

Marrow Stem Cells Shift Gene Expression and Engraftment Phenotype with Cell Cycle Transit

Jean-François Lambert,^{1,2} Meng Liu,³ Gerald A. Colvin,¹ Mark Dooner,¹ Christina I. McAuliffe,¹ Pamela S. Becker,⁵ Bernard G. Forget,⁴ Sherman M. Weissman,⁴ and Peter J. Quesenberry¹

¹Roger Williams Medical Center, Providence, RI 02908

²Division of Hematology, University Hospital, CH1211 Geneva 14, Switzerland

³Schepens Eye Research Institute, Harvard University, Boston, MA 02114

⁴Department of Genetics, Yale University School of Medicine, New Haven, CT 06520

⁵University of Massachusetts Cancer Center, Worcester, MA 01655

Abstract

We studied the genetic and engraftment phenotype of highly purified murine hematopoietic stem cells (lineage negative, rhodamine-low, Hoechst-low) through cytokine-stimulated cell cycle. Cells were cultured in interleukin (IL)-3, IL-6, IL-11, and steel factor for 0 to 48 h and tested for engraftment capacity in a lethally irradiated murine competitive transplant model. Engraftment showed major fluctuations with nadirs at 36 and 48 h of culture and recovery during the next G1. Gene expression of quiescent (0 h) or cycling (48 h) stem cells was compared with lineage positive cells by 3' end PCR differential display analysis. Individual PCR bands were quantified using a 0 to 9 scale and results were visually compared using color-coded matrices. We defined a set of 637 transcripts expressed in stem cells and not expressed in lineage positive cells. Gene expression analyzed at 0 and 48 h showed a major shift from "stem cell genes" being highly expressed at 0 h and turned off at 48 h, while "cell division" genes were turned on at 48 h. These observations suggest stem cell gene expression shifts through cell cycle in relation to cell cycle related alterations of stem cell phenotype. The engraftment defect is related to a major phenotypic change of the stem cell.

Key words: stem • cell • gene • expression • cycle

Introduction

Hematopoietic stem cells, despite being undifferentiated, can express specific stem cell functions, such as rapid homing to bone marrow (1, 2), cell surface molecule expression (3, 4), rapid motility (5, 6), and settlement in their final endosteal bone marrow niche (7). These cells are capable of quasi-infinite self-renewal and have a tremendous differentiation potential, a single to a few cells being capable of repopulating the entire hematopoietic system of a lethally irradiated recipient (8). Murine stem cells have been defined based on their undifferentiated characteristics (i.e., lineage negativity), as well as specific positive markers, such as Sca-1 and c-kit. Another approach was based on the relative quiescence of stem cells and used the DNA binding dye Hoechst 33342 and the mitochondrial binding dye

rhodamine 123. Isolation was based on a negative lineage selection using magnetic beads, followed by low Hoechst and low rhodamine expression (LRH; references 9 and 10).

Previous work with unseparated marrow has shown a loss of engraftment potential when whole marrow was cultured in cytokine cocktails such as IL-3, IL-6, IL-11, and Steel factor (11, 12) or Flt3, TPO, and stem cell factor (SCF)* (13). This defect was reversible and cell cycle mapping defined late S-early G₂ as the lowest engraftment period. In contrast to general dogma, primitive long-term renewing stem cells are not quiescent. BrdU incorporation experiments showed that more than 60% of stem cells had cycled at least once within 4 wk (10, 14, 15). They probably enter and exit cycle frequently, alternatively they may exist in a prolonged G₁ period. Furthermore, primitive stem cells are easily induced into active cell cycle after either in vivo transplantation or in vitro cytokine exposure (16, 17).

Address correspondence to Peter J. Quesenberry, Director, Department of Research, Roger Williams Medical Center, 825 Chalkstone Ave., Providence, RI 02908. Phone: 401-456-5770; Fax: 401-456-5759; E-mail: P.Quesenberry@rwmc.org

*Abbreviation used in this paper: SCF, stem cell factor (or steel factor).

Whole bone marrow stem cells probably cycle in an asynchronous fashion. Hematopoietic stem cells, purified by the Hoechst/rhodamine approach, are relatively quiescent, and when exposed to cytokines show a highly synchronous progression through cell cycle (17). We have now studied the engraftment function of highly purified LRH cells as they transit through cell cycle under cytokine stimulation.

To identify specific cell cycle related changes that could explain the engraftment defect, one needs to analyze in detail the pattern of stem cell genes expressed in noncycling and cycling cells. Using different sources of stem cells, several groups described stem cell gene expression analysis using subtracted libraries and array technology. Phillips, et al. (18) have reported on over 2,000 nonredundant gene products from fetal liver hematopoietic stem cells using a subtracted cDNA library to generate a micro array chip. They have identified several genes specific to fetal liver hematopoietic cells. In addition, when comparing fetal hematopoietic cells with adult hematopoietic cells (Rhodamine-123 low c-Kit⁺ Sca-1⁺) they found several genes that were coexpressed in fetal and adult stem cells, as well as genes specific for either fetal or adult stem cells. More recently, gene expression profiling of human and murine bone marrow, as well as other types of stem cells, has been reported (19, 20).

Park et al. (21) have also reported on murine hematopoietic stem cell gene profiling. They used a 5,000 cDNA array obtained by subtraction of cDNA from lineage positive cell populations and studied both hematopoietic adult stem cells and multipotent progenitors (with minimal self renewal capacity). Genes primarily expressed in stem cells were transcription factors, RNA binding proteins, chromatin modifiers, and protein kinases.

We have used differential display, which was developed for comparative gene expression studies, and allows for a systematic and nonbiased screening for molecular differences at the level of mRNA expression, between or among different cells or tissues. The comparisons use a gel-based method that employs the display of 3' end fragments of cDNA generated by cutting with specific restriction enzymes. The amplified cDNAs labeled with radioisotope are then distributed on a denaturing polyacrylamide gel and visualized by autoradiography (22). Side-by-side comparison of mRNA species from two or more related samples allows identification of both up- and down-regulated genes.

In this paper, we describe the effects of cell cycling on engraftment and transcript expression using highly purified hematopoietic stem cells cultured in IL-3, IL-6, IL-11, and steel factor.

Materials and Methods

Mice. 6 to 8 wk-old male or female BALB/c mice were purchased from Taconic Farms and housed in a conventional clean facility for at least 1 wk before experimental use. All experiments were approved by the University of Massachusetts and Roger Williams Medical Center Institutional Animal Care and Use Committee.

All mice received mouse chow and acidified water ad libitum.

Hematopoietic Stem Cell Purification. Bone marrow was isolated from iliac bones, femur, and tibiae of BALB/c mice 6 to 8 wk of age. A low-density fraction (<1.077 g/cm²) was isolated on Nycoprep 1.077A (Accurate Chemical and Scientific Corporation). These cells were lineage depleted by a magnetic bead separation with the following antibodies: Ter119, B220, Mac-1, GR-1, Lyt-2, L3t4, and YW25.12.7 and Dyna beads MW450 anti-rat IgG (Dynal). The lineage depleted cell were labeled with Rhodamine 123 at a concentration of 0.1 mg/ml and Hoechst 33342 at 10 mM. Cells were incubated in the dark for 30 min at 37°C, washed, and followed by an additional warm buffer (37°C) incubation for 20 min at 37°C to efflux the Rhodamine. This last incubation was performed twice in some experiments before sorting using FACS®. The 1st through the 13th percentiles of Rhodamine fluorescence and 1st through the 3rd percentiles of Hoechst fluorescence were isolated. This Lineage negative, Rhodamine low and Hoechst low fraction is abbreviated LRH.

Cytokine Culture. The LRH stem cells were cultured at an initial density of 5,000 cells/ml in Dulbecco's Modified Essential Medium low glucose (Life Technologies/GIBCO BRL) containing 15% heat-inactivated fetal calf serum (HyClone Laboratories), 1% penicillin (100 U/ml)/1% streptomycin (100 mg/ml), and 1% L-glutamine (100 mg/ml). The cytokine cocktail used was rmIL-3, 50 U/ml (collaborative), rmIL-6, 50 U/ml (R&D Systems), rhIL-11, 50 ng/ml (Genetics Institute), and rm steel factor, 50 ng/ml (SCF; R&D Systems). The LRH cells were cultured with the above cytokines for 24 to 48 h in nonadherent Teflon bottles in a humidified 5% CO₂, 37°C water-jacketed incubator.

Autoradiographic Detection of ³H-thymidine-labeled Nuclei. The number of cells in S phase was determined by autoradiography of the cells after pulse labeling with ³H-thymidine (17, 23). Briefly, duplicate aliquots of cells (500 to 1,000), at 6-h intervals, as they are progressing through cell cycle, were incubated with 2.5 µCi/ml of ³H-thymidine at 37°C in 5% CO₂ containing humidified incubator for 30 min. The incorporation of ³H-thymidine was terminated by cytospin centrifugation and immediately fixing with methanol/acetic acid (2:1 vol/vol), followed by three washes in methanol. Slides were allowed to air dry overnight and a thin film of Kodak nuclear track NTB3 emulsion (Eastman Kodak Co.) was applied. Slides were then incubated in the dark for 4 d, developed, and fixed with Kodak Decktol Developer and Kodak Fixer, respectively. Slides were washed extensively and stained with Giemsa. We determined the percentage of cells with labeled nuclei (representing cells in S phase) by counting 200 cells per slide. In addition, in order to estimate population doubling, cell counts were determined using a hemocytometer.

Irradiation and Transplant. Female mice were exposed to 10 Gy at a rate of 0.94–0.96 Gy/min in one fraction using a Cesium 137 gamma source (Gamma cell 40; MDS Nordian) at least two hours before transplant. Donor mice were killed by cervical dislocation. Bone marrow was isolated from femurs, tibias and iliac bones with cold PBS. The cells were counted in a hemocytometer, washed and resuspended for injection in PBS. For competitive transplant, 250,000 female marrow cells were mixed with a volume corresponding to 500 Lin⁻ Rh^{low} Ho^{low} cells initially introduced into the culture vial.

Southern Blot Analysis of Engraftment. DNA was extracted by lysis in 150 mM NaCl, 20 mM Tris (pH 7.5), 20 mM EDTA, 1% SDS, 0.5 mg/ml proteinase K, and 50 mg/ml pancreatic RNase A at 55°C overnight. Purification was completed by organic extraction with phenol-chloroform and ethanol precipitation. 5 µg of each DNA sample was digested with DraI (Boehringer), and

separated by gel electrophoresis in 0.8% agarose (GIBCO BRL). DNA fragments were alkaline transferred onto Zetaprobe nylon membranes (Bio-Rad Laboratories). The presence of Y chromosome-specific DNA sequences was assessed using a pY2-cDNA probe (24, 25; donated by Dr. I. Lemischka, Princeton University, Princeton, NJ). Sample loading variability was assessed and adjusted for by reprobing membranes with a cDNA for IL-3 (donated by J. Ihle, St. Jude Children's Research Hospital, Memphis, TN, and DNAX, Palo Alto, CA). Probes were labeled with ^{32}P using a random primed labeling kit (Boehringer), and autoradiography was performed using Kodak XRP x-ray film (Eastern Kodak Co.). Blots were exposed to PhosphorImaging plates (Molecular Dynamics), and the percentage of male and female DNA quantified after scanning the plates with a 400A PhosphorImager (Molecular Dynamics).

cDNA Preparation and PCR Amplification of cDNA 3' Ends. Poly A⁺ RNA was isolated from fresh or cultured cells using Oligotex (QIAGEN). cDNA was generated using the Superscript Choice System (GIBCO BRL; references 26 and 27). Briefly, 10 μg of PolyA⁺ RNA and 2 pmol of 1 of the 2-base anchored oligo(dT) primers with a heel sequence (20) were mixed with other components for first-strand synthesis reaction. This was followed by reverse transcription using 2 μl of Superscript reverse transcriptase (200 U/ml; Invitrogen/GIBCO BRL). Second-strand synthesis was then performed and the cDNAs were precipitated with ethanol. A Y-adaptor was assembled from 2 oligonucleotides with sequences TAGCGTCCGGCGCAGC-GACGCCAG and GATCCTGGCCGTCGGCTGTCTGTC-GGCAGC, respectively. The cDNA was digested with BglII and an aliquot of this reaction mixture (~ 4 ng) was then used for ligation to 100 ng of the Y-shaped adaptor. After ligation, the reaction mixture was diluted with water, T4 ligase was denatured at 65° and aliquots were made for PCR amplification (28). The primers used corresponded to the heel sequence for the 3' end and to one of the Y-adaptors for the 5' end (TAGCGTCCG-GCGCAGCAGC). The PCR products were identified on the display gel by mixing a small proportion of [$\gamma^{32}\text{P}$]ATP labeled 5' primer to the cold primer. PCR was performed with manual hot

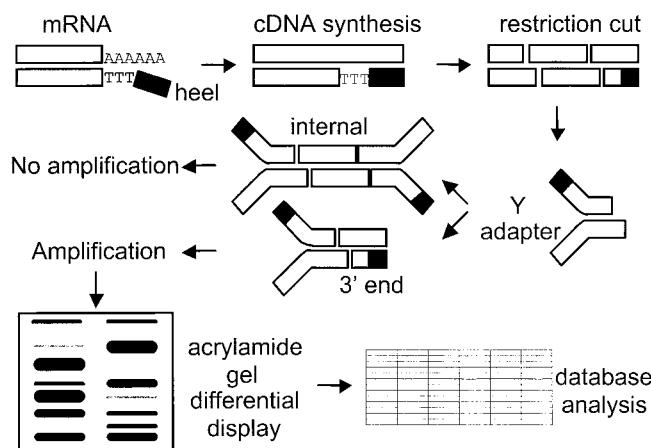


Figure 1. Schematic description of 3' end differential display. cDNA was prepared using a poly-T primer containing a 2-base anchor (5') and a terminal heel (3'). After restriction cut, the fragments were ligated to a Y-adaptor and then amplified using primers homologous to one strand of the Y-adaptor and to the heel sequence. The 3' end fragments are selectively amplified by PCR and resolved on polyacrylamide gels. Separate cell populations were directly compared and information on each band relative intensity was stored and analyzed in an Excel™ database.

start for 28–30 cycles of 94°C for 30 s, 56°C for 2 min, and 72°C for 30 s. PCR products (2.5 μl) were analyzed on a 6% polyacrylamide sequencing gel. Bands of interest were extracted from the display gels, reamplified using the 5' and 3' primers, and directly sequenced or subcloned into pCR-Script using the PCR-Script cloning kit (Stratagene). Sequencing was performed on an ABI automated sequencer. A schematic of the procedure is presented in Fig. 1.

Differential Display Analysis. Each PCR was repeated 2–3 times and bands of interest were given a relative intensity from 0 to 9 by visual evaluation. The radioactive signal being carried by the 5' primer only, the size of the PCR product did not influence the intensity grading. The information on each band was stored into an Excel 2000 spread sheet (Microsoft). The sequences of the bands were compared with public databases by BLAST® analysis (29) and information on matching gene/sequence was collected from NCBI website (30). The gene category was defined based on these similarities.

Database Comparison and Graphical Representation. The differential display of LRH, Lineage positive (Lin⁺), and LRH cultured for 48 h (LRH 48) was compared by subtraction of each band intensity and sorted according to the difference. Based on the intensity of expression, a graphical representation was constructed using color-coded squares, each representing a unique cDNA target. In this array, each gene was ordered according to the intensity of the difference between two groups. Thus, an individual gene can be visually compared between different cell populations, their position on each array being the same. In addition this color analysis allowed direct visual comparison of the entire subset of genes to identify patterns. Specific genes represented in this pattern analysis is available in supplementary data (see reference 30).

Data Availability. Data can be assessed on line as follows: open the following URL (http://info.med.yale.edu/genetics/weiss/Linksfor_Hematon.htm) and then go to the "stem cells" link. The total experimental information on gel analyses of RNA

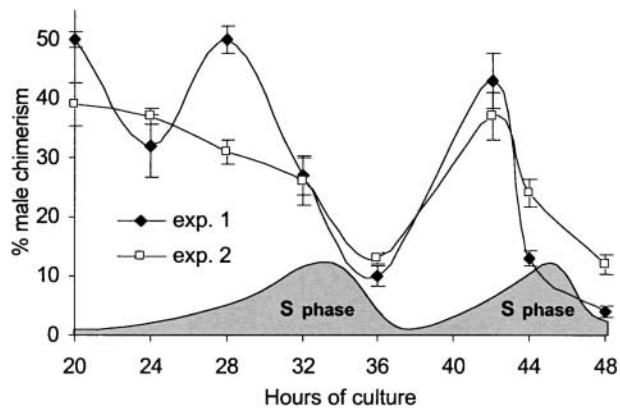


Figure 2. Engraftment defect through cell cycle. Male LRH cells were cultured in IL-3, IL-6, IL-11, and steel factor for 0 to 48 h and competitively transplanted with female bone marrow cells (ratio 1:500) in lethally irradiated female recipients. Chimerism was measured 8 wk after transplantation by Southern blotting using a Y-chromosome probe. In two experiments, nadirs were observed at 36 and 48 h while a recovery was observed at 40 h. These nadirs were statistically different compared with time 0 and 40 in both experiments (Exp. 1: $P < 0.01$; Exp. 2: $P < 0.05$). The cell cycle status as determined by ^3H -thymidine pulse labeling and cell counts and population doubling of cultured LRH cell is shown at the bottom. The two nadirs coincided with the late S/early G₂ phase of cell cycle.

expression is collected in a Microsoft Access database with a separate sheet for each gel band that was analyzed.

Results

Engraftment Defect Through Cell Cycle. In two separate experiments, LRH stem cells were evaluated for engraftability at 8 wk in a competitive transplant model. The initial engraftment level was $50 \pm 1\%$ and $39 \pm 4\%$ for fresh LRH cells. Two nadirs of engraftment were observed at 36 and 48 h of culture in IL-3, IL-6, IL-11, and steel factor. The chimerism was 10 ± 2 and 13 ± 1 at 36 h and 4 ± 1 and 12 ± 2 at 48 h. A recovery of engraftment was ob-

served at 40 h (Fig. 2). In a separate experiment, LRH stem cells, cultured in the same cytokines, were mapped for cell cycle by ^3H -thymidine pulse labeling and cell count doubling. Cell cycle status is shown in the bottom of Fig. 2. These data indicate that engraftment nadirs occurred during late S/early G₂.

LRH Specific Genes. PCR amplification of the cDNA 3' ends was performed using a Y-adaptor ligated to restriction fragments of the cDNA mixture (see Fig. 1). Gene expression of LRH stem cells and lineage positive (Lin⁺) fraction was compared by differential display. A total of 637 genes were expressed only in LRH cells. They were all subcloned and sequenced. Among these genes, 411

Table I. Stem Cell-specific cDNAs Expressed in LRH but Not In Lin⁺ Cells

Transcription factors	Translational Apparatus
Murine homeobox protein zhx-1	Human ribosomal protein L18A
Mouse TAX1 binding protein	Human RNA helicase-like protein
Mouse translational controlled 40 kDa polypeptide p40	Mouse protein synthesis elongation factor Tu
Mouse whn transcription factor	Mouse protein tyrosine kinase in Lin ⁻ CD34 ⁺ Sca ⁺ hematopoietic progenitors
R25908NOT a human immediate-early response gene closely related to the Σ transcription factors	Energy metabolism
Cytokine	Mouse NADH-ubiquinone oxidoreductase
Mouse hepatoma derived growth factor	Mouse cytoplasmic gamma-actin
Mouse heparin-binding EGF-like	
Growth factor (HB-EGF), exons 5-6	
Signaling pathway	RNA m
Mouse putative E1-E2 ATPase	Mouse hematopoietic lineage switch 2 (HLS2)
Mouse PSMB5	Human splicing factor arginine/serine-rich 7 (SFRS7)
Human CD9	
Surface proteins	Chromatin
Mouse bullous pemphigoid antigen 1-b (Bpag 1)	Mouse HMG1-related DNA binding protein
Cell cycle regulation	Membrane trafficking
Human hypothetical protein FLJ10439	Mouse eukaryotic translation
Mouse c-yes tyrosine protein kinase	(previous line)Initiation factor 3 (new item)
Mouse casein kinase II, beta subunit	rat RAB14 protein
Mouse Ercc-4 DNA repair gene	
Mouse G protein beta subunit homologue	Receptor
	Mouse inositol 1, 4, 5-triphosphate receptor
	Apoptosis regulation
	Mouse aspartate aminotransferase

These represent specific genes expressed in LRH at a score of 6 or above and not expressed in Lin⁺ cells.

The number of genes expressed in LRH at any score and not in Lin⁺ cells is as follows: transcription factors 22, protein synthesis 11, surface protein 11, mitochondrial sequence 10, RNA metabolism 10, signaling pathway 9, cytokine 8, membrane trafficking 8, cytoskeleton 7, chromatin 5, metabolism 5, cell cycle regulation 4, energy metabolism 4, adhesion molecule 3, apoptosis regulation 2, lineage switch 2, secreted protein 2, translational apparatus 2, acid base regulation 1.

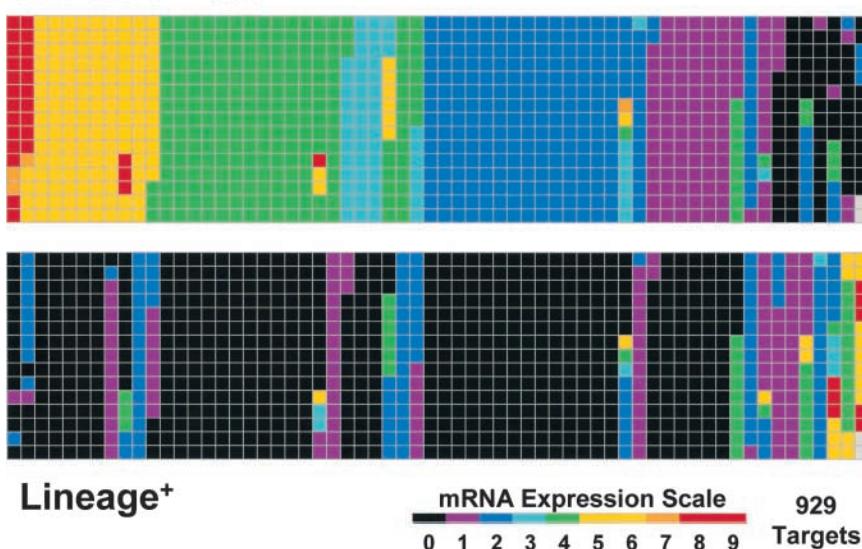
Lin⁻ Rho^{low} Ho^{low}

Figure 3. Dot matrix comparison of LRH and Lin⁺ cells. A total of 929 cDNA's were evaluated by direct comparison of LRH and Lin⁺ expression pattern by differential display. The intensity of each band was color-coded to represent each gene product as a squared-dot. The dot matrix is sorted according to the difference of intensity between LRH and Lin⁺. 637 genes were expressed only in LRH cells. Each specific dot can be further identified in the additional materials [www.jem.org] using the matrix coordinates (column A to BJ and row 1 to 15). For example, the red dot located in the first third of the LRH matrix at position W11 represent a cDNA homologous to the rat ribosomal protein S7 (GenBank accession no. X53377). Note that gray pixels represent blanks.

were unknown with no homologies to GenBank databases. There were 226 cDNA with homologies to GenBank databases and 126 of these corresponded to genes with known function. The large number of transcripts not associated with known genes is noteworthy. Very recent studies using genomic "tiling" arrays have shown that a much larger portion of the genome is transcriptionally active than would have been predicted from the presence of known or putative genes (31). The known genes belonged to 19 different gene categories (footnote, Table I).

Specific genes highly expressed in LRH (score of 6 or above) and not expressed in Lin⁺ are presented in Table I. An alignment of LRH and Lin⁺ genes is presented in Fig. 3.

LRH Gene Expression Shifts Through Cell Cycle. To evaluate gene expression through cytokine-induced cell

cycle, we prepared cDNA from LRH cells incubated in Teflon bottles for 0 or 48 h with IL-3, IL-6, IL-11, and steel factor (LRH48). 251 transcripts from fresh LRH cells or Lin⁺ cells were chosen for analysis based on their differential expression patterns. A major shift in gene expression was found with many genes active in LRH but turned off in LRH48 and other genes inactive in LRH turned on in LRH48 (Fig. 4). Analyzing the difference of gene expression between LRH and LRH48, we made a comparison of expression levels between baseline and 48 h of culture. Genes were sorted according to their difference in level of expression with a minimal difference of 4. A total 89 cDNA's were turned off at 48 h of culture with a residual expression level ≤ 2 . These genes, related to noncycling cells are defined as quiescent. Out of these cDNAs, we found 51 sequences with similarities to GenBank. Table II

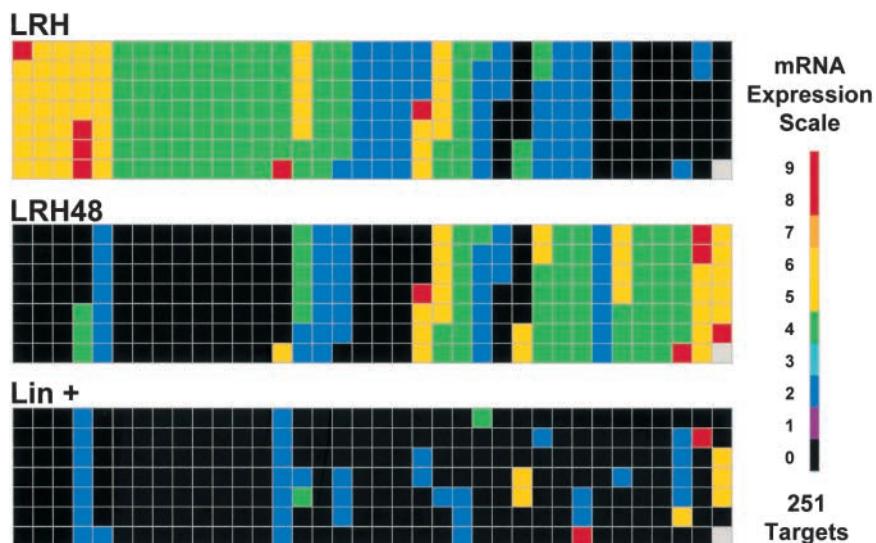


Figure 4. Matrix comparison of LRH, LRH48, and Lin⁺ gene expression. A total of 252 targets are presented. The approach is as outlined above in the legend for Fig. 3. Note that gray pixel represents a blank.

describes 28 genes whose name and function are known. A total of 39 cDNA were turned on at 48 h from a baseline expression level ≤ 2 . These genes, termed cycling represent 25 sequences with homologies to the GenBank database and 14 known genes (Table III). A few cDNA's (29) had a stable expression through cycle, while not expressed in Lineage⁺ cells. These represent 18 genes with similarities and 13 known genes (Table III, common genes). The gene shift seen here probably did not represent differentiation, as gene expression was dramatically different between the 48 h group and the differentiated lineage⁺ cells.

Discussion

Previous work by our laboratory has demonstrated that cytokine stimulated unseparated whole bone marrow cells developed an engraftment defect occurring during the late S/early G₂ of cell cycle. Others have also shown cell cycle associated changes in stem cell phenotype. Studying Lin⁻ Sca-1⁺ Thy1^{low} through cell cycle, Fleming et al. (32) showed decreased engraftment in S/G₂/M as compared with G₁. In a similar vein, Lin⁻ Sca-1⁺ cells, selected based on Hoechst staining, were found to have long term engraftment potential only when in G₀/G₁ and not when in S/G₂/M (33). We have also recently shown that purified stem cells (Lin⁻ Sca-1⁺) evidenced a homing defect coincident with the timing of an engraftment defect (2). In addition, work in collaboration with Dr. Pamela Becker, showed a major fluctuation of cell surface adhesion molecules on LRH cells during cell cycle passage (4). Here, using highly purified murine marrow hematopoietic stem cells, we have confirmed a reversible fluctuation of engraftment through cell cycle, with a comparable timing as was

seen with studies on unseparated marrow cells. The engraftment potential was followed for two cell divisions and fluctuation was reproducibly observed.

Using a nonselective approach with differential display analysis, we were able to identify over 600 stem cell genes, most of these being actively expressed in relatively "quiescent" stem cells selected on the base of their quiescent status. The gene machinery is highly active. Previous studies have also shown that LRH cells can move extremely rapidly, thus further negating the dogma that these cells are metabolically inactive (6).

The 48 h culture time point, showing a major engraftment defect, was chosen for analysis of the pattern of LRH gene expression. A major shift of gene expression was demonstrated. Compared with time zero (i.e., fresh LRH), the cells cultured for 48 h had turned down most of the highly expressed genes and turned on most of the genes initially not or faintly expressed. From these data, we can postulate that many genes necessary for engraftment or initial homing are turned off during cell division, thus possibly explaining the observed engraftment defect. A number of genes were turned on and it is unknown whether these genes participate in altering homing and engraftment. In contrast, many genes were comparably expressed in both LRH and LRH48 cells, defining a pattern of stem cell specific genes whose expression does not seem to be modified by cycling. When comparing the categories of genes expressed in LRH and in LRH48 cells, the "quiescent" genes are mainly transcription factors and protein synthesis genes, while "cycling genes" are related to cell cycle regulation and chromatin remodeling.

When comparing the same panel of genes with Lin⁺ cells, we noted a completely different picture; the genes turned on during cell cycle were not expressed in Lin⁺ cells with a few exceptions such as Histone H2A gene (see Table III), active in both cycling LRH48 and Lin⁺ but not in fresh LRH. This pattern thus suggests cell cycle specificity and is fundamentally different from lineage differentiation. This weighs strongly against the hypothesis that cell cycle engraftment defect could be related to lineage differentiation. However, it must be acknowledged that some of the genes turned on and expressed at 48 h could represent an early onset of differentiation, as the gene expression profile of cells early in differentiation might be different from that seen in fully differentiated cells such as are represented by lineage positive cells. This seems unlikely to us, but will only be resolved with experiments showing reversibility of these gene changes.

These results show a major shift in gene expression of purified marrow stem cells at a time when engraftment is markedly and reversibly depressed. It is difficult to assign specific roles to individual genes, given the relatively large number that are turned on or off and given the presence of a number of unknowns. Genes turned on at 48 h of culture include those involved in DNA damage repair (34–36), chromatin modification (37, 38), RNA splicing (39), and intracellular signaling (40, 41). Several genes could be involved in cell-cell and cell-matrix interactions, adhesion

Table II. Differential Expression Comparing cDNAs from LRH and LRH 48 Cells

Genes with high expression in LRH		Genes with high expression in LRH48	
Gene category	No.	Gene category	No.
Transcription factors	4	Cell cycle regulation	4
Protein synthesis	4	Chromatin	3
Mitochondrial seq.	4	Lineage differentiation	2
Cytoskeleton	3	Transcription factors	2
RNA metabolism	3	Apoptosis	1
Metabolism	3	DNA replication	1
Surface protein	2	Membrane trafficking	1
Cytokine	1	RNA maturation	1
Membrane trafficking	1	Secreted protein	1
Receptor	1		
Signaling pathway	1		
Translation apparatus	1		

Table III. Known Genes Turned Off During Cycle (Quiescent), Conserved through Cycle (Stem Cell Specific), or Turned On at 48 h of Culture (Cycling Genes)

ID no.	GenBank match	Access no.	Size	LRH*	LRH48**
Quiescent genes					
2626	Human TBP-associated factor 170	AJ001017	186	6	0
2606	Mouse antigen peptide transporter 1	AF027865	331	6	0
2683	Mouse aspartate aminotransferase	X07309	130	6	0
2687	Mouse A-X actin	J04181	149	6	0
2742	Mouse bullous pemphigoid antigen 1-b (Bpag1)	AF396879	344	6	0
2597	Mouse elongation factor 1-alpha	X13661	118	6	0
2634	Mouse inositol 1,4,5-trisphosphate receptor	Z71173	234	6	0
2604	Mouse PSMB5	AB003306	265	6	0
2663	Mouse spermophilus tridecemlineatus 26s proteasome	U36395	391	6	0
2717	Mouse translation initiation factor 4E	M61731	162	6	0
2718	Human splicing factor, arginine/serine-rich 7 (SFRS7)	L41887	178	6	2
2681	Mouse putative E1-E2 ATPase	AF011336	470	6	2
2648	Human memd	U31000	350	4	0
2602	Human splicing factor Sip1	AF030234	249	4	0
2654	Mouse 84 kD heat shock protein	M18186	350	4	0
2698	Mouse cholesterol 7-a-hydroxylase exon 1	Z18860	174	4	0
2588	Mouse GU protein	AA272436	253	4	0
2689	Mouse heat shock protein 70 cognate	U27129	196	4	0
2585	Mouse inositol 1,4,5-triphosphate receptor 5	Z33908	237	4	0
2771	Mouse interleukin-5	X06271	292	4	0
2600	Mouse mitochondrial 12S ribosomal RNA	X84382	210	4	0
2772	Mouse mitochondrial genome	V00711	77	4	0
2719	Mouse pim-1 proto-oncogene	M13945	192	4	0
2624	Mouse protein synthesis elongation factor Tu	M22432	364	4	0
2799	Mouse retinoblastoma binding protein 2	AC018559	153	4	0
2615	Mouse ribosomal protein S12	X15962	142	4	0
2806	Mouse signal recognition particle receptor beta subunit	AA419748	220	4	0
2647	Mouse T cell receptor gamma locus (gamma 2 and 4 gene clusters)	AF021335	267	4	0
2761	Rat androgen-binding protein	M19993	272	4	0
Common genes					
2677	Mouse Ercc-4 DNA repair gene	AC004155	365	6	4
2805	Human ribosomal protein L18A	L05093	41	6	4
2594	Mouse TIE receptor tyrosine kinase	X73960	364	6	4
2571	Mouse ribosomal protein S20	X51537	150	6	4
2573	Mouse jerky mRNA	U35730	164	8	8
2633	Mouse casein kinase II, beta subunit	X80685	189	6	6
2744	Human CD9	L08115	156	6	6
2595	Mouse protein synthesis elongation factor Tu	M22432	375	6	6
2608	Mouse antigen peptide transporter 1	AF027865	372	6	6
2582	Rat matrin cyclophilin	AF043642	203	4	4
2569	Mouse ribosomal protein S12	X15962	146	4	4
2727	Rat 3-hydroxyiso- butyrate	J04628	270	4	6
2671	Mouse heat shock protein 70 cognate	M19141	587	4	6
Cycling genes					
2674	Mouse excision repair cross-complementing rodent repair deficiency, complementation group 2	L47235	223	0	4
2631	SWI/SNF related transcription termination factor, RNA polymerase II	AA311008	275	0	4
2568	Rat matrin cyclophilin	AF043642	149	0	4
2575	CCR4-NOT transcription complex, subunit 7	AA816074	198	0	4
2705	Mouse G-utrophin	X83506	450	0	4
2711	Human HS1 hematopoietic protein	X16663	123	0	4
2596	Myeloid cell-specific leucine-rich glycoprotein (CD14)	AC087795	390	2	8
2644	Mouse transcription elongation factor S-II-T1	D86081	206	0	6
2696	Mouse beta-1,4-galactosyltransferase	D37791	136	0	6
2572	Mouse MAP1	AA396150	156	0	6
2743	Mouse cell division control protein 19	D86725	363	0	6
2750	Rat basement membrane-associated chondroitin proteoglycan Bamacan	U82626	272	0	6
2754	H2A Histone family, member Y	C75971	304	2	8
2592	MUS81 endonuclease	AA014278	280	0	8

Relative expression in LRH cells* or LRH48 cells**.

and cell migration. These are rat basement membrane-associated chondroitin proteoglycan BAMacan (42), mouse cell division control protein 19 (43), and mouse β -1,4-galactosyltransferase (44, 45). The latter appears to be important in cytoskeletal assembly and lamellipodia stability and “mesenchymal cell migration on basal lamina”. Expression of these particular genes could indicate changes in stem cell migration/homing patterns which could negatively effect marrow stem cell engraftment. In a similar vein, genes which are turned off could adversely effect stem cell engraftment into the marrow cavity. Many maintenance genes are turned off and their effects on proliferation and survival could determine ultimate engraftment outcomes. Human MEMD is turned off, and this gene appears to play a role in cell–cell interactions and in migration of mobile cells through tissues (46). Thus, turning off of this gene could also have effects in influencing marrow homing. Altogether the observed engraftment defect is likely to be due to polygenic changes.

Recent studies in our laboratory have indicated that when marrow homing is depressed, stem cells are diverted to other nonmarrow tissues, such as lung (47). Alterations of expression of different migration/adhesion factors thus may be involved in such stem cell diversions. This is, of course, only one of many possibilities. A fuller understanding will have to await knockout (embryonic or siRNA adult) studies of individual gene function and identification of the unknown genes.

The modulation of gene expression is consistent with previous observations of modulation of both adhesion proteins and cytokine receptor expression (4, 48). The observations that primitive stem cells are continuously, if intermittently, passing through cell cycle suggests that their phenotype is probably continuously changing.

During cell cycle progression, chromatin is remodeled. In previous studies, chromatin modulations at the β -globin and lysozyme gene loci were evaluated (49–52). Myeloid specific cis-regulatory elements showed a specific chromatin pattern at the lysozyme locus in myelomonocytic cells at different differentiation stages. This chromatin pattern was also found in multipotent hematopoietic progenitors, but was no longer apparent with erythroid differentiation.

Studies of multipotent hematopoietic stem cell chromatin structure showed that the lineage associated genes, globin, myeloperoxidase, IgH, and CD3 δ have accessible control regions before unilineage commitment (50–54). Other studies suggest that chromatin remodeling factors recruited in one phase of cell cycle may determine ultimate action in a later phase. This in turn would determine changes in transcriptional programs (55). A reasonable sequence of events for stem cell phenotype regulation is chromatin remodeling with cytokine induced cell cycle passage, leading to varying levels of transcription factor access to DNA, followed by alteration in gene expression. Thus, these events would prime a stem cell to respond to a specific signal. If the signal was not delivered, the stem cells would again change phenotype. For example, Notch is probably an important primitive stem cell surface receptor.

If Notch expression was augmented or present and the stem cell did not see the appropriate ligand, the moment of opportunity would pass and the cell would then be responsive to different signals (56, 57). If, however the cell saw its ligand, ragged-1, on a stromal cell, a sequence of events would follow with activation of PU.1 and induction of myeloid differentiation (58, 59).

In summary, using highly purified murine HSC, we have shown a major shift of gene expression between two specific functional states. Thus, noncycling HSC selectively express mainly transcription regulators and protein synthesis factors, while they are fully capable of repopulating a myeloablated transplant recipient. In contrast, cells in S/G₂ have turned down most of the originally active genes, and now express cell cycle related as well as chromatin remodeling genes. This indicates a stem cell phenotype that is continuously changing its potential over time, while HSC slowly travel through the cell cycle.

We are grateful to N.V. Baskaran and H. Habibian for expert technical help.

This study was supported by grants from the National Heart, Lung, and Blood Institute (P0-1 HL56920) and the National Institutes of Diabetes and Digestive and Kidney Diseases (P0-1 DK50222) and (R0-1 DK27424).

Submitted: 9 January 2003

Revised: 8 April 2003

Accepted: 16 April 2003

References

- Hendrikx, P.J., C.M. Martens, A. Hagenbeek, J.F. Keij, and J.W. Visser. 1996. Homing of fluorescently labeled murine hematopoietic stem cells. *Exp. Hematol.* 24:129–140.
- Cerny, J., M. Dooner, C. McAuliffe, H. Habibian, K. Stencl, V. Berrios, J. Reilly, J. Carlson, A.M. Cerny, L. D'Hondt, J.-F. Lambert, G. Colvin, and P.J. Quesenberry. 2002. Homing of purified murine lymphohematopoietic stem cells: a cytokine-induced defect. *J. Hematother. Stem Cell Res.* 11:913–922.
- Sato, T., J.H. Laver, and M. Ogawa. 1999. Reversible expression of CD34 by murine hematopoietic stem cells. *Blood*. 94:2548–2554.
- Becker, P.S., S.K. Nilsson, Z. Li, V.M. Berrios, M.S. Dooner, C.L. Cooper, C.C. Hsieh, and P.J. Quesenberry. 1999. Adhesion receptor expression by hematopoietic cell lines and murine progenitors: modulation by cytokines and cell cycle status. *Exp. Hematol.* 27:533–541.
- Wright, D.E., A.J. Wagers, A.P. Gulati, F.L. Johnson, and I.L. Weissman. 2001. Physiological migration of hematopoietic stem and progenitor cells. *Science*. 294:1933–1936.
- Frimberger, A.E., C.I. McAuliffe, K.A. Werme, R.A. Tuft, K.E. Fogarty, B.O. Benoit, M.S. Dooner, and P.J. Quesenberry. 2001. The fleet feet of haematopoietic stem cells: rapid motility, interaction and proteopodia. *Br. J. Haematol.* 112: 644–654.
- Nilsson, S.K., H.M. Johnston, and J.A. Coverdale. 2001. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood*. 97: 2293–2299.

8. Lemishka, I.R., D.H. Raulet, and R.C. Mulligan. 1986. Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell.* 45:917–927.
9. Wolf, N.S., A. Kone, G.V. Priestley, and S.H. Bartelmez. 1993. In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. *Exp. Hematol.* 21:614–622.
10. Bradford, G.B., B. Williams, R. Rossi, and I. Bertoncello. 1997. Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. *Exp. Hematol.* 25:445–453.
11. Peters, S.O., E.L. Kittler, H.S. Ramshaw, and P.J. Quesenberry. 1996. Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. *Blood.* 87:30–37.
12. Habibian, H.K., S.O. Peters, C.C. Hsieh, J. Wuu, K. Vergilis, C.I. Grimaldi, J. Reilly, J.E. Carlson, A.E. Frimberger, F.M. Stewart, and P.J. Quesenberry. 1998. The fluctuating phenotype of the lymphohematopoietic stem cell with cell cycle transit. *J. Exp. Med.* 188:393–398.
13. Colvin, G.A., J.E. Carlson, J.-F. Lambert, C.I. McAuliffe, and P.J. Quesenberry. 2000. Hematopoietic stem cells in microgravity. *Exp. Hematol.* 28(Suppl. 1):118. (Abstr.)
14. Chesire, S.H., S.J. Morrison, X. Liao, and I.L. Weissman. 1999. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA.* 96:3120–3125.
15. Pang, L., P.V. Reddy, and P.J. Quesenberry. 1999. Are bone marrow stem cells quiescent? *Exp. Hematol.* 27:106. (Abstr.)
16. Nilsson, S.K., M.S. Dooner, and P.J. Quesenberry. 1997. Synchronized cell-cycle induction of engrafting long-term repopulating stem cells. *Blood.* 90:4646–4650.
17. Reddy, G.P.V., C.Y. Tiarks, L. Pang, and P.J. Quesenberry. 1997. Synchronization and cell cycle analysis of pluripotent hematopoietic progenitor stem cells. *Blood.* 90:2293–2299.
18. Phillips, R.L., R.E. Ernst, B. Brunk, N. Ivanova, M.A. Mahan, J.K. Deanehan, K.A. Moore, G.C. Overton, and I.R. Lemischka. 2000. The genetic program of hematopoietic stem cells. *Science.* 288:1635–1640.
19. Ramalho-Santos, M., S. Yoon, Y. Matsuzaki, R.C. Mulligan, and D.A. Melton. 2002. “Stemness”: transcriptional profiling of embryonic and adult stem cells. *Science.* 298:597–600.
20. Ivanova, N.B., J.T. Dimos, C. Schaniel, J.A. Hackney, K.A. Moore, and I.R. Lemischka. 2002. A stem cell molecular signature. *Science.* 298:601–604.
21. Park, I.K., Y. He, F. Lin, O.D. Laerum, Q. Tian, R. Bumgarner, C.A. Klug, K. Li, C. Kuhr, M.J. Doyle, et al. 2002. Differential gene expression profiling of adult murine hematopoietic stem cells. *Blood.* 99:488–498.
22. Prashar, Y., and S.M. Weissman. 1996. Analysis of differential gene expression by display of 3' expression by display of 3' restriction fragments of cDNAs. *Proc. Natl. Acad. Sci. USA.* 93:659–663.
23. Reddy, G.P. 1989. Compartmentation of deoxypyrimidine nucleotides for nuclear DNA replication in S phase mammalian cells. *J. Mol. Recognit.* 2:75–83.
24. Lamar, E.E., and E. Palmer. 1984. Y-encoded, species-specific DNA in mice: evidence that the Y chromosome exists in two polymorphic forms in inbred strains. *Cell.* 37:171–177.
25. Hawkins, A.L., R.J. Jones, B.A. Zehnbauer, M.S. Zicha, M.J. Collector, S.J. Sharkis, and C.A. Griffin. 1992. Fluorescence in situ hybridization to determine engraftment status after murine bone marrow transplant. *Cancer Genet. Cytogenet.* 64:145–148.
26. Degar, B.A., N. Baskaran, R. Hulspas, P.J. Quesenberry, S.M. Weissman, and B.G. Forget. 2001. The homeodomain gene Pitx2 is expressed in primitive hematopoietic stem/progenitor cells but not in their differentiated progeny. *Exp. Hematol.* 29:894–902.
27. Liu, M., Y.V. Subramanyam, and N. Baskaran. 1999. Preparation and analysis of cDNA from a small number of hematopoietic cells. *Methods Enzymol.* 303:45–55.
28. Liang, P., and A.B. Pardee. 1995. Recent advances in differential display. *Curr. Opin. Immunol.* 7:274–280.
29. Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.
30. Available at URL: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide>.
31. Kapranov, P., S.E. Cawley, J. Drenkow, S. Bekiranov, R.L. Strausberg, S.P. Fodor, and T.R. Gingeras. 2002. Large-scale transcriptional activity in chromosomes 21 and 22. *Science.* 296:916–919.
32. Fleming, W.H., E.J. Alpern, N. Uchida, K. Ikuta, G.J. Spangrude, and I.L. Weissman. 1993. Functional heterogeneity is associated with the cell cycle status of murine marrow cells. *J. Cell Biol.* 122:897–902.
33. Orschell-Traycoff, C.M., K. Hiatl, R.N. Dagher, S. Rice, M.C. Yoder, and E.F. Srour. 2000. Homing and engraftment potential of Sca-1+ lin- cells fractionated on the basis of adhesion molecule expression and position in cell cycle. *Blood.* 96:1380–1387.
34. Interthal, H., and W.D. Heyer. 2000. MUS81 encodes a novel helix-hairpin-helix protein involved in the response to UV- and methylation-induced DNA damage in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 263:812–827.
35. Nishino, T., K. Morikawa. 2002. Structure and function of nucleases in DNA repair: shape, grip and blade of the DNA scissors. *Oncogene.* 21:9022–9032.
36. Tanaka, K., S. Kamiuchi, Y. Ren, R. Yonemasu, M. Ichikawa, H. Murai, M. Yoshino, S. Takeuchi, M. Saijo, Y. Nakatsu, H. Miyauchi-Hashimoto, and T. Horio. 2001. UV-induced skin carcinogenesis in xeroderma pigmentosum group A (XPA) gene-knockout mice with nucleotide excision repair-deficiency. *Mutat. Res.* 477:31–40.
37. Marushige, Y., K. Marushige. 1995. Disappearance of ubiquitinated histone H2A during chromatin condensation in TGF beta 1-induced apoptosis. *Anticancer Res.* 15:267–272.
38. Rogakou, E.P., W. Nieves-Neira, C. Boon, Y. Pommier, and W.M. Bonner. 2000. Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. *J. Biol. Chem.* 275:9390–9395.
39. Mortillaro, M.J., and R. Berezney. 1998. Matrin CYP, an SR-rich cyclophilin that associates with the nuclear matrix and splicing factors. *J. Biol. Chem.* 273:8183–8192.
40. Hutchcroft, J.E., J.M. Slavik, H. Lin, T. Watanabe, and B.E. Bierer. 1998. Uncoupling activation-dependent HS1 phosphorylation from nuclear factor of activated T cells transcriptional activation in Jurkat T cells: differential signaling through CD3 and the costimulatory receptors CD2 and CD28. *J. Immunol.* 161:4506–4512.
41. Hubacek, J.A., and R. Pledne. 1999. The common cDNA and amino acid sequences of the Cd14 (myeloid cell-specific

- leucine-rich glycoprotein) receptor. *Physiol. Res.* 48:323–326.
42. Ghiselli, G., R.V. Iozzo. 2000. Overexpression of bamacan/SMC3 causes transformation. *J. Biol. Chem.* 275:20235–20238.
43. Martin, P.R., A.A. Watson, T.F. McCaul, and J.S. Mattick. 1995. Characterization of a five-gene cluster required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 16:497–508.
44. Appeddu, P.A., and B.D. Shur. 1994. Molecular analysis of cell surface beta-1,4-galactosyltransferase function during cell cycle. *Proc. Natl. Acad. Sci. USA.* 91:2095–2099.
45. Appeddu, P.A., and B.D. Shur. 1994. Control of stable lamellipodia formation by expression of cell surface beta 1,4-galactosyltransferase cytoplasmic domains. *J. Cell Sci.* 107: 2535–2545.
46. Degen, W.G., L.C. van Kempen, E.G. Gijzen, J.J. van Groningen, Y. van Kooyk, H.P. Bloemers, and G.W. Swart. 1998. MEMD, a new cell adhesion molecule in metastasizing human melanoma cell lines, is identical to ALCAM (activated leukocyte cell adhesion molecule). *Am. J. Pathol.* 152:805–813.
47. Cerny, J., M. Dooner, C. McAuliffe, H. Habibian, K. Stencil, V. Berrios, J. Reilly, J. Carlson, A.M. Cerny, L. D'Hondt, et al. 2002. Homing of purified murine lymphohematopoietic stem cells: a cytokine-induced defect. *J. Hematother. Stem Cell Res.* 11:913–922.
48. Reddy, G.P., C.I. McAuliffe, L. Pang, P.J. Quesenberry, and I. Bertoncello. 2002. Cytokine receptor repertoire and cytokine responsiveness of Ho^{dull}/Rh^{dull} stem cells with differing potentials for G1/S phase progression. *Exp. Hematol.* 30:792–800.
49. Bonifer, C. 1999. Long-distance chromatin mechanisms controlling tissue-specific gene locus activation. *Gene.* 2238:277–289.
50. Maes, J., L.P. O'Neill, P. Cavelier, B.M. Turner, F. Rougeon, and M. Goodhardt. 2001. Chromatin remodeling at the Ig loci prior to V(D)J recombination. *J. Immunol.* 167: 866–874.
51. Jimenez, G., S.D. Griffiths, A.M. Ford, M.F. Greaves, and T. Enver. 1992. Activation of the beta-globin locus control region precedes commitment to the erythroid lineage. *Proc. Natl. Acad. Sci. USA.* 89:10618–10622.
52. Kontaraki, J., H.H. Chen, A. Riggs, and C. Bonifer. 2000. Chromatin fine structure profiles for a developmentally regulated gene: reorganization of the lysozyme locus before transactivator binding and gene expression. *Genes Dev.* 14:2106–2122.
53. Hu, M., D. Krause, M. Greaves, S. Sharkis, M. Dexter, C. Heyworth, and T. Enver. 1997. Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev.* 11:774–785.
54. Ford, A.M., C.A. Bennett, L.E. Healy, E. Navarro, E. Spooncer, and M.F. Greaves. 1992. Immunoglobulin heavy-chain and CD3 delta-chain gene enhancers are Dnase I-hypersensitive in hemopoietic progenitor cells. *Proc. Natl. Acad. Sci. USA.* 89:3424–3428.
55. McConnell, S., and C. Kaznowski. 1991. Cell cycle dependence of laminar determination in developing neocortex. *Science.* 254:282–285.
56. Schroeder, T., and U. Just. 2000. Just mNotch1 signaling reduces proliferation of myeloid progenitor cells by altering cell-cycle kinetics. *Exp. Hematol.* 28:1206–1213.
57. Kumano, K., S. Chiba, K. Shimizu, T. Yamagata, N. Hosoya, T. Saito, T. Takahashi, Y. Hamada, and H. Hirai. 2001. Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. *Blood.* 98:3283–3289.
58. Reddy, V.A., A. Iwama, G. Iotzova, M. Schulz, A. Elsasser, R.K. Vangala, D.G. Tenen, W. Hiddemann, and G. Behre. 2002. Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. *Blood.* 100:483–490.
59. Olson, M.C., E.W. Scott, A.A. Hack, G.H. Su, D.G. Tenen, H. Singh, and M.C. Simon. 1995. PU.1 is not essential for early myeloid gene expression but is required for terminal myeloid differentiation. *Immunity.* 3:703–714.