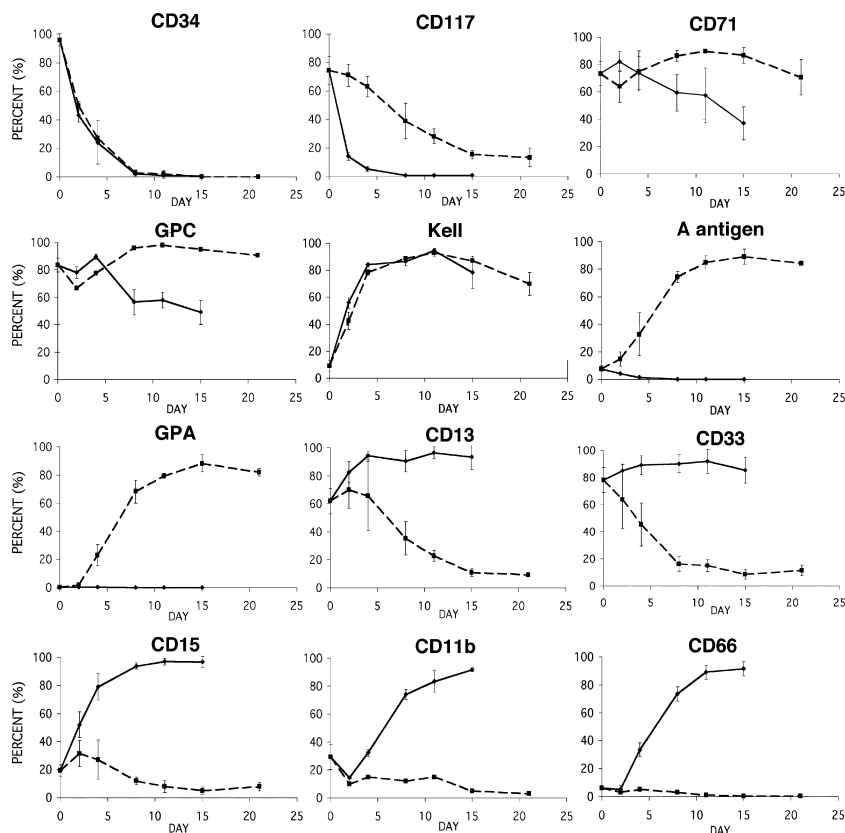


Fig 3. Development of surface markers. Percentage of cells in liquid culture positive for CD34, CD117, CD71, GPC, Kell, A antigen, GPA, CD13, CD33, CD15, CD11b and CD66 at day 0, 2, 4, 7, 10 and 15 in neutrophil culture (unbroken line) and at day 0, 2, 4, 8, 11, 15, and 21 in erythroid culture (broken line). The figure shows mean \pm SD of three to five experiments, except for CD11b and CD66 in erythroid culture, which shows one experiment.

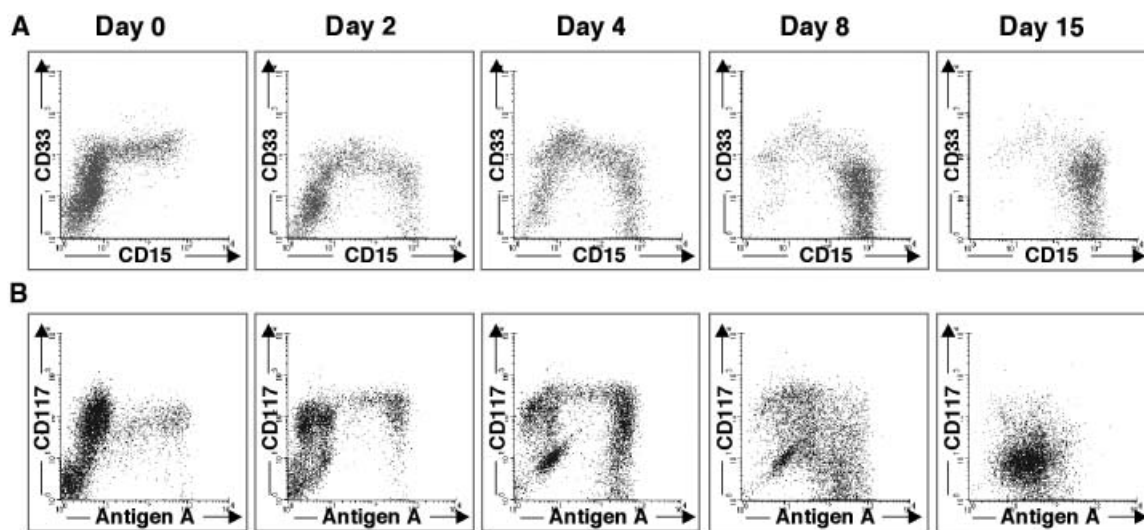


Fig 4. Differentiation reflected in the evolution of CD15/CD33 and A/CD117. Neutrophil and erythroid differentiation are reflected in the continuous evolution of surface markers CD15 versus CD33, and A antigen versus CD117 respectively. The figure shows one of five representative sample of neutrophil (A) and erythroid (B) liquid culture analysed on day 0, 2, 4, 8 and 15.

simultaneously with the appearance of surface A antigen. Notably, at day 5, the ABO gene expression decreased in the late CD117-negative erythroid population, although the surface expression remained. Kell glycoprotein had low or no mRNA expression in early populations and increased markedly

with erythroid development. The even later erythroid markers, GPA, AHSP and β -globin, were expressed almost exclusively in erythroid development.

The neutrophil-/monocyte-associated transcription factors C/EBP α and PU.1 were most highly expressed in the early

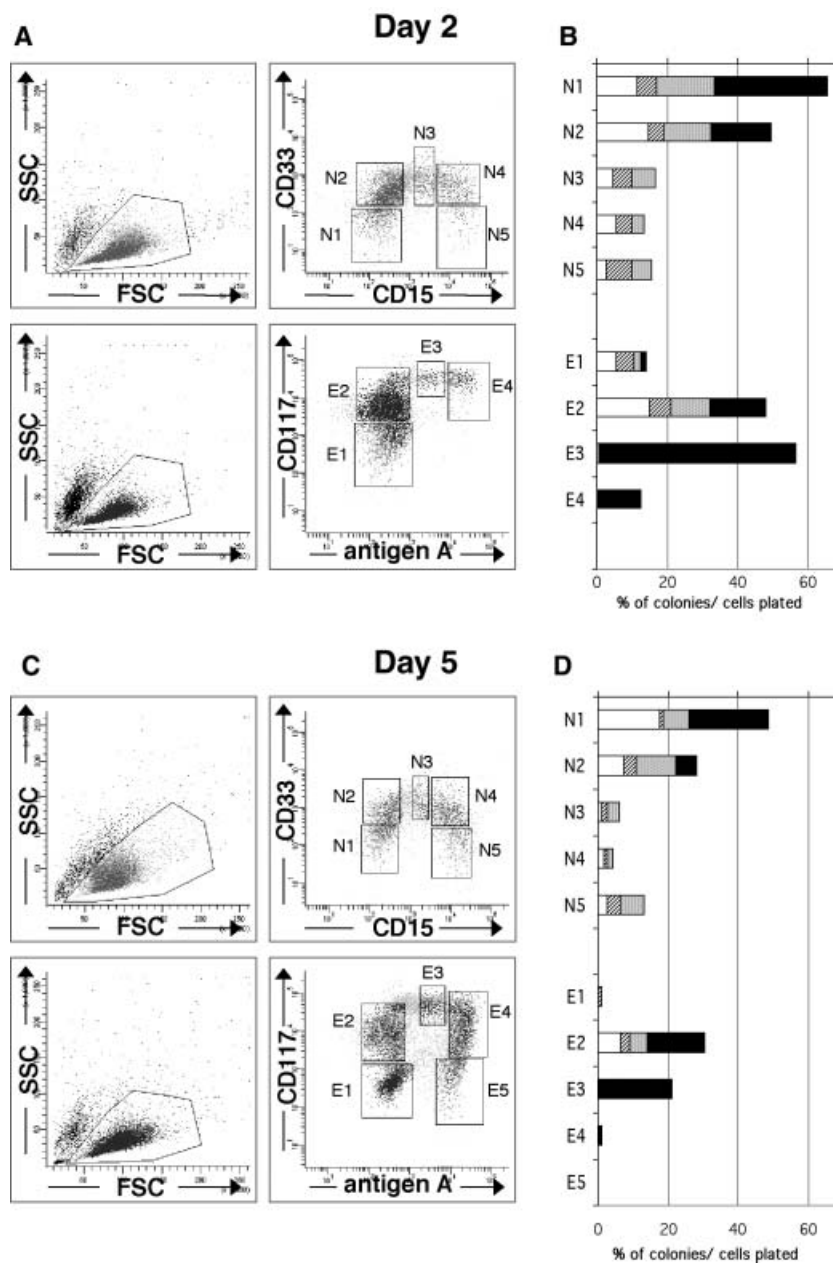


Fig 5. Relationship between clonogenic capacity and surface markers. Cells representing different stages of maturation in neutrophil and erythroid cultures as represented by surface expression of CD15/CD33 and A antigen/CD117 were sorted into methylcellulose on day 2 (A and B) and day 5 (C and D) of liquid culture to assess colony-forming capacity. Cells within a region defined by forward scatter (FSC) and side scatter (SSC) (left dot plots) containing <1% dead cells (4',6-diamidino-2-phenylindole, DAPI-positive) were further divided into four to five gates on the basis of CD15/CD33, numbered N1–N5, and A antigen/CD117 expression, numbered E1–E5 as shown (right dot plots). Ten cells per well and 12 wells per population were sorted to 96-well plates containing 100 μ l medium per well. Colonies were scored in an inverted microscope on day 14. Panels B and D show the cloning capacity for each sorted population and the colony-type composition sorted regions. Unfilled bars: CFU-G, striped bars: CFU-M, shaded bars: CFU-GM, and filled bars: BFU-E and CFU-E. The figure shows one of five representative experiments. Note the five-decade fluorescence scales.

stages of neutrophil differentiation and retained some expression, albeit slightly lower, in CD15⁺/CD33⁻ cells, while expression of these factors almost disappeared from erythroid differentiation after A antigen surface expression. The expression of the G-CSFR gene showed a unique pattern in the sense that there was little difference between the differentiation stages on day 2 and a marked up-regulation in all sorted fractions on day 5 of neutrophil culture. In erythroid culture, G-CSFR gene expression disappeared with the surface expression of A antigen. The late-acting transcription factor C/EBP ϵ increased during neutrophil differentiation with the highest expression in CD15⁺/CD33⁻ cells on day 5, while it disappeared early during erythroid culture. Expression of the primary granule protein PR3 increased during neutrophil

differentiation and appeared earlier than expression of the secondary granule protein lactoferrin, which was expressed almost exclusively in CD15⁺/CD33⁺ cells on day 5.

Discussion

Previously, the importance of specific transcription factors and gene expression patterns during maturation has been studied mostly in differentiation-inducible cell lines, transduced cells and by gene targeting in mice. However, the use of recombinant growth factors has allowed the development of defined *in vitro* cultures, using normal human haematopoietic cells. In this study, we used two simple *in vitro* culture systems, which resulted in mature neutrophils and the late stages of erythroid

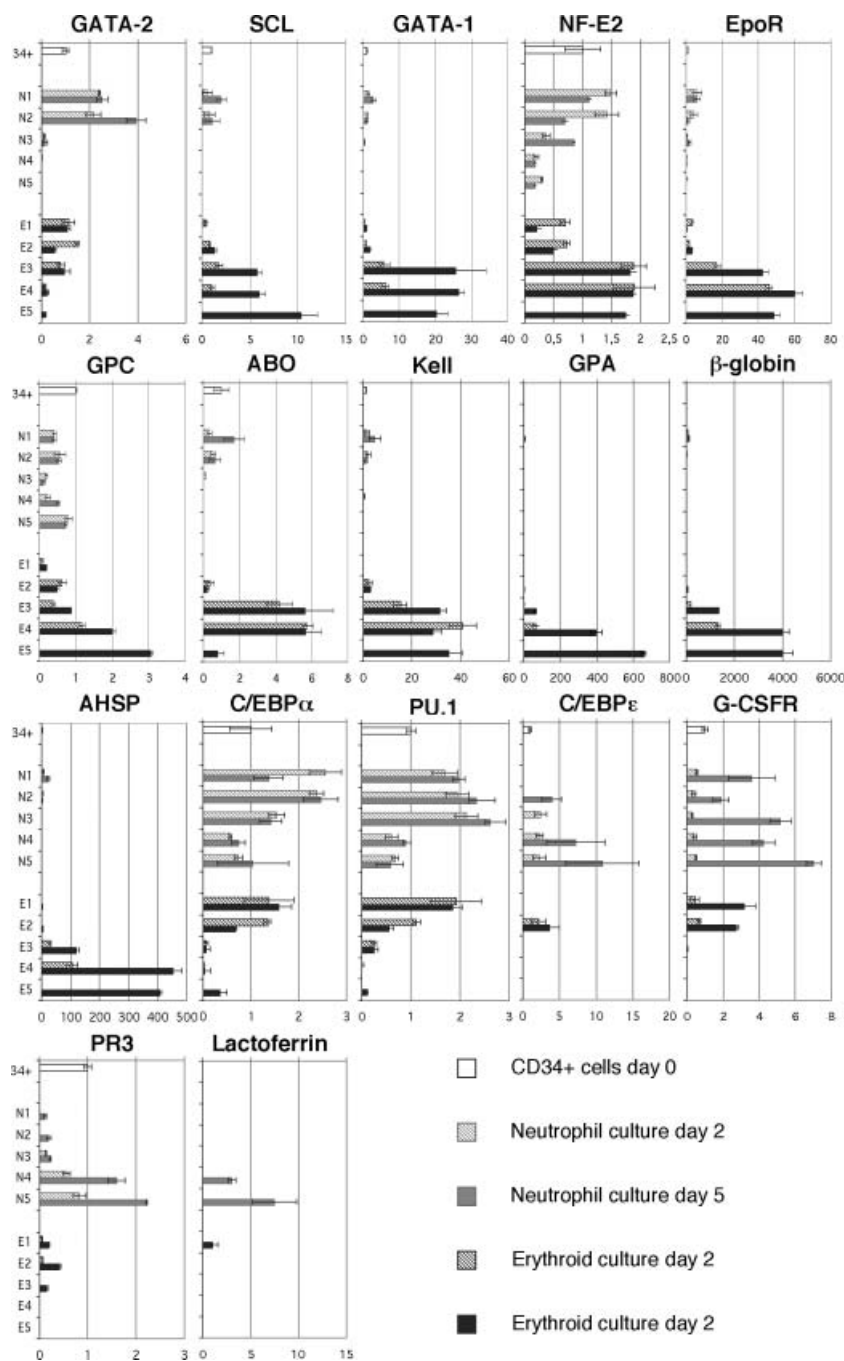


Fig 6. Gene expression analysis of neutrophil and erythroid subpopulations. The same subpopulations of neutrophil and erythroid differentiation stages as shown in Fig 5 were sorted directly to polymerase chain reaction (PCR)-tubes containing lysis buffer (500 cells/tube) and immediately reverse-transcribed. The graphs show *n*-fold differences in gene expression relative to CD34⁺ cells day 0, except for glycoprotein A (GPA) and lactoferrin, which are not expressed in fresh CD34⁺ cells; GPA and lactoferrin instead were compared with the lowest positive value. The bars are oriented identical with Fig 5B and D, and N1–N5 and E1–E5 refer to the sorted populations shown in Fig 5. Each value represents mean and SD, and results from one representative marrow are shown. The same pattern was observed for four marrow samples. Hatched bars show results from day 2 and solid bars from day 5 of neutrophil and erythroid cultures respectively.

differentiation, producing total cell expansion and maturation comparable with previous reports (Panzenböck *et al*, 1998; Scicchitano *et al*, 2003). The attainment of terminal neutrophil and erythroid differentiation was evident from the morphological analysis, showing cells in advanced stages of differentiation, but also by changes in surface marker expression. At the end of neutrophil culture nearly all cells expressed CD15 and CD13 and other neutrophil markers were highly expressed as well, and in erythroid culture cells expressing GPA and A antigen constituted more than 90% of the cells after 2 weeks. It is therefore conceivable that these cultures reflect normal maturation.

However, CD34⁺ human bone marrow cells constitute a fairly heterogeneous population. Consequently, the cultured cells were also heterogeneous and samples from any day of culture contained cells of different stages of maturation although the majority of cells had reached a certain level of maturation. Therefore, it is important to further separate the cells before analysis to couple clonogenicity and gene expression patterns to a certain phenotype. Here, FACS with a cloning device enabled the rapid separation of phenotypically defined subpopulations directly into methylcellulose cultures or lysis buffer for RT-PCR. Although the cultures developed

over 2–3 weeks, changes in gene expression and clonogenicity were most evident during the early stages of the culture period, representing the processes of lineage commitment when the cells still have clonogenic capacity. Since the clonogenic capacity rapidly declines after day 4–5 of culture, we chose to study the relationship between phenotype, gene expression and clonogenicity on day 2 and day 5 to represent the early and late stages of commitment respectively. In a sensitive assay, such as real-time PCR, even a small fraction of cells at another differentiation stage than the majority of cells in the sample may skew the results obtained for differentiation-associated gene expression patterns. To minimize this problem we used the surface expression of CD15/CD33 and A antigen/CD117 to separate the neutrophil and erythroid differentiation stages respectively. By using a sorting strategy that produced several adjacent gates it was possible to achieve cell populations representing the whole spectrum of differentiation on that particular day of culture, although it was impossible to achieve 100% purity for two adjacent gates, even with extensive cell sorting, because of the fact that the phenotypic differentiation pattern described a continuum without clear-cut boundaries (Figs 4 and 5). With these limitations in mind, we still believe that our results from gene expression and clonogenic capacity demonstrated the validity of this strategy. The clonogenic assays demonstrated that cell populations negative for CD15 or A antigen retained potential for both lineages, thus containing immature and uncommitted cells. However, the expression of CD15 and A antigen marked lineage-restricted commitment, since cells expressing CD15 could only form neutrophil/monocytic colonies and cells fully expressing A antigen could only form erythroid colonies. Unlike Sieff *et al* (1982), we found no CFU-GM potential among the cells expressing A antigen. Hence, the clonogenic assays confirmed the applicability of the CD15/CD33 and antigen A/CD117 combinations for illustrating differentiation, and these were accordingly used in the analysis of differentiation-associated changes in gene expression.

All transcription factors investigated here, except for C/EBP- ϵ , showed some expression during the earliest stages of culture before lineage restriction, supporting the notion that transcription factors associated with two or more lineages are expressed at low levels prior to commitment (Cheng *et al*, 1996; Manz *et al*, 2002; Miyamoto *et al*, 2002). The GATA-2 transcription factor is believed to be important mainly in early haematopoiesis (Tsai *et al*, 1994; Perry & Soreq, 2002) and not required for terminal differentiation (Tsai & Orkin, 1997). We noted a minor up-regulation of GATA-2 during the early stages of neutrophil culture that may be a reflection of cell proliferation rather than differentiation, since, with the appearance of CD15 surface expression, GATA-2 was down-regulated and became undetectable. GATA-2 and GATA-1, can inhibit PU.1, but the reverse is also true depending on the relative concentrations of the two transcription factors (Zhang *et al*, 1999). Thus, the up-regulation of PU.1 in neutrophil differentiation until the cells acquire CD15, could possibly

explain the down-regulation of GATA-2 (and GATA-1) at this differentiation stage. However, the continued expression in committed erythropoietic cells suggests a prolonged role for GATA-2 even after the appearance of A antigen. GATA-1 has a well-recognized role in erythropoiesis, and studies in mice and cell lines have indicated a major role in the erythroid commitment process and in the following erythroid differentiation (Cantor & Orkin, 2002; Perry & Soreq, 2002). In accordance with this, we registered a major up-regulation of GATA-1 concomitantly with surface expression of A antigen, but no expression in CD15⁺ neutrophil-committed cells. A very similar expression pattern was observed for SCL, which agreed with its postulated role in erythroid differentiation (Cantor & Orkin, 2002; Zhu & Emerson, 2002). The up-regulation of SCL before that of GPA probably reflects the determining role of SCL in GPA gene expression (Lahlil *et al*, 2004).

The NF-E2, a basic-leucine zipper transcription factor, has an important role in erythropoiesis in the regulation of α - and β -globin gene expression and controlling the gene expression of two of the enzymes involved in haem synthesis (Andrews, 1998). Surprisingly, deletion of the NF-E2 gene has little effect on erythropoiesis, but results in severe thrombocytopenia, reflecting the crucial role of NF-E2 in the development of platelet-producing megakaryocytes (Shivdasani, 2001). Considering its role in globin gene expression, it was expected that NF-E2 would be up-regulated during erythroid differentiation in our culture system, preceding that of β -globin and AHSP. However, we also noted a sustained expression of NF-E2 throughout neutrophil differentiation including CD15⁺ cells, suggesting an unexplored role for NF-E2 in granulocytic/monocytic differentiation. It is conceivable that this may reflect the role of NF-E2 in haem synthesis, since the haem-containing myeloperoxidase is a major constituent of primary granules formed during early neutrophil development.

Several markers have been used for the characterization of erythroid differentiation and some of them have been claimed to be erythro-specific. GPA is truly erythro-specific but appears late during maturation and the GPA gene was expressed almost exclusively in erythroid culture and in cells expressing A antigen and paralleled the expression of the β -globin and AHSP genes. CD71 is expressed in both neutrophil and erythroid cultures, but has a much higher expression level in the latter. Although a population of CD71^{hi} cells is certainly enriched with erythroid progenitors, it is seldom purely erythroid (Mayani *et al*, 1993). CD36, a receptor for thrombospondin, collagens and oxidized lipoprotein, has been used as an early erythroid marker (Ziegler *et al*, 1999; Scicchitano *et al*, 2003), but its broad distribution on non-erythroid cells, including platelets and monocytes, limits its specificity. Other suggested erythro-specific surface markers are the Kell glycoprotein and sialated GPC (Bony *et al*, 1999; Southcott *et al*, 1999; Daniels & Green, 2000). In concordance with previous reports we found an early expression of GPC and Kell glycoprotein in erythroid maturation (Southcott *et al*, 1999; Daniels & Green, 2000; Kie *et al*, 2003), but also an early and

lasting surface expression of GPC and Kell in neutrophil maturation. With regard to GPC this was paralleled with GPC gene expression at all of the differentiation stages. It has been shown that the GPC molecule on erythroid cells is separable from the GPC expressed in other tissues, based on an erythro-specific sialic acid epitope (Le Van Kim *et al*, 1989; Villeval *et al*, 1989). However, our results suggest that all of the sialated epitopes are not erythro-specific, implying that GPC is not suitable as an erythro-specific marker. Regarding the Kell glycoprotein, its presumed erythro-specificity has been contradicted by both clinical and experimental studies in recent years (Russo *et al*, 2000; Wagner *et al*, 2000) and our findings showed that its surface expression does not differ between neutrophil and erythroid differentiation. Considering the high surface expression of Kell in neutrophil culture, its low gene expression was unexpected, and a possible explanation could be an early production of Kell in myeloid cells and an ensuing preservation of the surface expression on neutrophil cells through little or no turnover. Alternatively, the Kell gene could give rise to two separate transcripts through alternative splicing (Russo *et al*, 2000; Camara-Clayette *et al*, 2001), of which only one was detected by our TaqMan-probe.

In our hands, the A antigen was found to be the most reliable early erythro-specific marker. It was already expressed on a subfraction of fresh CD34⁺ bone marrow cells and increased during culture before GPA (Wada *et al*, 1990; Okumura *et al*, 1992; Bony *et al*, 1999). The ABO gene expression increased several-fold concomitantly with attainment of surface A antigen. However, in the A+/117⁻ cells (day 5) the expression decreased substantially, although the surface expression remained high. This supports the hypothesis postulated by Hosoi *et al* (2003), suggesting that although high ABO mRNA expression is necessary to obtain A antigen expression, it is not needed to maintain it. There was no detectable surface expression of A antigen on day 5 in neutrophil culture, but the ABO gene was still expressed in the CD15-negative populations, which is best explained by the fact that these populations contain early erythroid progenitors. The EpoR gene expression was up-regulated many-fold during erythroid differentiation as anticipated, but was also present during neutrophil differentiation, although at decreasing levels. This observation was in agreement with a report of surface as well as gene expression of the EpoR in neutrophils (Sela *et al*, 2001). The function of this neutrophil EpoR is unknown.

The transcription factors C/EBP α and PU.1 are crucial for neutrophil and monocyte development, both in commitment to a bipotent progenitor and in the following bifurcation of the two lineages (Ward *et al*, 2000; Friedman, 2002; Zhu & Emerson, 2002). Accordingly, they were highly expressed in fresh CD34⁺ cells and increased further during early neutrophil culture. There is a complex interplay between C/EBP α and PU.1 since C/EBP α can activate the promoter of the PU.1 gene (Kummalue & Friedman, 2003), but it can also block the function of PU.1 by displacing the co-activator c-Jun from PU.1 (Reddy *et al*, 2002), thereby blocking monocyte

development in favour of neutrophil maturation. An explanation for these opposing effects is provided by the findings that the ratio between C/EBP α and PU.1 probably determines whether the cells will mature into neutrophils or monocytes (Dahl & Simon, 2003; Dahl *et al*, 2003). Both transcription factors were still expressed in CD15⁺ cells on day 2 and 5 of neutrophil culture, which probably reflects the fact that 10–20% of these cells were capable of producing either mixed neutrophil/monocyte colonies or colonies of either cell type alone. The expression of C/EBP α and PU.1 genes during the early phases of erythroid culture (before A antigen acquisition) may be derived from the neutrophil/monocyte progenitors still present in these cell fractions, but it cannot be excluded that C/EBP α and PU.1 have a role in erythropoiesis, in view of our finding of low expression of both transcription factors even in late A+/CD117⁻ cells and recent reports suggesting a role for PU.1 in regulating the proliferation of erythroid progenitors (Back *et al*, 2004; Fisher *et al*, 2004).

The C/EBP α and PU.1 induce the G-CSFR gene (Smith *et al*, 1996) and C/EBP α ($-/-$) mice do not have G-CSF receptors and do not produce mature neutrophils (Zhang *et al*, 1997). It has also been proposed that G-CSFR signalling may induce C/EBP α , thus creating a co-operative autocrine loop between C/EBP α and G-CSFR activities (Ward *et al*, 2000). This relationship between C/EBP α and G-CSFR was illustrated in the neutrophil culture where the expression of the G-CSFR increased several-fold from day 2 to day 5 in all sorted fractions, concomitant with a high expression of C/EBP α . Also in erythroid culture, the G-CSFR gene expression was up-regulated from day 2 to day 5, but only in the A antigen-negative fractions still containing neutrophil/monocyte progenitors, whereas A antigen-positive cells showed no expression at all.

Formation of primary and secondary granules during neutrophil maturation constitutes the basis for the morphological distinction between myeloblasts, promyelocytes and myelocytes. PR3 is a primary granule constituent synthesized in promyelocytes, and it has been demonstrated that PR3 gene expression is up-regulated by G-CSF, mainly mediated by PU.1 and probably also involving c-Myb and C/EBP (Lutz *et al*, 2000, 2001). The time course of PU.1 and PR3 gene expression in neutrophil culture is a reflection of this relationship, as demonstrated by the up-regulation of PU.1 on day 2, whereas the up-regulation of PR3 gene expression was delayed and most prominent on day 5. C/EBP ϵ is believed to be expressed exclusively in the neutrophil lineage (Morosetti *et al*, 1997; Lekstrom-Himes, 2001). C/EBP ϵ -deficient mice lack secondary and tertiary granules and mutations within the C/EBP ϵ gene have been found in humans with neutrophil-specific granule deficiency (Lekstrom-Himes *et al*, 1999). Thus, it is evident that C/EBP ϵ plays an important role in late neutrophil maturation. As then would be expected, it had a later expression than C/EBP α in our neutrophil cultures, its expression increased with differentiation and preceded the expression of the lactoferrin gene, lactoferrin being a marker of neutrophil secondary granules produced in myelocytes.

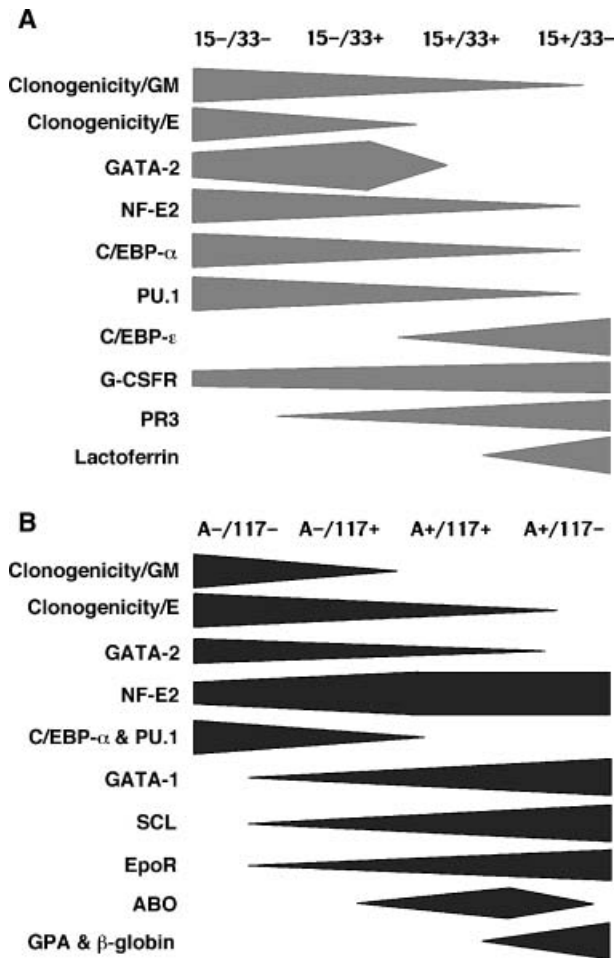


Fig 7. Schematic illustration of the relationship between clonogenicity, gene expression and phenotype. Based on the data presented in Figs 3–6, this figure shows principal changes in clonogenicity and gene expression of some of the central transcription factors and marker proteins for each differentiation pathway in relation to the phenotypic development. Panel (A) shows neutrophil maturation represented by the development of CD15/CD33 expression and panel (B) erythroid maturation represented by A antigen/CD117 expression.

In conclusion, we have used *in vitro* cultures to obtain cells of advanced neutrophil and erythroid differentiation from adult human CD34⁺ bone marrow cells, and mapped differentiation-associated changes in surface antigen and gene expression as well as clonogenic capacity. The relationship between these three parameters is summarized in Fig 7. Our parallel studies of neutrophil and erythroid differentiation of normal human progenitors extend previous findings primarily obtained from studies of leukaemic or transduced cell lines and transgenic mouse models.

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