

ORIGINAL ARTICLE

Gene expression profiles of AML derived stem cells; similarity to hematopoietic stem cells

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Tumors contain a fraction of cancer stem cells that maintain the propagation of the disease. The CD34+CD38– cells, isolated from acute myeloid leukemia (AML), were shown to be enriched leukemic stem cells (LSC). We isolated the CD34+CD38– cell fraction from AML and compared their gene expression profiles to the CD34+CD38+ cell fraction, using microarrays. We found 409 genes that were at least twofold over- or under-expressed between the two cell populations. These include underexpression of DNA repair, signal transduction and cell cycle genes, consistent with the relative quiescence of stem cells, and chromosomal aberrations and mutations of leukemic cells. Comparison of the LSC expression data to that of normal hematopoietic stem cells (HSC) revealed that 34% of the modulated genes are shared by both LSC and HSC, supporting the suggestion that the LSC originated within the HSC progenitors. We focused on the Notch pathway since Jagged-2, a Notch ligand was found to be overexpressed in the LSC samples. We show that DAPT, an inhibitor of gamma-secretase, a protease that is involved in Jagged and Notch signaling, inhibits LSC growth in colony formation assays. Identification of additional genes that regulate LSC self-renewal may provide new targets for therapy.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease with variations in the cell markers, chromosomal aberrations, mutations, response to therapy and prognosis. Increasing evidence suggest that AML and other malignancies are sustained by a minor tumor subpopulation with self-renewal potential, referred to as 'cancer stem cells' or 'leukemic stem cells' (LSC). These cells drive the growth and dissemination of the leukemia.^{1–4} Recent studies have extended this model to other types of cancers such as breast cancer⁵ and Glioblastoma multiforme.⁶ The advances in the analysis of the origin of the leukemic cells came from studies on engraftment of patient's derived leukemia into the immunodeficient NOD-SCID mice,^{7,8} and following the propagation of the disease.⁹ These studies led to the identification of CD34+CD38– cells as the LSC fraction of AML. In addition, a high percentage of CD34+CD38– stem

cells at diagnosis was directly correlated with poor survival and significantly correlated with a high minimal residual disease frequency, especially after chemotherapy.¹⁰

An important property that is shared by both LSC and normal HSC is their ability of self-renewal, which enables the maintenance of the leukemic clone on the one hand or the normal hematopoiesis on the other hand.^{3,4,11,12} Some molecular mechanisms responsible for self renewal, like Bmi-1, Notch and Wnt signaling pathways, were found to be shared by both HSC and LSC.^{4,13,14} This suggests that the origin of LSC may be at the hematopoietic stem cell level.² The existence of cancer stem cells is of major clinical relevance since their unique properties, such as slow mitosis, increased multidrug resistance and lower expression of *Fas/Fas-L* and *Fas*-induced apoptosis, may enable them to escape therapy¹⁵ that is based on markers or phenotype of the entire AML population. We therefore sought to examine the gene expression profiles of the LSC within the AML cell population to further understand their biology and identify new target genes that maintain and characterize LSC.

For this purpose we isolated CD34+CD38– and CD34+CD38+ cell populations from five AML patients and analyzed their gene expression profiles using Affymetrix Hu133A microarrays. We found 409 genes that were over or under expressed between the two populations, sorting the modulated genes showed a clear distinction between the cell populations. The GO (Gene Ontology) analysis of the modulated genes in LSC revealed a decreased expression of DNA repair, signal transduction and cell cycle regulating genes and an increased expression of genes related to protein degradation, cell adhesion, transcription and the Notch pathway. We therefore tried to inhibit the growth of LSC colonies in methyl cellulose by inhibiting the Notch pathway, an approach that may be of clinical value. Understanding the mechanism of action of genes involved in LSC has significant implications for future research and will potentially lead to new targets for therapy and diagnostics.

Materials and methods

Sample collection and isolation of LSC

Leukemic peripheral blood (PB) samples from five AML patients were collected after obtaining informed consent. Samples were used in accordance with the procedures approved by the human experimentation and ethics committee of the Chaim Sheba Medical Center. Disease diagnosis and classification were

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according to French-American-British (FAB) criteria. Mononuclear cells (MNC) were purified from the samples by Ficoll–Hypaque density gradient centrifugation, resulting in approximately $1\text{--}3 \times 10^8$ cells per sample. To determine the CD34 and CD38 content, cells were labeled with anti-human CD34–fluorescein isothiocyanate (FITC) anti-CD38–phycoerythrin (PE) and IgG isotype control monoclonal antibodies (Miltenyi Biotec, Auburn, CA, USA) and the CD34 and CD38 expression levels were determined for each sample using FACS-Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). Background levels were determined with PE-FITC labeled IgG control. Data acquisition and analysis were performed with CellQuest software (Becton Dickinson). Each phenotype was generated by analysis of 10 000 AML cells. Cell populations CD34 + CD38– and CD34 + CD38+ were sorted using a FACS Vantage flow cytometer (Becton Dickinson) after which the cells were spun down, resuspended by vigorous pipeting in Trizol (200 μ l/ 10^6 cells) and stored at -70°C until RNA extraction.

RNA extraction and microarray hybridization

Total RNA (4–10 μ g) of the CD34 + CD38– and the CD34 + CD38+ cell populations of each patient was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The quality of the total RNA were analyzed using an agarose gel. All experiments were performed using Affymetrix Human Hu133A oligonucleotide arrays containing 22 215 probe sets (PS) as described in the Affymetrix human_datasheet.pdf (http://www.affymetrix.com/support/technical/datasheets/human_datasheet.pdf) (Affymetrix, Santa Clara, CA, USA). Total RNA from each sample was used to prepare biotinylated target cRNA, with minor modifications from the manufacturer's recommendations (Affymetrix Expression Manual). The target cRNA generated from each sample was processed according to the manufacturer's recommendation using an Affymetrix GeneChip Instrument System (Affymetrix Expression Manual). Arrays were then washed and stained with streptavidin-phycoerythrin before being scanned on an Affymetrix GeneChip scanner. When scaling the expression values to a target intensity of 150, scaling factors for all arrays were within acceptable limits (0.855–1.533). Scanned output files were analyzed by the probe level analysis package MAS 5.0.

Data analysis

The expression data for each AML patient is represented by pairs of samples, CD34 + CD38– and CD34 + CD38+, respectively. The complete gene expression data is available at: <http://www.weizmann.ac.il/physics/complex/compphys/downloaddata.htm>. Gene expression values <10 were adjusted to 10 to eliminate noise from the data and subsequently all values were log₂-transformed. The expression ratio for each gene in the pair of samples, CD34 + CD38– and CD34 + CD38+, was determined for each AML patient. Only genes that were 'Present' in the 'Present/Absent' call provided by the Affymetrix program, in at least one sample were selected, remaining with 14 125 valid genes. Valid genes with expression ratios above or below twofold in at least three out of five patients were selected. This resulted in 148 and 261 genes, showing over- or underexpression in LSC, respectively.

The statistical significance of the lists of modulated genes was tested by a random model, described in detail in the Supplementary Information (Method S1 and Figure S1). Briefly, in this model, a binary matrix of modulated gene expression ratios was randomized such that the total number of matrix

elements remained unchanged. This randomization was repeated 10^5 times, yielding 10^5 lists of modulated genes, above or below twofold, in three (or more) out of five samples. The maximal list-size of those 10^5 lists of over- (and under-) expressed genes generated by the random model was smaller than the list-sizes of 148 over- and 261 underexpressed genes obtained from the data, and thus provide an upper bound (10^{-5}) for the *P*-values for our findings. An estimate of the *P*-values was obtained by an approximate analytical calculation (see Supplementary Method S1 and Figure S1 for more details). The genes were classified into functional categories according to the David¹⁷ and GeneCards databases.¹⁸ Before data analysis, we performed normalization to eliminate noise that is due to similarities within the pairs of samples of each patient. We performed this normalization by scanning all the genes and subtracting from each pair of samples (i.e., CD34 + CD38– and CD34 + CD38+) the mean expression (over the pair of samples) of the particular gene. We then applied the SPIN (Sorting Points Into Neighborhoods) algorithm, an unsupervised analysis tool for organization and visualization of the data.¹⁹

Quantitative real time-polymerase chain reaction

QRT-PCR assays were used to determine the expression of eight modulated genes: *CD38*, *BUB1B*, *IGBP7*, *CCL4*, *HES1*, *BCL11A*, *RBPMS* and *LIMK2*. Reactions were performed using the SYBR Green PCR Master mix with the 7900HT ABI platform (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA), as described previously.²² Primers (Danyel Biotech, Rehovot, Israel) were designed according to Primer-Express software. The primers used appear in supplementary Table S5. Samples were normalized to the housekeeping gene beta-2-microglobulin ($\beta 2M$), whose levels of expression were not changed significantly according to the microarray data (data not shown).

Colony formation in methyl cellulose

The LSC (CD34 + CD38–) cells from sample 4 were isolated by magnetic beads. This AML sample contained very low levels of CD38+ cells (4.6%) and after separation with anti-CD34 + beads, the isolated fraction was 94.9% CD34 + CD38– (similar results were obtained when separation was done with FACS). Cells (1×10^5) were plated in 35 mm plates, with varying concentrations of DAPT, in a medium of 0.9% methyl cellulose, RPMI medium supplemented with penicillin-streptomycin, 10% FCS, 2 mmol/l L-glutamine, and a mixture of 1% BSA with 5 U/ml human erythropoietin, 10 ng/ml GM-CSF, 10 ng/ml IL-3 and 100 ng/ml SCF. Assays were performed in duplicates, and colonies or inhibition of colony growth were scored, and photographed microscopically on day 14.

Comparisons with other HSC profiles

We compared our LSC gene expression data with normal HSC populations, obtained from three previous microarray studies; Georgantas *et al.*,²⁰ Ivanova *et al.*²¹ and Toren *et al.*²² Georgantas *et al.*²⁰ described modulated genes in human CD34 + CD38– cells, from bone marrow (BM), cord blood (CB), and PB. These lists include a total of 2205 and 1999 over- and underexpressed genes, respectively, which met the twofold threshold criteria when compared with the CD34 + CD38+ cells. Ivanova *et al.*²¹ described 822 human homologs for murine HSC-related genes that are expressed in fetal liver. Analysis by Toren *et al.*²² of the Ivanova *et al.*²¹ data generated a list of 1905 human HSC-related genes, as described

previously.²² Toren et al.²² determined lists of HSC-related genes in CD133+ cells. They obtained 244 over- and 224 underexpressed genes, by at least twofold in gene expression profiles of HSC CD133+, compared with the differentiated CD133- that were derived from CB and mobilized PB. The HSC lists from these three studies were compared with the 148 over- and 261 underexpressed genes, obtained from our study (Supplementary Tables S1 and S2). The hypergeometric distribution²³ was used to obtain the chance probability of observing the number of overlapping genes between our lists and the HSC data sets.

Results

Heterogeneity of the AML samples

The karyotypic and surface markers of the five samples, as shown in Table 1, illustrate the heterogeneity of the AML samples. Nevertheless, all samples contain a variable-size

fraction (3–36%) of CD34+CD38- cells that represent the LSC fraction. The AML samples were sorted into highly purified CD34+CD38- and CD34+CD38+ cell fractions that were subsequently subjected to microarray analysis. Sorting results of three AML samples and the purity of the isolated fractions are shown in Figure 1. The content and purity of each fraction, for all AML patients, is shown in Table 1.

Gene expression profiling of LSC reveals similarity with HSC

Human Affymetrix Hu133A oligonucleotide arrays were used to compare the gene expression profiles of the highly enriched LSC, CD34+CD38-, to their differentiated counterparts, CD34+CD38+ cells, obtained from the AML patients. Using filtering criteria, which selected genes that were modulated by at least twofold in the CD34+CD38- versus CD34+CD38+ cell fractions, in at least three out of five AML patients, we remained with a total of 409 genes. Of these 409 genes 148

Table 1 Characterization of AML samples and expression of CD34 and CD38

No.	Sample/FAB subtype	Sex	CD34+ in MNC (%)	CD34+CD38- in MNC (%)	Purity of sorted CD34+CD38- (%)	Karyotype
1	AML/M4	F	71	3	>90	inv(16)(p13q22)
2	AML/M4	F	81	12	>90	t(9;11)(p22;q23)
3	AML/M4	M	23	2.8	>90	trisomy 8
4	AML/M2	F	48	36	86	ND
5	AML/M2	F	62	29	>90	t(3;8)

Abbreviations: FAB, French-American-British criteria for subtypes; MNC, mononuclear cells; ND, not determined.

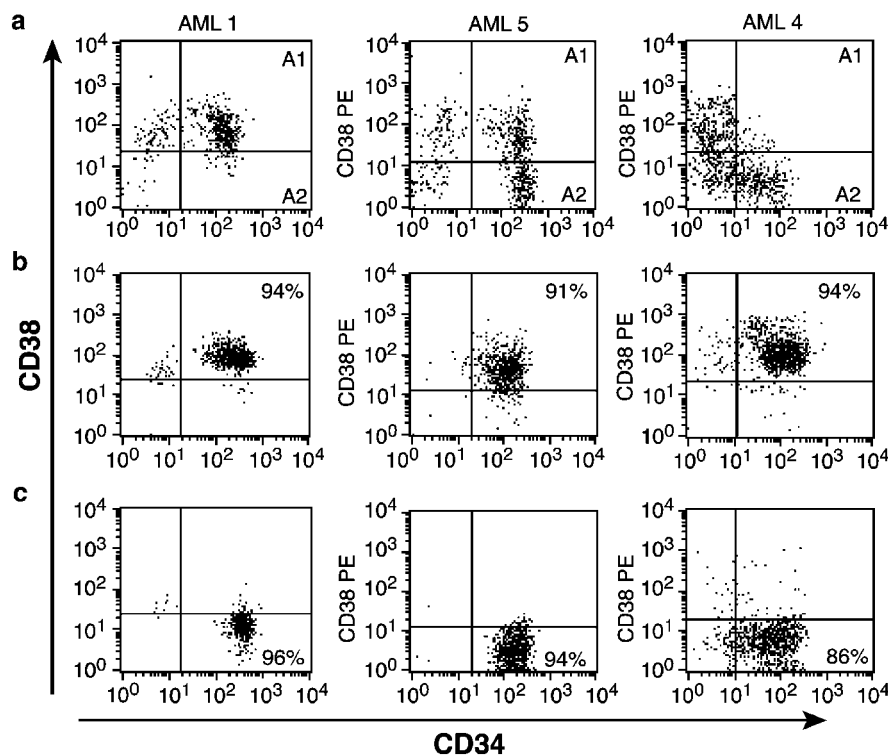


Figure 1 Representative analysis of FACS isolated AML subpopulations. AML cells were fractionated and analyzed for CD34 and CD38 content. (a) CD34 and CD38 content of three AML samples, prior to sorting. A1 and A2 quadrants containing the cell fractions CD34+CD38+ and CD34+CD38-, respectively. (b) Analysis of the sorted CD34+CD38+ populations. (c) Analysis of the sorted CD34+CD38- populations. % Indicates the purity of the sorted fractions.

were overexpressed and 261 were underexpressed in the LSC fraction, respectively (Supplementary Tables S1 and S2).

The statistical significance of obtaining this number of over- and underexpressed genes, as calculated by an analytic approximation to the distribution generated by the random model (see Materials and methods) is extremely low; $P \sim 3.2 \times 10^{-65}$ and $P \sim 9.8 \times 10^{-223}$, with FDR¹⁶ of 27 and 19%, for the over- and underexpressed genes, respectively. This means that the number of genes that show modulated expression between the LSC and non-stem cell populations of AML is highly significant and cannot be obtained by chance. Overexpressed genes (73%) and 81% of the underexpressed genes are not due to chance (for more details, see Supplementary Method S1 and Figure S1).

The results of the microarray hybridization were further validated by QRT-PCR analysis of a group of eight genes, selected from the 409 modulated genes. A good correlation between the two methods was observed, as shown in Supplementary Figure S2.

For the visualization of the data, we applied the SPIN (Sorting Points Into Neighborhoods) analysis tool¹⁹ on the filtered genes and the results are summarized in the expression matrix of Figure 2. The expression matrix shows a clear distinction between the LSC (CD34+CD38-), and their differentiated counterpart CD34+CD38+ cells. We searched for genes that are modulated in LSC in our data and compared them with three

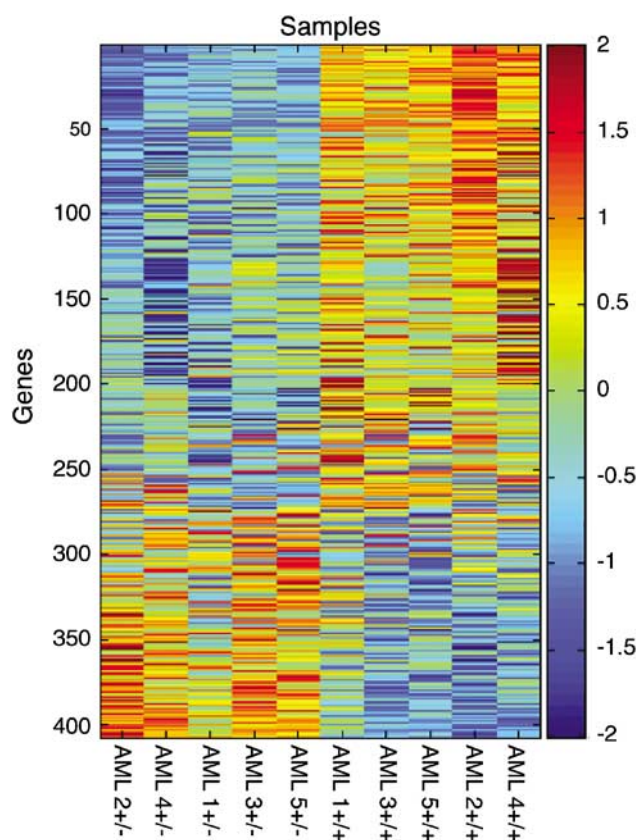


Figure 2 Expression matrix of the 409 modulated genes. Each row represents a single gene, and each column represents the CD34+CD38- (+/-) or the CD34+CD38+ (+/+) cell population of a particular AML patient (AML1–AML5). The colors indicate the relative expression levels of the genes in the AML samples, according to the color code shown on the right. The expression matrix shows the partitioning of the LSC and the non-stem populations (CD34+CD38+) in all AML patients.

data sets of gene expression, obtained from normal HSC.^{20–22} We found that 34 (23%) out of the 148 genes overexpressed in the LSC, were reported in different data sets to be typical to normal HSC (Table 2). The probability of observing this overlap by chance using hypergeometric distribution²³ was found to be low ($P = 5.5 \times 10^{-3}$). Similarly, 104 (39%) out of the 261 genes that were underexpressed in LSC (i.e. over-expressed in CD34+CD38+) showed similar behavior in normal HSC (Supplementary Table S2), with a chance probability of $P = 8.6 \times 10^{-12}$. Thirty selected genes out of the 104 genes are presented in Table 3. These findings suggest that some of the ‘signature’ genes of LSC are consistent with the cell stage of HSC or progenitor cells.

LSC show underexpression of cell cycle and DNA repair genes

Analysis of the 409 modulated genes in the AML samples by GO annotation using DAVID¹⁷ revealed underexpression in genes controlling signal transduction pathways, DNA repair and cell cycle genes (Figure 3). These genes include: *Cyclin A2*, *Cyclin B2*, *Cyclin B1*, *CDC20*, *CDC2*, *CDC48*, *CDC6*, *CDC45A*, *CDC25C*, *BUB1* and *BUB1B* (Supplementary Table S2). In spite of the overexpression of *cyclin D1* which is known to be activated in a variety of tumors, the underexpression of many cell cycle genes is in line with the known slow cell division rate (quiescence) characteristic of stem cells.²⁴ This is also consistent with the underexpression of several transcription factors like *E2F1*, *E2F2*, *E2F8*, *EGR2* and the differentiation antigen *MNDA* (Supplementary Table S2). Interestingly, several genes known to be oncogenic are overexpressed, such as *ETS1*, *MAF*, *MLL*, *GATA-1* and *BCL11A* (Supplementary Table S1). Another characteristic of LSC gene expression is the under-expression of important DNA repair genes such as: *LIG1*, *FEN1*, *RAD51*, *POLM* and *TOP2A* (Supplementary Table S2). These findings are consistent with the increasing chromosomal aberrations and mutations that are typical of AML.

The heterogeneity of AML does not yet permit a list of signature genes typical of AML. However, it is possible to identify some genes that may be responsible for the stemness of the malignant stem cells. Interestingly, such genes may also be expressed in both LSC and normal HSC. For example, *IGF1R*, *MLL*, *VEGFB* and *JAG2* are part of such a group (Table 2). Jagged-2, a ligand of the Notch receptor was found to be highly expressed in our LSC population. Notch pathway has been linked with the self-renewal and maintenance of HSC stem cells.^{25–27} Notch proteins are mutated in a variety of cancers, including leukemia progenitors.²⁸ It has been shown that other Notch ligands such as Jagged-1 and Delta-1 affect the growth and differentiation of primary AML cells²⁹ and are important factors in the malignancy of a variety of cancers including intestinal and hematopoietic cancers.³⁰ This result suggests that the inhibition of the Notch pathway may affect the properties and self-renewal of the LSC. To further study the role of this pathway, we analyzed the effect of inhibitors of Notch in AML samples.

Effects of inhibitor of Notch pathway on LSC colony growth

The Notch transmembrane receptor interacts with the transmembrane ligands Delta and Jagged, resulting in proteolytic cleavage by the secretase family that facilitate the signaling by the Notch pathway. Several enzymes are involved in this cleavage, one of which is the gamma secretase complex, which

Table 2 Genes overexpressed in leukemic CD34+CD38– versus CD34+CD38+, and in HSC versus hematopoietic differentiated cells

Gene Name	Symbol	HSC data source ^a	Fold change ^b				
			AML1	AML2	AML3	AML4	AML5
Insulin-like growth factor 1 receptor	<i>IGF1R</i>	I	2.47	5.67	2.04	1.53	1.39
Neurotrophic tyrosine kinase, receptor 3	<i>NTRK3</i>	I	1.06	2.85	2.78	5.25	2.67
G protein-coupled receptor 1	<i>GPR1</i>	G	1.00	3.07	0.35	4.36	2.04
Neurofibromin 2	<i>NF2</i>	I	2.91	2.98	1.12	2.61	2.89
Mitogen-activated protein kinase kinase 5	<i>MAP2K5</i>	I	0.84	0.98	2.09	2.11	2.20
MAD, homolog 6 (<i>Drosophila</i>)	<i>MADH6</i>	I	0.46	3.91	1.00	2.99	3.78
Interferon regulatory factor 6	<i>IRF6</i>	I	3.17	18.55	2.65	1.00	1.77
Myeloid/lymphoid or mixed-lineage leukemia	<i>MLL</i>	G	2.02	0.81	2.40	0.44	2.06
B-cell CLL/lymphoma 11A	<i>BCL11A</i>	G, T	2.34	0.90	2.01	1.97	2.30
Jagged 2	<i>JAG2</i>	I	2.04	3.34	4.21	1.21	0.31
Vascular endothelial growth factor B	<i>VEGFB</i>	I	0.85	3.85	3.12	1.24	2.74
Slit homolog 2 (<i>Drosophila</i>)	<i>SLIT2</i>	I	0.39	2.51	5.38	2.25	3.13
Amelogenin (amelogenesis imperfecta 1, X linked)	<i>AMELX</i>	G	0.73	0.51	4.54	2.29	3.83
Actin binding LIM protein 1	<i>ABLIM1</i>	G	2.46	15.53	1.68	2.13	1.92
LIM domain kinase 2	<i>LIMK2</i>	G	7.86	1.13	4.80	0.89	2.24
Discs, large homolog 3 (<i>Drosophila</i>)	<i>DLG3</i>	I	4.65	4.06	4.32	1.14	4.82
Bullous pemphigoid antigen 1	<i>BPAG1</i>	G, T	2.51	0.68	0.41	4.30	2.46
Integrin, beta 4	<i>ITGB4</i>	I	1.08	1.59	3.97	2.43	3.11
Bridging integrator 1	<i>BIN1</i>	I	1.37	2.18	3.96	2.88	1.00
Myosin X	<i>MYO10</i>	I	1.01	3.74	1.00	2.41	2.35
Dihydrolipoamide branched chain transacylase	<i>DBT</i>	I	0.77	2.83	3.03	1.45	3.04
Aldo-keto reductase family 1, member C1	<i>AKR1C1</i>	G	1.73	0.18	2.77	2.20	2.69
Topoisomerase (DNA) III beta	<i>TOP3B</i>	I	2.22	2.56	0.92	2.59	1.02
Cyclin D1	<i>CCND1</i>	I	0.30	6.04	2.15	1.00	2.96
RNA binding protein with multiple splicing	<i>RBPMS</i>	I, G, T	2.21	2.55	1.48	1.31	3.53
PFTAIRE protein kinase 1	<i>PFTK1</i>	I	0.93	2.69	2.61	2.22	0.95
Protein kinase, DNA-activated, catalytic polypeptide	<i>PRKDC</i>	I	3.43	0.25	0.51	3.23	2.15
B4Gal-T4	<i>B4GALT4</i>	I, G	0.37	0.79	7.14	2.11	4.44
Hypothetical protein MGC14817	<i>MGC14817</i>	G	1.94	0.36	2.42	3.57	2.44
Hypothetical protein FLJ11000	<i>FLJ11000</i>	G	3.72	0.41	0.59	3.29	5.67
Cathepsin Z	<i>CTSZ</i>	I	1.18	5.21	2.30	0.64	2.67
Peroxisome biogenesis factor 1	<i>PEX1</i>	I, T	2.95	1.82	1.61	2.50	2.11
ATP synthase lipid-binding protein, mitochondrial precursor	<i>ATP5G2</i>	I	2.17	2.70	2.29	1.58	0.38
Breast epithelial mucin-associated antigen	<i>BPHL</i>	I	2.42	1.31	2.41	0.41	2.17

Abbreviations: HSC, hematopoietic stem cells; LSC, leukemic stem cells.

^aData sets were obtained from: G, Georgantas;²⁰ I, Ivanova;²¹ T, Toren.²²
^bThe indicated fold change is the ratio of CD34+CD38– versus CD34+CD38+ cell populations of each AML patient.

cleaves the Notch receptor within the membrane. Inhibition of this enzyme intervenes with the Notch signaling pathway and many inhibitors of gamma-secretase are being used mainly in Alzheimer's disease. We attempted to affect the growth of LSC colonies in methyl cellulose by the gamma-secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenyl glycine *t*-butyl ester).³¹ The LSC from AML sample 4 were isolated by magnetic beads and 1×10^5 cells were mixed with methyl cellulose, medium and cytokines and incubated for 14 days, as described in Materials and methods. Several photographs were taken at similar position from each plate and the comparison of colonies with and without DAPT is shown in Figure 4. It is shown that the treatment with DAPT affected the number of colonies and reduced the size of the large colonies. This effect was dose dependent and reached approximately 50% inhibition at 1.6×10^{-5} M (Figure 4a).

Discussion

The 'cancer-stem-cell hypothesis' implies that tumors are composed of a heterogeneous population of cells, within which resides a small population of cancer stem cells that are responsible for the maintenance and propagation of the tumors. These cells possess the stem cell properties of self-renewal and

of inhibited differentiation, but lost the controls operating in normal stem cells, or gained mutations that endowed them with tumorigenicity. In this model, in the case of AML, the LSC exhibit similar phenotype to HSC (e.g. CD34+CD38–), but also show differences from HSC such as the expression of CD123 (IL3 receptor) that is present only on LSC.³² Hence, LSC may represent transformed HSC or progenitor cells that acquire self-renewal by this transformation.¹² It was indeed demonstrated recently that some leukemia oncogenes, generated by chromosomal translocations are able, upon transfection, to confer self-renewal to hematopoietic progenitors and convert them into leukemia cells like AML.³³ An important question is what is the similarity between LSC and HSC? What may be the cellular program that confers selfrenewal property on somatic cells that are normally destined for differentiation but change course towards cancer? It is expected that therapies would have to be targeted to the cancer stem cells for successful treatment of cancer. Currently failure of cancer treatment may be due to the fact that therapies are aimed at the bulk of the cancer cells and not specifically at cancer stem cells and that LSC are more resistant to such chemotherapy. Indeed, poor survival of cancer patients was correlated with high stem cell frequency in AML.¹⁰

In this study, we aimed to identify the gene expression profile of leukemic stem cells by comparing the gene expression of CD34+CD38– cells to that of CD34+CD38+ from AML

Table 3 Genes under expressed in leukemic CD34+CD38– versus CD34+CD38+, and in HSC versus hematopoietic differentiated cells

Gene Name	Symbol	HSC data Source ^a	Fold Change ^b				
			AML1	AML2	AML3	AML4	AML5
CD14 antigen	CD14	T	–2.4	–2.8	–1.0	–106.4	–3.2
Myeloid cell nuclear differentiation antigen	MNDA	G, T	–2.1	–5.1	–0.7	–10.1	–33.9
Elastase 2, neutrophil	ELA2	G, T	–9.5	–24.0	–4.7	–0.5	–2.9
CCAAT/enhancer binding protein (C/EBP), delta	CEBPD	G	–1.1	–4.7	–3.2	–14.3	–0.7
Lamin B1	LMNB1	G	–1.0	–13.8	–3.4	–0.8	–2.0
Ribonucleotide reductase M2 polypeptide	RRM2	G	–2.6	–3.2	–13.4	–3.2	–2.4
Insulin-like growth factor binding protein 7	IGFBP7	G, I	–1.2	–2.0	–11.9	–10.1	–1.6
Chemokine (C–C motif) ligand 4	CCL4	T	–3.3	–3.6	–2.5	–8.0	–1.5
Cyclin B2	CCNB2	G	–3.4	–0.7	–7.8	–2.5	–3.1
Topoisomerase (DNA) II alpha 170 kDa	TOP2A	G	–4.6	–3.8	–4.8	–7.3	–1.2
Baculoviral IAP repeat-containing 5 (survivin)	BIRC5	G, I	–6.8	–0.2	–5.3	–5.0	–1.5
CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	CD86	T	–6.6	–5.0	–1.0	–1.8	–3.4
BUB1 budding uninhibited by benzimidazoles 1, beta (yeast)	BUB1B	G	–1.7	–6.5	–2.4	–6.0	–1.0
CDC45 cell division cycle 45-like (<i>S. cerevisiae</i>)	CDC45L	I	–1.4	–6.4	–3.8	–2.7	–2.7
Leukocyte immunoglobulin-like receptor, B4	LILRB4	T	–2.5	–5.9	–2.5	–3.0	–2.0
Chemokine (C–C motif) ligand 5	CCL5	T	–3.7	–1.2	–2.8	–2.6	–5.1
G protein-coupled receptor 65	GPR65	G, T	–3.0	–3.1	–0.4	–5.1	–0.4
G protein-coupled receptor 109B	GPR109B	G	–2.6	–5.1	–1.3	–1.5	–2.8
CDC20 cell division cycle 20 homolog (<i>S. cerevisiae</i>)	CDC20	G, I	–3.0	–1.0	–2.8	–3.2	–4.7
CD38 antigen (p45)	CD38	G	–2.8	–4.4	–1.6	–2.3	–3.3
Growth differentiation factor 3	GDF3	I	–1.5	–4.4	–3.4	–0.4	–2.8
Ligase I, DNA, ATP-dependent	LIG1	G, I	–1.3	–2.8	–2.5	–4.4	–3.0
BUB1 budding uninhibited by benzimidazoles 1 (yeast)	BUB1	G	–2.0	–0.4	–4.2	–2.8	–0.8
Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	FGR	T	–2.5	–2.8	–0.9	–4.1	–1.5
Polymerase (DNA-directed), alpha	POLA2	G, I	–1.3	–2.6	–2.3	–4.0	–1.1
RAD51 homolog (RecA homolog, <i>E. coli</i>) (<i>S. cerevisiae</i>)	RAD51	G, I	–1.3	–2.4	–3.0	–1.0	–3.7
Cyclin B1	CCNB1	G	–2.3	–3.0	–3.4	–2.5	–0.8
Cyclin-dependent kinase inhibitor 3	CDKN3	G	–2.0	–1.5	–3.3	–2.5	–0.7
Endothelial cell growth factor 1 (platelet-derived)	ECGF1	T	–2.6	–3.2	–2.2	–1.0	–1.0
Mitogen-activated protein kinase 13	MAPK13	I	–2.2	–2.0	–0.7	–2.9	–3.0

Abbreviations: LSC, leukemic stem cells; HSC, hematopoietic stem cells.

^aData sets were obtained from: G, Georgantas;²⁰ I, Ivanova;²¹ T, Toren.²²

^bThe indicated fold change is the ratio of CD34+CD38– versus CD34+CD38+ cell populations of each AML patient. The ‘–’ sign indicates under expression.

The full list of underexpressed genes in LSC and HSC (104 genes) is shown in Supplementary Table S2.

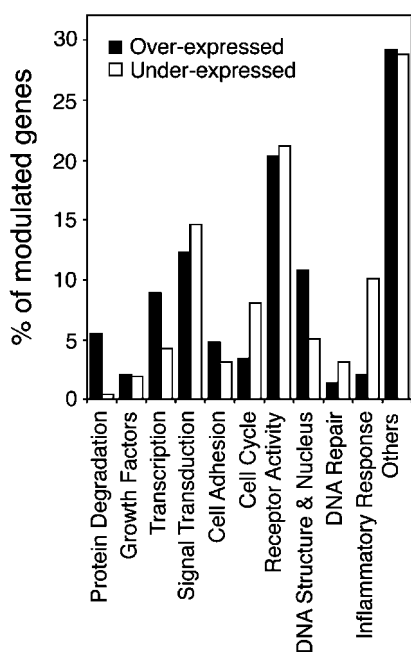


Figure 3 Comparison between the over- and underexpressed genes in LSC, derived from five AML patients. Functional classification was performed according to GO.^{17,18} The percentage of each functional group was derived with respect to the total number of over- or underexpressed genes.

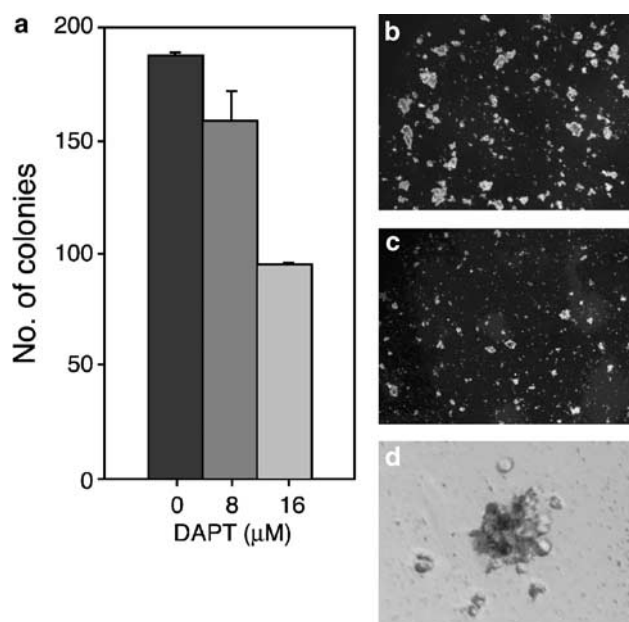


Figure 4 The effect of DAPT on colony formation of CD34 + CD38– cells. (a). The effects of DAPT concentrations on colony formation. (b, c) Representative photomicrograph of colonies, formed by 1×10^5 CD34 + CD38– cells, plated in methyl cellulose, in the absence (b) or in the presence (c) of DAPT (1.6×10^{-5} M). (d) A single colony of CD34 + CD38– cells at high magnification ($\times 200$).

patients. We found 409 genes that were modulated between the two populations and of these 148 and 261 were over-, or underexpressed, respectively. By comparing these lists with three datasets that defined the genes expressed in normal HSC,^{20–22} we found that 23% of the overexpressed and 39% of the underexpressed genes in LSC were present in normal HSC (Tables 2 and 3).

Classification of the modulated genes in LSC revealed decreased expression of DNA repair genes, which is consistent with the increasing chromosomal aberrations that are typical to AML. Furthermore, a large number of cell cycle genes were found to be underexpressed in LSC (Supplementary Table S2). This is in line with the slow division rate, characteristic of adult stem cells.²⁴ A relevant point is the sensitivity of stem cells to chemotherapy, which is directed against rapidly dividing cells. For example, 5-fluorouracil treatment of human leukemia which destroy most of the cells, spare a small fraction of resistant cells. Upon transplantation into SCID mice, these spared cells can reconstitute the leukemia, indicating the resistance of LSC to chemotherapy.³⁴ Additionally in other systems, normal stem cells were found to be mitotic quiescent and differentiation-inhibited.²⁴

Analysis of LSC gene expression may identify new targets for therapy that may be directed at the stem cell fraction. We focused on the Jagged-2 gene, a ligand of the Notch signaling pathway that was found to be overexpressed in LSC in our data. The oncogenic effect of Notch signaling is well known and was reviewed recently.³⁵ Jagged-1, a Notch ligand that is similar to Jagged-2 was shown to transform fibroblasts³⁶ and is considered to be involved in self-renewal of HSC through the activity of the Notch pathway. We use DAPT, the gamma-secretase inhibitor of the Notch pathway, to study the effect of such inhibition on leukemic stem cells colony formation. We show that the treatment with DAPT reduced the number of colonies in LSC and reduced the size of large colonies, probably by inhibiting their proliferation. Previous experiments demonstrated that various inhibitors of the Notch pathway and particularly DAPT analogs, suppress the growth of T-ALL cell lines.^{28,37} Furthermore, activating Notch1 mutations occur in more than 50% of some leukemias,³⁸ underscoring the involvement of Notch1 in the self-renewal mechanism of LSC.

Clinical studies provide many examples of a positive response of tumors to drugs without statistically significant improvements in survival.³⁹ A well known example is the case of CML (chronic myelogenous leukemia) and imatinib (STI571 Gleevec, Novartis, 2001). Although imatinib is highly active against differentiated CML progenitors, this drug may have a limited activity against the CML stem cells and therefore will not prevent relapse.⁴⁰ Similar examples are known for a variety of other cancers³⁹ and indicate the need for expanding the study of cancer stem cell population as targets for new therapy. Our work and that of others shows that the AML stem cells are different from the majority of the leukemic cells and may respond differently to therapy. Understanding the mechanism of action of genes involved in LSC has significant implications for future research on leukemia and may lead to identifying novel targets for therapy and diagnosis.

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)