The EMBO Journal Vol.18 No.20 pp.5559–5566, 1999

HLS7, a hemopoietic lineage switch gene homologous to the leukemia-inducing gene *MLF1*

James H.Williams[1,2], Louise N.Daly[1,2], Evan Ingley[1,2], Jennifer G.Beaumont[1,2], Peta A.Tilbrook[1,2], Jean-Philippe-Lalonde[1,2], Justin P.Stillitano[1,2] and S.Peter Klinken[1,2,3]

[1]Laboratory for Cancer Medicine, Department of Biochemistry, Royal Perth Hospital, Perth, WA 6001 and [2]Department of Biochemistry, University of Western Australia, Nedlands 6907, Australia

[3]Corresponding author
e-mail: pklinken@cyllene.uwa.edu.au

Hemopoietic lineage switching occurs when leukemic cells, apparently committed to one lineage, change and display the phenotype of another pathway. cDNA representational difference analysis was used to identify myeloid-specific genes that may be associated with an erythroid to myeloid lineage switch involving the murine J2E erythroleukemic cell line. One of the genes isolated (*HLS7*) is homologous to the novel human oncogene myeloid leukemia factor 1 (*MLF1*) involved in the t(3;5)(q25.1;q34) translocation associated with acute myeloid leukemia. Enforced expression of HLS7 in J2E cells induced a monoblastoid phenotype, thereby recapitulating the spontaneous erythroid to myeloid lineage switch. HLS7 also inhibited erythropoietin- or chemically-induced differentiation of erythroleukemic cell lines and suppressed development of erythropoietin-responsive colonies in semi-solid culture. However, intracellular signaling activated by erythropoietin was not impeded by ectopic expression of HLS7. In contrast, HLS7 promoted maturation of M1 monoblastoid cells and increased myeloid colony formation *in vitro*. These data show that HLS7 can influence erythroid/myeloid lineage switching and the development of normal hemopoietic cells.

Keywords: erythroleukemia/hemopoiesis/intracellular signaling/lineage switching

Introduction

Mature cells for each of the hemopoietic lineages develop from pluripotent stem cells (Metcalf, 1989). As hemopoietic cells gradually mature, the capacity for proliferation is lost. Leukemias develop when cells maintain self-renewal at the expense of differentiation (Metcalf, 1989). Most leukemias display the phenotypic features of one particular lineage; however, there are examples where leukemic cells have changed phenotype suddenly to display features of another lineage. This could be due to the 'bi-phenotypic' nature of the original transformed cell, or to a phenomenon described as 'lineage switching' (Greaves *et al*., 1986; Klinken *et al*., 1988a). The latter observations suggest a degree of plasticity within transformed cells that enables them to reprogram patterns of gene expression and traverse lineages.

One example of hemopoietic reprograming involved B lymphoma cells from Eμ-*myc* transgenic mice becoming macrophages following introduction of the *raf* oncogene (Klinken *et al*., 1988a). This demonstrated that the introduction of a single gene into committed B lymphoma cells could promote lineage switching. Similarly, murine erythroleukemic J2E cells, transformed with the *raf* and *myc* oncogenes (Klinken *et al*., 1988b), have on rare occasions spontaneously produced variants with phenotypic features of immature monocytic cells (Keil *et al*., 1995). Normally, erythropoietin (epo) induces J2E cells to differentiate into mature, hemoglobin-synthesizing erythroid cells (Klinken *et al*., 1988b; Busfield and Klinken, 1992; Tilbrook *et al*., 1997). These cells display erythroid, but not myeloid or lymphoid, cell surface proteins and express erythroid-specific genes. J2E cells produce only compact colonies in semi-solid culture and, within the limitations of these assays, show no bi-phenotypic characteristics. Significantly, karyotypic abnormalities were present in each of the monocytic cell lines derived from either J2E cells (Keil *et al*., 1995) or Eμ-*myc* B lymphoma lines (Klinken *et al*., 1988a).

J2E-m2 cells are one myeloid variant of the J2E erythroid line that emerged from a spontaneous lineage switch (Keil *et al*., 1995). Unlike the parental J2E line, J2E-m2 cells are adherent, do not respond to epo and morphologically resemble immature monocytic cells. The J2E-m2 cells do not express epo receptors or the erythroid-specific surface marker Ter 119, and have greatly reduced levels of erythroid-restricted transcription factors GATA-1, NF-E2 and EKLF. Unlike other lines that had undergone erythroid/myeloid lineage switching, these cells do not express surface markers of mature monocytes/macrophages (Keil *et al*., 1995). We hypothesized that J2E-m2 cells may still express genes associated with the lineage switch, but fewer structural/functional genes of more differentiated cells.

In an attempt to isolate genes involved in the lineage switch, cDNA representational difference analysis (RDA) was performed on the parental erythroleukemic J2E cells and the modified J2E-m2 monocytoid cells. Hemopoietic lineage switch (HLS) 7 was one myeloid-specific gene isolated by this polymerase chain reaction (PCR)-based subtractive hybridization technique. Intriguingly, *HLS7* is homologous to myeloid leukemia factor 1 (*MLF1*), a gene associated with acute myeloid leukemia (Yoneda-Kato *et al*., 1996). When introduced into J2E cells, HLS7 imposed a monoblastoid phenotype upon these erythroid cells; furthermore, HLS7 blocked the differentiation of erythroleukemic and normal erythroid cells and favored maturation of a monoblastoid cell line as well as myeloid colony formation. From these studies, it was concluded that

```
MFRMLSSSFEDDPFFADSFLAHRESMRNMMRSFSEPLGRDLLSISDGRGR
     *          **  .      *  . *       .
MFRMLNSSFEDDPFFSESILAHRENMRQMIRSFSEPFGRDLLSISDGRGR

THNRRERDDGEDSLTHADVNPFQTMDRMMANMRSGIQELQRNFGQLSMDP
.    ..*          .  *.     * **   *.* * *       *
AHNRRGHNDGEDSLTHTDVSSFQTMDQMVSNMRNYMQKLERNFGQLSVDP

NGHSFCSSSVMTYSKVGDEPPKVFQASTQTRRAPGGVKETRKAMRDSDSG
                *                          *
NGHSFCSSSVMTYSKIGDEPPKVFQASTQTRRAPGGIKETRKAMRDSDSG

LERMAVGHHIHDRGHVIRKSKYNKTGDEEVNQEFINMNESDAHAFDDEWQ
  *  *         .   *  ..                        *
LEKMAIGHHIHDRAHVIKKSKNKKTGDEEVNQEFINMNESDAHAFDEEWQ

NEVLKYKSIGRS_GHTGMRSVGHEHPGSRELKRREKIHRNSAIESGRRSN
*       ..... * .        *           ..**.    .
SEVLKYKPGRHNLGNTRMRSVGHENPGSRELKRREKPQQSPAIEHGRRSN

VFVDKLNVKGSPVKITKK
 ..   **   .  ..
VLGDKLHIKGSSVKSNKK
```

Fig. 1. HLS7 is homologous to MLF1. Amino acid sequence alignment of murine HLS7 (upper) and human MLF1 (lower). Sequence analysis revealed that the predicted *HLS7* open reading frame had 79% identity and 90% homology to *MLF1*. Asterisks (*) represent conservative substitutions, dots (.) represent non-conservative changes. The consensus 14-3-3 binding site (RSXSXP) present in both HLS7 and MLF1 is underlined.

HLS7 can influence erythroid/myeloid lineage switching as well as normal hemopoietic differentiation.

Results

Isolation of genes associated with lineage switching

Myeloid-specific cDNAs from J2E-m2 cells were isolated following three rounds of subtraction by RDA. Eight discrete bands observed on ethidium bromide-stained agarose gels were termed *HLS 1–8*. Five of the eight fragments were expressed only by the J2E-m2 line and not by parental J2E cells. *HLS1* and *HLS6* were macrophage-restricted genes cyclo-oxygenase 2 and GDP dissociation inhibitor-D4, respectively (DeWitt *et al*., 1990; Adra *et al*., 1993), which validated the RDA approach for isolating myeloid-restricted genes. When originally isolated, *HLS2* and *HLS5* had no homology to any characterized species in the DNA databases.

Interestingly, murine *HLS7* had 79% amino acid identity and 90% homology with human *MLF1* (Yoneda-Kato *et al*., 1996) (Figure 1). Thus, *HLS7* is most probably the murine homolog of *MLF1*, or a closely related member of a family that now includes *MLF2* (Kuefer *et al*., 1996). *MLF1* was identified as the C-terminus of a fusion protein with nucleophosmin, produced by the t(3;5)(q25.1;q34) translocation associated with myelodysplastic syndrome and acute myeloid leukemia (Yoneda-Kato *et al*., 1996). A close inspection of the *HLS7* sequence identified a characteristic RSXSXP binding motif for 14-3-3 proteins (Muslin *et al*., 1996). However, no other obvious functional domains could be demonstrated for *HLS7*.

Expression of HLS7

As *HLS7* was identified following an erythroid to myeloid lineage switch, and *MLF1* is a putative leukemia-inducing

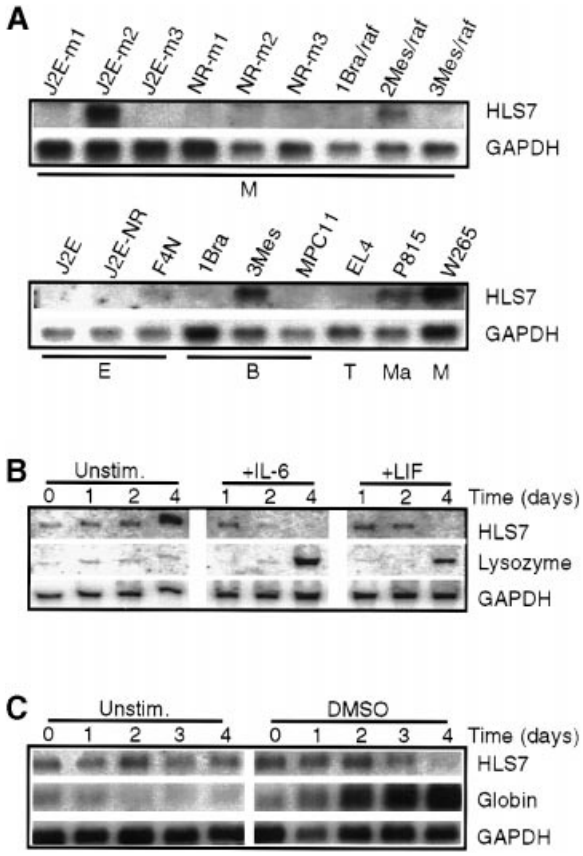


Fig. 2. Expression of HLS7. (**A**) Northern blot of poly(A)$^+$ RNA isolated from myeloid (M), erythroid (E), B-, T- and mast (Ma) cell lines probed with ^{32}P-labeled *HLS7* cDNA. *HLS7* was expressed in the J2E-m2 tester cell line but not the J2E erythroid cell line used for the RDA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the loading control. (**B** and **C**) Poly(A)$^+$ Northern blots of differentiating M1 monoblastoid and 707 erythroleukemic cells, respectively. M1 cells were stimulated with 4 ng/ml IL-6 or 32 ng/ml LIF, while 707 cells were induced to differentiate with 1.5% dimethylsulfoxide (DMSO), whereas control cultures (unstim.) were not treated with these differentiation agents. Induction of lysozyme and globin mRNAs served as markers of myeloid and erythroid differentiation, respectively.

gene (Yoneda-Kato *et al*., 1996), we investigated the expression of *HLS7* in a panel of murine hemopoietic cell lines (Figure 2A). Northern blotting showed that *HLS7* produced a single 1.2 kb transcript and expression was generally restricted to less mature cell lines. *HLS7* RNA was produced by immature myeloid cells J2E-m2, W265, 2Mes/*raf* (Figure 2A) and M1 (Figure 2B); in addition, transcripts were detected in mast cells (P815) and early B cells (1-Bra, 3Mes), as well as in undifferentiated murine erythroleukemia cell lines F4N (Figure 2A) and 707 (Figure 2C).

To explore the notion that *HLS7* was expressed principally by immature cell types, *HLS7* mRNA levels were monitored during the differentiation of M1 monoblastoid cells and 707 erythroleukemic cells. Figure 2B shows that *HLS7* transcripts decreased markedly as M1 cells developed into macrophages. Conversely, lysozome mRNA (a marker of macrophage maturity) increased as the M1 cells differentiated. *HLS7* transcripts also fell as erythroid 707 cells differentiated, while globin mRNA rose appreciably (Figure 2C). These data supported the

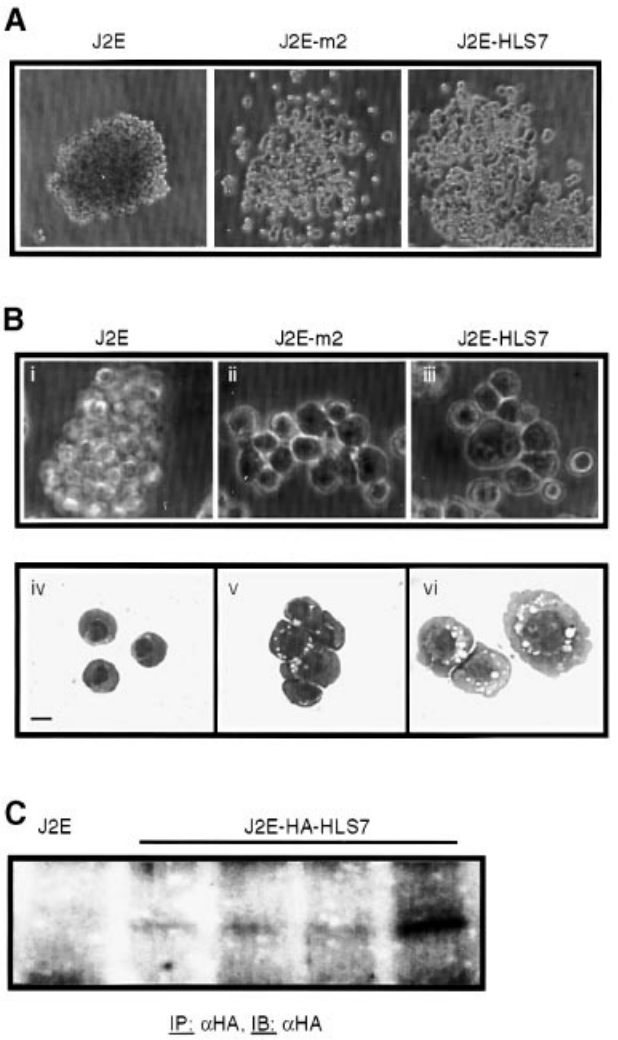


Fig. 3. Phenotypic changes in J2E erythroid cells induced by HLS7. (**A**) J2E, J2E-m2 and J2E-HLS7 colonies in methylcellulose. J2E cells expressing HLS7 had a dispersed (myeloid) colony morphology compared with the compact (erythroid) colonies of the parental line. (**B**) J2E, J2E-m2 and J2E-HLS7 cell lines in culture (i–iii) and Wright-stained cytocentrifuge preparations (iv–vi). Each J2E-HLS7 clone was adherent in culture (iii) and had a monocytoid morphology (vi) that closely resembled the J2E-m2 myeloid cell line (ii and v). Bars in (iv–vi) represent 10 μm. (**C**) Immunoprecipitation of HA-tagged HLS7 from 5 mg of protein isolated from four clones of retrovirally infected J2E cells. Cells infected with HA-HLS7 displayed phenotypic characteristics identical to wild-type HLS7.

proposition that *HLS7* is expressed primarily in less differentiated hemopoietic cells.

HLS7 expression alters the morphology of J2E cells

To determine the consequences of constitutive HLS7 expression in J2E cells, the full-length cDNA was introduced by amphotropic retroviral infection. Strikingly, HLS7 expression produced a dramatic change in the morphology of J2E cells (Figure 3). In semi-solid media, parental J2E cells had a compact colony morphology, whereas cells expressing HLS7 (J2E-HLS7) formed dispersed colonies that closely resembled those generated by J2E-m2 myeloid cells, from which *HLS7* was isolated (Figure 3A). Instead of growing in large clumps in suspension, each of the four J2E-HLS7 clones became adherent and displayed the morphology of monocytoid cells (Figure 3B); moreover, the volume of J2E-HLS7 clones was 4- to 5-fold greater than J2E cells. The presence of HLS7 protein in these cells was demonstrated by immunoprecipitation and immunoblotting (Figure 3C). In contrast, clones expressing the antisense *HLS7* construct (J2E-αHLS7) maintained a normal erythroblastoid phenotype (data not shown). Although cell surface expression of epo and transferrin receptors decreased slightly in J2E-HLS7 cells, they did not display mature myeloid surface markers Mac-1, Ia or Gm3.2 (data not shown). Thus, the phenotypic features imposed on J2E erythroid cells by HLS7 resembled the characteristics of myeloid J2E-m2 cells (Keil *et al*., 1995). It is worth noting that the altered phenotype of the J2E-HLS7 cells was not accompanied by chromosomal alterations seen in J2E-m2 cells (Keil *et al*., 1995).

HLS7 inhibits erythroid differentiation

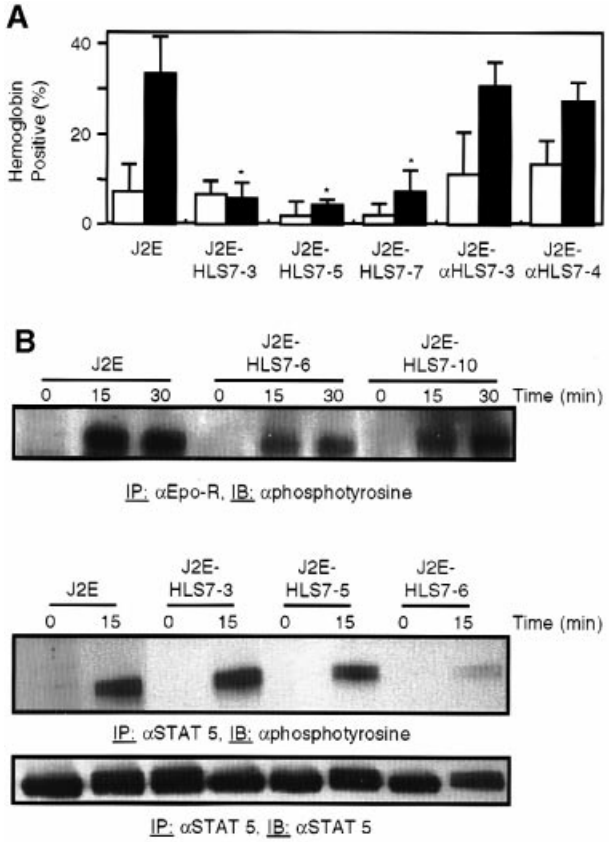
As HLS7 significantly altered the erythroid phenotype of J2E cells, the impact on epo-induced differentiation was examined by benzidine staining for hemoglobin synthesis. All J2E-HLS7 clones were significantly restricted in their capacity to synthesize hemoglobin after epo stimulation (Figure 4A), and morphological maturation along the erythroid pathway was non-existent (data not shown). Although J2E-HLS7 cells had fewer epo receptors, immunoprecipitation and immunoblotting revealed that the receptor was still phosphorylated upon ligand binding (Figure 4B); epo-initiated phosphorylation of STAT 5 (Figure 4B) and mitogen-activated protein (MAP) kinase (data not shown) was also unaffected by ectopic HLS7 expression. In addition, these cells contained amounts of GATA-1, EKLF and NF-E2 similar to J2E cells (Figure 4C). Therefore, the inability to synthesize hemoglobin was not caused by a failure to activate epo signaling cascades, or a lack of erythroid transcription factors.

Having observed a marked reduction in epo-stimulated hemoglobin production by J2E cells, the effect of HLS7 expression on murine erythroleukemia cell maturation was then investigated. Figure 5A shows that erythroleukemic 707 cells overexpressing HLS7 (707-HLS7) were also restricted in their capacity to manufacture hemoglobin during differentiation induced by dimethylsulfoxide. However, unlike J2E cells, expression of antisense *HLS7* in 707 cells (707-αHLS7) consistently produced a modest increase in benzidine-positive cells. This is most probably due to reduced levels of endogenous HLS7 in these cells.

To determine the effect of HLS7 on normal erythroid progenitors, cells from 12-day-old fetal livers were isolated and infected with an *HLS7*-containing retrovirus. Figure 5B shows that HLS7 prevented the development of epo-responsive BFU-E (burst-forming units-erythroid). Thus, constitutive expression of HLS7 suppressed erythroid terminal differentiation in three model systems—not only were two erythroleukemic cell lines inhibited in their response to epo or chemical inducers, but normal red cell colony formation was also impeded (Figures 4 and 5).

HLS7 expression enhances myeloid differentiation

The effect of increasing HLS7 expression in myeloid cells was examined by overexpressing the gene in the M1 monoblastoid cell line. In contrast to erythroid cells,



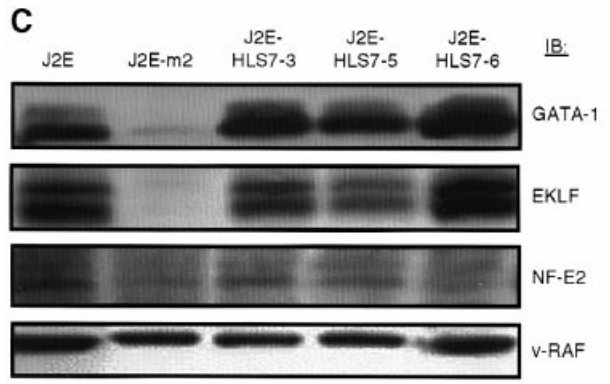


Fig. 4. HLS7 suppresses J2E differentiation without affecting epo signaling. (**A**) J2E cells expressing HLS7 were retarded in their ability to differentiate in response to epo (5 U/ml). Cells were untreated (open bars) or stimulated with epo (solid bars) for 72 h. Hemoglobin production was determined by benzidine staining cells from J2E, J2E-HLS7 and J2E-αHLS7 (antisense HLS7) cultures. Each result is the mean ± SD ($n = 3$) and * represents $P < 0.01$ (one-tailed *t*-test). (**B**) J2E-HLS7 cells displayed normal activation of the epo receptor (epo-R) and STAT 5 in response to epo stimulation. Cells were stimulated with epo for the times indicated and protein lysates (1 mg) were immunoprecipitated (IP) with either α-epo-R or α-STAT 5 antibodies and immunoblotted (IB) with an α-phosphotyrosine antibody to detect phosphorylated forms of the proteins. (**C**) Protein lysates (1 mg) from various cell lines were immunoblotted with antibodies against several erythroid-restricted transcription factors. Each J2E-HLS7 clone expressed amounts of the transcription factors similar to the parental J2E lines. In contrast, J2E-m2 cells had reduced levels of these factors. v-RAF served as a loading control.

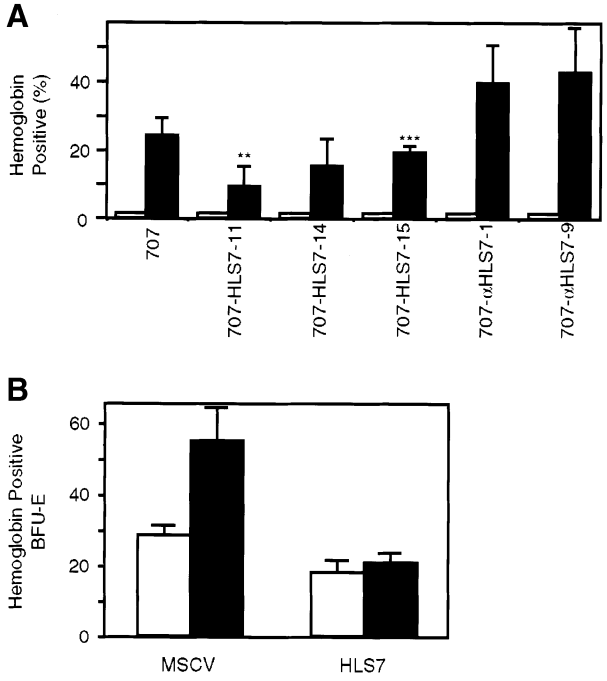


Fig. 5. HLS7 blocks erythroleukemic and normal erythroid differentiation. (**A**) 707, 707-HLS7 and 707-αHLS7 cells were unstimulated (open bars) or exposed to 1.5% dimethylsulfoxide (solid bars) for 48 h. Cells were stained with benzidine and each value represents the mean ± SD ($n = 3$), with ** and *** representing $P < 0.025$ and 0.05, respectively. (**B**) HLS7 reduced epo-responsive benzidine-positive BFU-E from day 12 fetal liver cells. Cells were co-cultivated with packaging lines producing either MSCV or MSCV-HLS7 retroviruses and infected cells were plated in methylcellulose containing geneticin in the presence (solid bars) or absence of epo (5 U/ml). Each result is the mean ± SD ($n = 3$).

overexpression of HLS7 in M1 cells promoted myeloid maturation. Figure 6A shows that expression of the mature macrophage surface marker Mac-1 was elevated in M1-HLS7 cells prior to hormonal stimulation. Higher levels of Mac-1 expression were also seen in M1-HLS7 cultures exposed to sub-maximal concentrations of interleukin 6 (IL-6) (Figure 6A) or leukemia inhibitory factor (LIF) (data not shown). This effect was observed with four independent M1-HLS7 clones.

To study the effect of HLS7 in the myeloid compartment of non-transformed cells, fetal liver cells were infected and development of myeloid colonies enumerated. Data presented in Figure 6B demonstrate that normal hemopoietic precursors infected with HLS7 produced 30% more myeloid colonies. Taken together, the results on inappropriate expression of HLS7 in hemopoietic cells demonstrate that this molecule is able to inhibit erythroid development and favor myeloid differentiation.

HLS7 localizes to the nucleus and cytoplasm

Previously, the human homolog MLF1 was reported to localize exclusively to the cytoplasm of transfected COS cells (Yoneda-Kato *et al.*, 1996). Indirect immunofluorescence and confocal microscopy were used to determine the intracellular localization of HLS7 in hemopoietic cells. However, the levels of endogenous HLS7 were too low to detect in J2E-m2 and M1 cells; moreover, retrovirally produced HLS7 in J2E or M1 cells could not be observed by this technique. These observations are consistent with

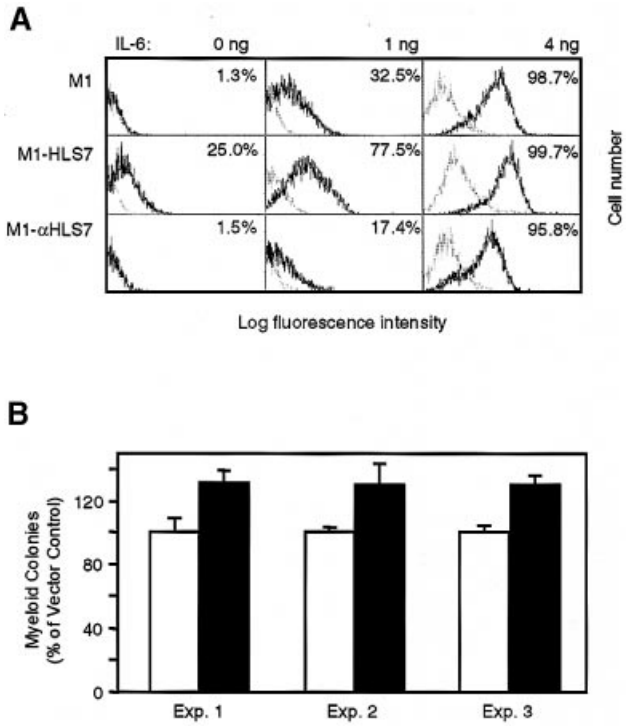


Fig. 6. HLS7 enhances myeloid differentiation. (**A**) HLS7 enhanced the differentiation of the M1 monoblastoid cell line. Expression of Mac-1 cell surface marker by M1 cell lines was determined by flow cytometry. Cells were stimulated with 0–4 ng/ml IL-6 for 4 days. Percentages represent the number of gated positive cells. The data presented are typical of three experiments performed on four separate M1-HLS7 (sense) and M1-αHLS7 (antisense) clones. (**B**) HLS7 increased the numbers of myeloid colonies produced from day 12 fetal liver cells. Myeloid colonies produced in semi-solid media from fetal liver cells infected with either MSCV (open bars) or MSCV-HLS7 (closed bars) retroviruses were enumerated. Results from three independent experiments are shown as percentages relative to the MSCV control (mean ± SE; Exp. 1, $n = 2$; Exp. 2 and Exp. 3, $n = 3$).

the large amount of protein needed to detect ectopic HLS7 by immunoprecipitation (Figure 3C). Consequently, COS cells were transiently transfected with a pcDNA3-HLS7 construct, and the protein product detected with a polyclonal antibody raised against the full-length protein. Figure 7 shows that HLS7 was found primarily in the cytoplasm, as expected of a protein that contains a consensus 14-4-3 binding site, with some concentration in the perinuclear region. However, HLS7 was also observed in discrete bodies within the nucleus. These punctate nuclear foci are reminiscent of nuclear bodies associated with hnRNP U (Eggert *et al*., 1997), or transcription factors GATA-1 and PML (Elefanty *et al*., 1996; Hodges *et al*., 1998). Thus, HLS7 protein is present in both the cytoplasm and nucleus of transfected COS cells.

Discussion

Here we have shown that HLS7, the murine homolog of the putative oncogene product MLF1 (Yoneda-Kato *et al*., 1996), is activated during erythroid to myeloid lineage switching. Data presented here demonstrate that HLS7 could impose a monocytoid phenotype on J2E erythroleukemic cells, recapitulating the original lineage switch. Moreover, HLS7 influenced cytokine-mediated maturation

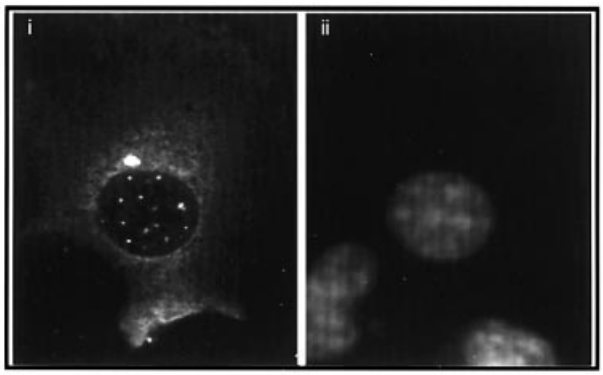


Fig. 7. HLS7 localizes to both cytoplasm and nucleus. COS cells were transiently transfected with pCDNA3-HLS7, stained with α-HLS7 polyclonal antiserum then visualized with a FITC-conjugated secondary antibody and a laser scanning confocal microscope (**i**). Nuclei were counterstained with Hoechst 33258 (**ii**).

of transformed cell lines and normal hemopoietic progenitors. Thus, the levels of HLS7 in normal or transformed cells can have a significant impact on their phenotype.

The capacity of HLS7 to alter the phenotype of J2E cells is reminiscent of the reprograming of hemopoietic cells by GATA-1. Kulessa *et al.* (1995) introduced GATA-1 into avian myelomonocytic cells, which produced a dramatic change in phenotype, generating eosinophils, thromboblasts and erythroblasts. Significantly, this phenomenon was concentration dependent. Similarly, overexpression of GATA-1 in M1 monoblastoid cells generated erythroid and megakaryocytic progeny (Yamaguchi *et al*., 1998). In contrast, PU.1 dictates myeloid lineage commitment in transformed multipotent cells by suppressing GATA-1 (Nerlov and Graf, 1998) and blocks erythroid differentiation via direct interaction with GATA-1 (Rekhtman *et al*., 1999), while GATA proteins repress PU.1 transactivation of myeloid target genes (Zhang *et al*., 1999). These examples support the concept that plasticity within the hemopoietic system can be manipulated by the levels of transcriptional regulators.

HLS7 inhibited epo-induced differentiation of J2E cells and BFU-E, as well as chemically initiated maturation of murine erythroleukemic cells. In J2E cells, this was achieved despite phosphorylation of the epo receptor, STAT 5 and MAP kinase, and normal levels of erythroid transcription factors GATA-1, NF-E2 and EKLF. Thus, HLS7 behaves like transcription factors PU.1, myc, myb, mafB and LMO2 (Coppola and Cole, 1986; Todokoro *et al*., 1988; Sieweke *et al*., 1996; Visvader *et al*., 1997; Rekhtman *et al*., 1999) by suppressing erythroid differentiation. This inhibition of red cell maturation mediated by overexpression of HLS7 may account for the disproportionate number of erythroleukemias associated with the t(3;5) translocation (Raimondi *et al*., 1989). Conversely, HLS7 promoted the IL-6- or LIF-induced maturation of M1 monoblastoid cells into macrophages and enhanced myeloid colony formation by fetal liver cells. These data suggest that persistent expression of HLS7 favors maturation along the myeloid pathway and impedes erythropoietic progress.

The presence of HLS7 in both the cytoplasm and nucleus (Figure 7) is intriguing. It is, however, consistent with nuclear and cytoplasmic binding partners we have

identified in a yeast two-hybrid screen (J.H.Williams *et al.*, manuscript in preparation). Predictably, the cytoplasmic 14-3-3 associated with HLS7 in this screen. In addition, HLS7 bound the murine homolog of the nuclear protein chURP, a molecule related to hnRNP U (Lodge *et al.*, 1999). Association with chURP, together with the appearance in discrete nuclear foci, indicates that HLS7 may have a functional role within the nucleus. Similar nuclear staining patterns have been observed for the protein product of the *PML* gene, which is involved in the t(15:17) translocation of promyelocytic leukemia (Borrow *et al.*, 1990; de The *et al.*, 1990; Longo *et al.*, 1990). Transcription factor GATA-1 has comparable punctate nuclear localization (Elefanty *et al.*, 1996), as does hnRNP U (Eggert *et al.*, 1997). With mounting evidence that these nuclear bodies have specific functions (Hodges *et al.*, 1998), further studies are under way to elucidate the role of the HLS7 bodies in the nucleus.

The importance of 14-3-3 family members in controlling the subcellular localization of key regulatory molecules has recently become evident. Phosphorylated forms of these proteins may be held in the cytoplasm by 14-3-3 before release and translocation to other organelles, e.g. cdc25 and FKHRL1 to the nucleus (Peng *et al.*, 1997; Brunet *et al.*, 1999; Lopez-Girona *et al.*, 1999; Yang *et al.*, 1999) or Bad to mitochondria (Zha *et al.*, 1996). It is conceivable that HLS7 is retained in the cytoplasm by 14-3-3 but may translocate to form complexes in nuclear bodies. Since the nucleophosmin–MLF1 fusion protein in the t(3;5) translocation appears entirely in the nucleus (Yoneda-Kato *et al.*, 1996), it may bypass the circuitry involving 14-3-3ζ. Therefore, the subcellular localization of HLS7/MLF1 is likely to play an important role in the normal function of the protein.

Materials and methods

cDNA RDA

Poly(A)$^+$ RNA was isolated from J2E driver and J2E-m2 tester cell lines using the PolyATract mRNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. Double-stranded cDNA was synthesized, digested with *Sau*3AI and subjected to three cycles of RDA. The RDA procedure used was the same as previously published protocols (Hubank and Schatz, 1994), except that R-Bgl adaptors were not removed from driver cDNA prior to subtraction and the final difference product (DP3) was amplified for 35 cycles without mung bean nuclease treatment. Oligonucleotide adaptor sequences were R-Bgl-12, 5′-GATCTGCGGTGA-3′; R-Bgl-24, 5′-AGCACTCTCCAGCCTCTCACCGCA-3′; J-Bgl-12, 5′-GATCTGTTCATG-3′; J-Bgl-24, ACCGACGTCGACTATCCATGAACA-3′; N-Bgl-12, GATCTTCCCTCG-3′; and N-Bgl-24, AGGCAACTGTGCTATCCGAGGGAA-3′. Fragments enriched by this procedure were subcloned into pGEM-T (Promega) for further analysis.

cDNA library screening

A mouse 11-day embryo 5′-STRETCH PLUS cDNA library in λgt11 (Clontech, Palo Alto, CA) was screened for full-length *HLS7* using the 446 bp HLS7 RDA fragment as probe. Positive phage were isolated, and cDNA inserts were excised and subcloned into pBlueScript (Stratagene, La Jolla, CA) for sequencing.

Amphotropic viral infection of cell lines

PCR was used to introduce unique *Xho*I sites in the 5′- and 3′-untranslated regions of *HLS7*. Oligonucleotides used were HLS7-5P (5′-CACCTCGAGACGATGTTCCGGATGCTGAGC-3′) and HLS7-3P (5′-GTGCTCGAGTTATTTTTTGGTGATTTTCAC-3′). The 0.816 kbp PCR product was digested with *Xho*I and ligated into the *Xho*I site of the pMSCV-neo 2.2 retroviral vector (Hawley *et al.*, 1994) generating both sense and antisense constructs. A hemagglutinin (HA)-tagged *HLS7* retroviral construct was prepared by insertion of the 0.816 kb *HLS7* PCR product into the *Xho*I site of the pMSCV-HA vector which was generated by ligation of an HA linker into the *Bgl*II site of pMSCV. HA linker sense strand, 5′-GATCGCATGGCCTACCCTTATGATGTGCCAGATTATGCC-3′; HA linker antisense strand, 5′-GATCCGGCATAATCTGGCACATCATAAGGGTAGGCCATG-3′. The PA317 packaging cell line was then transfected with the linearized constructs and J2E, 707 and M1 cells were infected with the amphotropic viral supernatants as described previously (Tilbrook *et al.*, 1997). Individual clones were isolated from methylcellulose. Unique viral integration sites were determined by Southern blot analysis and expression of exogenous HLS7 transcripts was determined by Northern blotting as well as immunoprecipitation of the HA-tagged form of the protein.

Infection of hemopoietic cells

For infection of liver cells from 12-day fetal CBA mice, pMSCV and pMSCV-HLS7 constructs were transfected into the ψ2 ecotropic packaging line (Klinken *et al.*, 1988b). Typically, 8×10^5 fetal liver cells were co-cultivated for 2 days with irradiated (3000 Rads) virally producing packaging lines in the presence of 10% WEHI 3B D$^-$ conditioned media and 50 μg/ml transferrin. The cells were washed extensively and then 20 000 cells per 30-mm dish were cultured in Iscove's modified Dulbecco's medium containing 0.9% methylcellulose, 18.75% fetal calf serum (FCS) and 50 μg/ml transferrin in the presence or absence of 10% WEHI 3B D$^-$ conditioned media, 5 U/ml epo and 1 mg/ml geneticin (Sigma, St Louis, MO) to select for virally infected clones. Viral integration was confirmed by PCR analysis of individual colonies. Hemoglobin-positive BFU-E, visualized by benzidine staining (Tilbrook *et al.*, 1997), or dispersed myeloid colonies were scored 7 days later as we have described previously (Klinken *et al.*, 1989; Principato *et al.*, 1990; Keil *et al.*, 1995).

Immunoprecipitations and immunoblotting

For detection of HA-tagged HLS7 present in infected cells, immunoprecipitations were performed on 5 mg of protein with anti-HA (HA.11; BabCo, Richmond, CA) and protein A–Sepharose. Cells used in signaling experiments were serum starved for 5 h and stimulated with 5 U/ml epo for 15 min. Lysates (1 mg) were immunoprecipitated with either anti-epo receptor (Yoshimura *et al.*, 1990) or anti-STAT 5 (sc-835; Santa Cruz Biotechnology). Immunoprecipitated proteins or cell lysates (100 μg) were separated by SDS–PAGE, transferred to nitrocellulose membranes and analyzed by immunoblotting with either anti-GATA 1, anti-phosphotyrosine, anti-STAT 5, anti-raf, anti-myc (sc-7020, sc-835, sc-1019, sc-265, sc-133, sc-41; Santa Cruz Biotechnology), anti-EKLF (Miller and Bieker, 1993), anti-NF-E2 (Andrews *et al.*, 1993) or with anti-HA (HA.11; BabCo) followed by horseradish peroxidase-conjugated antibodies. Visualization was by enhanced chemiluminescence (Amersham, Bucks, UK).

Flow cytometry

To assess Mac-1 expression, cells were washed in phosphate-buffered saline (PBS) containing 2% FCS, incubated with anti-Mac-1 antibody (Springer *et al.*, 1979) (1:200 dilution in PBS with 2% FCS) for 30 min on ice. Following washes, fluorescein isothiocyanate (FITC)-conjugated sheep anti-rat secondary antibody was added (Silenus; 1:100 in PBS + 2% FCS) for 30 min on ice in the dark. Cells were washed and resuspended in 1 ml of PBS + 2% FCS containing 1 mg/ml propidium iodide before analysis on a Beckman-Coulter Epics XL/MCL flow cytometer.

Production of anti-HLS7 antiserum

Full-length recombinant HLS7, N-terminally tagged with six histidine residues, was expressed in *Escherichia coli* using the pET expression system (Novagen, Madison, WI) and affinity-purified by metal chelate affinity chomatography according to the manufacturer's instructions. Two New Zealand White rabbits were immunized with 200 μg of recombinant protein three times at 4 week intervals and the resultant antiserum was tested for specificity by Western blotting of recombinant protein and protein lysates from COS cells transiently transfected with HLS7.

Confocal microscopy

cDNA encoding *HLS7* was subcloned into the pCDNA3 eukaryotic expression vector (Invitrogen, San Diego, CA) for transient expression in COS7 cells. Cells (1×10^4) were seeded onto coverslips and transfected with 0.5 μg of plasmid using the DEAE–dextran method as

described previously (Lowe and Goeddel, 1987). After 24 h, cells were fixed with 50% acetone/50% methanol for 5 min at 4°C and blocked for 30 min with 3% bovine serum albumin (BSA) in Tris-buffered saline–Tween-20 (TBS-T: 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20). Rabbit anti-HLS7 antiserum was added (diluted 1:1000 in TBS-T) for 1 h. After washing (3× 5 min, 3% BSA–TBS-T), FITC-conjugated sheep anti-rabbit secondary antibody was applied (1:200 in 3% BSA–TBS-T) for 60 min in the dark. Following washing as above, coverslips were mounted in 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Fluka, NSW, Australia) containing 0.00005% Hoechst 33258 (Calbiochem, La Jolla, CA). Fluorescence was visualized on a Bio-Rad MRC 1024 UV Laser Scanning Confocal Microscope using a Nikon Fluor 40× 1.15 NA Water Immersion objective lens.

Accession number

The *HLS7* sequence data have been deposited in the DDBJ/EMBL/GenBank under accession No. AF009515.

Acknowledgements

Recombinant human epo (Eprex) was a generous gift from Dr J.Adams (Jansen-Cilag, Sydney, Australia). The authors are extremely grateful to Drs N.Nicola and D.Hilton (Walter and Eliza Hall Institute, Victoria, Australia) for their generous gifts of murine growth factors. The authors would also like to thank Dr S.Morris for helpful discussions. This work was supported by grants from the National Health and Medical Research Council of Australia (#970095), AMRAD, Cancer Foundation of WA and the Medical Research Foundation of Royal Perth Hospital. N.D. is a Richard Walter Gibbon Fellow.

References

Adra,C.N., Ko,J., Leonard,D., Wirth,L.J., Cerione,R.A. and Lim,B. (1993) Identification of a novel protein with GDP dissociation inhibitor activity for the ras-like proteins CDC42Hs and rac I. *Genes Chromosomes Cancer*, **8**, 253–261.

Andrews,N.C., Erdjument-Bromage,H., Davidson,M.B., Tempst,P. and Orkin,S.H. (1993) Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature*, **362**, 722–728.

Borrow,J., Goddard,A.D., Sheer,D. and Solomon,E. (1990) Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. *Science*, **249**, 1577–1580.

Brunet,A. *et al*. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, **96**, 857–868.

Busfield,S.J. and Klinken,S.P. (1992) Erythropoietin-induced stimulation of differentiation and proliferation in J2E cells is not mimicked by chemical induction. *Blood*, **80**, 412–419.

Coppola,J.A. and Cole,M.D. (1986) Constitutive c-*myc* oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. *Nature*, **320**, 760–763.

de The,H., Chomienne,C., Lanotte,M., Degos,L. and Dejean,A. (1990) The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor α gene to a novel transcribed locus. *Nature*, **347**, 558–561.

DeWitt,D.L., el-Harith,E.A., Kraemer,S.A., Andrews,M.J., Yao,E.F., Armstrong,R.L. and Smith,W.L. (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.*, **265**, 5192–5198.

Eggert,M., Michel,J., Schneider,S., Bornfleth,H., Baniahmad,A., Fackelmayer,F.O., Schmidt,S. and Renkawitz,R. (1997) The glucocorticoid receptor is associated with the RNA-binding nuclear matrix protein hnRNP U. *J. Biol. Chem.*, **272**, 28471–28478.

Elefanty,A.G., Antoniou,M., Custodio,N., Carmo-Fonseca,M. and Grosveld,F.G. (1996) GATA transcription factors associate with a novel class of nuclear bodies in erythroblasts and megakaryocytes. *EMBO J.*, **15**, 319–333.

Greaves,M.F., Chan,L.C., Furley,A.J., Watt,S.M. and Molgaard,H.V. (1986) Lineage promiscuity in hemopoietic differentiation and leukemia. *Blood*, **67**, 1–11.

Hawley,R.G., Lieu,F.H., Fong,A.Z. and Hawley,T.S. (1994) Versatile retroviral vectors for potential use in gene therapy. *Gene Ther.*, **1**, 136–138.

Hodges,M., Tissot,C., Howe,K., Grimwade,D. and Freemont,P.S. (1998) Structure, organization and dynamics of promyelocytic leukemia protein nuclear bodies. *Am. J. Hum. Genet.*, **63**, 297–304.

Hubank,M. and Schatz,D.G. (1994) Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.*, **22**, 5640–5648.

Keil,U., Busfield,S.J., Farr,T.J., Papadimitriou,J., Green,A.R., Begley,C.G. and Klinken,S.P. (1995) Emergence of myeloid cells from cultures of J2E erythroid cells is linked with karyotypic abnormalities. *Cell Growth Differ.*, **6**, 439–448.

Klinken,S.P., Alexander,W.S. and Adams,J.M. (1988a) Hemopoietic lineage switch: v-*raf* oncogene converts Eμ-*myc* transgenic B cells into macrophages. *Cell*, **53**, 857–867.

Klinken,S.P., Nicola,N.A. and Johnson,G.R. (1988b) *In vitro*-derived leukemic erythroid cell lines induced by a *raf*- and *myc*-containing retrovirus differentiate in response to erythropoietin. *Proc. Natl Acad. Sci. USA*, **85**, 8506–8510.

Klinken,S.P., Rapp,U.R. and Morse,H.C.,III (1989) *raf/myc*-infected erythroid cells are restricted in their ability to terminally differentiate. *J. Virol.*, **63**, 1489–1492.

Kuefer,M.U. *et al*. (1996) cDNA cloning, tissue distribution and chromosomal localization of myelodysplasia/myeloid leukemia factor 2 (MLF2). *Genomics*, **35**, 392–396.

Lodge,A.P., Walsh,A., McNamee,C.J. and Moss,D.J. (1999) Identification of chURP, a nuclear calmodulin-binding protein related to hnRNP-U. *Eur. J. Biochem.*, **261**, 137–147.

Longo,L. *et al.* (1990) Rearrangements and aberrant expression of the retinoic acid receptor α gene in acute promyelocytic leukemias. *J. Exp. Med.*, **172**, 1571–1575.

Lopez-Girona,A., Furnari,B., Mondesert,O. and Russell,P. (1999) Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature*, **397**, 172–175.

Lowe,D.G. and Goeddel,D.V. (1987) Heterologous expression and characterization of the human R-*ras* gene product. *Mol. Cell. Biol.*, **7**, 2845–2856.

Metcalf,D. (1989) The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature*, **339**, 27–30.

Miller,I.J. and Bieker,J.J. (1993) A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. *Mol. Cell. Biol.*, **13**, 2776–2786.

Muslin,A.J., Tanner,J.W., Allen,P.M. and Shaw,A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, **84**, 889–897.

Nerlov,C. and Graf,T. (1998) PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev.*, **12**, 2403–2412.

Peng,C.Y., Graves,P.R., Thoma,R.S., Wu,Z., Shaw,A.S. and Piwnica-Worms,H. (1997) Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science*, **277**, 1501–1505.

Principato,M., Cleveland,J.L., Rapp,U.R., Holmes,K.L., Pierce,J.H., Morse,H.C.D. and Klinken,S.P. (1990) Transformation of murine bone marrow cells with combined v-*raf*–v-*myc* oncogenes yields clonally related mature B cells and macrophages. *Mol. Cell. Biol.*, **10**, 3562–3568.

Raimondi,S.C., Dube,I.D., Valentine,M.B., Mirro,J.,Jr, Watt,H.J., Larson,R.A., Bitter,M.A., Le Beau,M.M. and Rowley,J.D. (1989) Clinicopathologic manifestations and breakpoints of the t(3;5) in patients with acute nonlymphocytic leukemia. *Leukemia*, **3**, 42–47.

Rekhtman,N., Radparvar,F., Evans,T. and Skoultchi,A.I. (1999) Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev.*, **13**, 1398–1411.

Sieweke,M.H., Tekotte,H., Frampton,J. and Graf,T. (1996) MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation. *Cell*, **85**, 49–60.

Springer,T., Galfre,G., Secher,D.S. and Milstein,C. (1979) Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur. J. Immunol.*, **9**, 301–306.

Tilbrook,P.A., Ingley,E., Williams,J.H., Hibbs,M.L. and Klinken,S.P. (1997) Lyn tyrosine kinase is essential for erythropoietin-induced differentiation of J2E erythroid cells. *EMBO J.*, **16**, 1610–1619.

Todokoro,K., Watson,R.J., Higo,H., Amanuma,H., Kuramochi,S., Yanagisawa,H. and Ikawa,Y. (1988) Down-regulation of c-*myb* gene expression is a prerequisite for erythropoietin-induced erythroid differentiation. *Proc. Natl Acad. Sci. USA*, **85**, 8900–8904.

Visvader,J.E., Mao,X., Fujiwara,Y., Hahm,K. and Orkin,S.H. (1997) The LIM-domain binding protein Ldb1 and its partner LMO2 act as negative regulators of erythroid differentiation. *Proc. Natl Acad. Sci. USA*, **94**, 13707–13712.

Yamaguchi,Y., Zon,L.I., Ackerman,S.J., Yamamoto,M. and Suda,T. (1998) Forced GATA-1 expression in the murine myeloid cell line M1: induction of c-Mpl expression and megakaryocytic/erythroid differentiation. *Blood*, **91**, 450–457.

Yang,J., Winkler,K., Yoshida,M. and Kornbluth,S. (1999) Maintenance of G_2 arrest in the *Xenopus* oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. *EMBO J.*, **18**, 2174–2183.

Yoneda-Kato,N., Look,A.T., Kirstein,M.N., Valentine,M.B., Raimondi, S.C., Cohen,K.J., Carroll,A.J. and Morris,S.W. (1996) The t(3;5) (q25.1;q34) of myelodysplastic syndrome and acute myeloid leukemia produces a novel fusion gene, NPM-MLF1. *Oncogene*, **12**, 265–275.

Yoshimura,A., Longmore,G. and Lodish,H.F. (1990) Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. *Nature*, **348**, 647–649.

Zha,J., Harada,H., Yang,E., Jockel,J. and Korsmeyer,S.J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X (L). *Cell*, **87**, 619–628.

Zhang,P., Behre,G., Pan,J., Iwama,A., Wara-Aswapati,N., Radomska, H.S., Auron,P.E., Tenen,D.G. and Sun,Z. (1999) Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proc. Natl Acad. Sci. USA*, **96**, 8705–8710.

Received June 16, 1999; revised and accepted August 27, 1999