Identification of a role for the nuclear receptor EAR-2 in the maintenance of clonogenic status within the leukemia cell hierarchy

CV Ichim\(^1,2\), HL Atkins\(^3\), NN Iscove\(^1,4,5,6,9\), and RA Wells\(^1,2,7,8,9\)

\(^1\)Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada
\(^2\)Discipline of Molecular and Cellular Biology, Sunnybrook Research Institute, Toronto, Ontario, Canada
\(^3\)Center for Innovative Cancer Research, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada
\(^4\)Department of Molecular and Cell Biology, Ontario Cancer Institute, Toronto, Ontario, Canada
\(^5\)McEwen Center for Regenerative Medicine, Toronto, Ontario, Canada
\(^6\)Campbell Family Institute for Cancer Research, Toronto, Ontario, Canada
\(^7\)Department of Medicine, University of Toronto, Toronto, Ontario, Canada
\(^8\)Department of Medical Oncology, Myelodysplastic Syndromes Program, Odette Cancer Centre, Sunnybrook Health Sciences Centre, Toronto, Ontario, Canada

Abstract

Identification of genes that regulate clonogenicity of acute myelogenous leukemia (AML) cells is hindered by the difficulty of isolating pure populations of cells with defined proliferative abilities. By analyzing the growth of clonal siblings in low passage cultures of the cell line OCI/AML4 we resolved this heterogeneous population into strata of distinct clonogenic potential, permitting analysis of the transcriptional signature of single cells with defined proliferative abilities. By microarray analysis we showed that the expression of the orphan nuclear receptor EAR-2 (NR2F6) is greater in leukemia cells with extensive proliferative capacity than in those that have lost proliferative ability. EAR-2 is expressed highly in long-term hematopoietic stem cells, relative to short-term hematopoietic stem and progenitor cells, and is downregulated in AML cells after induction of differentiation. Exogenous expression of EAR-2 increased the growth of U937 cells and prevented the proliferative arrest associated with terminal differentiation, and blocked differentiation of U937 and 32Dcl3 cells. Conversely, silencing of EAR-2 by short-hairpin RNA initiated terminal differentiation of these cell lines. These data identify EAR-2 as an important...
factor in the regulation of clonogenicity and differentiation, and establish that analysis of clonal siblings allows the elucidation of differences in gene expression within the AML hierarchy.

Keywords
clonogenicity; hierarchy; NR2F6; EAR-2; differentiation; leukemia stem cell

Introduction
Despite many advances made in the understanding of leukemia biology, therapy for acute myelogenous leukemia (AML) has remained essentially unchanged for 40 years and is, too often, debilitating and ineffective especially in older patients. Development of new therapies depends upon the discovery of novel pathways and genes that drive the cardinal features of this disease. Thus, elucidation of gene sets that regulate growth, clonogenicity and differentiation within the population of leukemia cells is of great clinical interest.

In individual patients with AML, the blast population is heterogeneous in its clonogenic capacity, the capacity to proliferate sufficiently to form a colony in vitro. The majority of blasts are not clonogenic, although only a tiny fraction can proliferate extensively. The leukemia stem cell (LSC) model explains this heterogeneity by proposing that the population of leukemia cells is composed of stem and non-stem leukemia cells. Once a leukemia stem cell becomes a non-stem leukemia cell, it initiates a program of aberrant differentiation, culminating in terminally differentiated leukemia cells that, while remaining functionally immature, can no longer divide.

The clinical impetus has been intense to determine those genes that govern clonogenicity or inversely, the differentiation of clonogenic leukemia cells into terminal non-proliferating leukemia blast cells, as such analyses promise to lead to the identification of targets for novel leukemia therapeutics. However, identification of such genes would depend on means of resolving and prospectively isolating pure populations of clonogenic cancer cells. Fluorescence-activated cell sorting based on cellular immunophenotype has yielded leukemia cells highly enriched for clonal longevity, but clonogenic AML cells remain a small minority within such populations complicating gene expression analysis. The characterization of the transcriptome of clonogenic cancer cells has therefore awaited the development of techniques and approaches that permit the study of homogeneous populations of clonogenic versus non-clonogenic cells.

To address the issue of homogeneity we employed a single cell approach. We compared gene expression in pure populations of clonogenic vs non-clonogenic cells drawn from low-passage cultures of OCI/AML4, a cell line whose heterogeneity mimics that of a primary AML cell population. Microarray analysis performed on pools of single cells of uniform clonogenicity revealed 14 candidate expression differences. One of these candidates, v-erb A related-2 (EAR-2, NR2F6), is an orphan nuclear receptor with no previously characterized role in hematopoiesis, and a homolog of Drosophila svp, a gene that has a well-characterized role in cell-fate decisions of primitive neural cells. EAR-2 has previously been shown to interact in vitro with the key hematopoietic transcription factor Runx1/AML1. Here we
show that EAR-2 drives the clonal longevity of leukemia cells and is a negative regulator of
the differentiation of hematopoietic and leukemia cells. These results validate our
experimental approach and identify a potential role for EAR-2 as a modulator of
hematopoiesis.

Materials and methods

Cell lines

OCI/AML-4, obtained from Dr MD Minden, University Health Network, Toronto, Canada,
is a continuous cell line derived from the blast cells of a patient with AML secondary to the
treatment of Hodgkin’s disease. The cell line was established from a peripheral blood
sample taken in 1987. After an unrecorded initial passage history, cells were frozen. They
were thawed in 1990, passaged once and frozen again, and cells for the present study,
designated ‘low passage’, were used from the latter stock. OCI/AML-4 cells designated
‘continuous passage’ had been continuously passaged since 1987. U937 and 32Dcl3 cells
were purchased from ATCC (Manassas, VA, USA). The 293GPG retroviral packaging cell
line was the gift from Richard Mulligan, Harvard University. Culture conditions and
methods for growth factor dropout experiments are described in Supplementary Methods.

Subclones

Subclones were generated from early passages of OCI-AML4 cells by plating cells at
limiting dilution in culture conditions described in Supplementary Methods. Although the
majority of cells were unable to sustain proliferation, a very small proportion of the cells
were able to proliferate long term and establish subclones. The frequency of clonogenic cells
was also assessed in a subline of the AML-4 cell line that had been passaged continuously
over numerous years (late passage AML-4). Frequencies and 95% confidence intervals were
determined by culturing cells at limiting dilutions and analyzing data using the PoissonMax
program for maximum likelihood statistics available at http://www.uhnresearch.ca/iscovelab/
homebrew.html.

Analysis of growth potential of clonal siblings

Clones consisting of four AML siblings were obtained by culturing early passages of OCI/
AML-4 at limiting dilutions for 48–72 h (five cells per well). Clusters of four cells were then
identified and individual cells from a cluster were transferred into separate wells. Single
cells were co-cultured on an irradiated OP9 feeder cell layer. Wells were regularly monitored
for growth with weekly medium changes and split as required. Cultures were maintained
until cells ceased proliferating. A total of 115 OCI/AML-4 clones were analyzed.

Single cell global reverse transcriptase PCR

Clusters of four cells were identified and three clonal siblings were plated in individual wells
under standard culture conditions, whereas the fourth cell was taken for global amplification
of its mRNA. Global single cell reverse transcriptase (RT)-PCR was performed as previously
described. The integrity of complementary (c)DNAs generated by the global single cell RT-
PCR was validated by hybridization to the L27 housekeeping probe (Figure 3c).
Microarray analysis

Labeling of cDNA was conducted using aminoallyl-dUTP as described. Labeled targets were hybridized to 1.7K cDNA microarrays obtained from the University Health Network Microarray Center (http://www.microarray.ca). Because the global RT-PCR amplification strategy captured only extreme 3′ transcript ends, many of which were not covered on the available oligonucleotide arrays, we elected to use cDNA arrays. Microarrays were processed as described. The primary analytic approach is described in Supplementary Methods. The data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO series accession number GSE28612 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28612).

Real-time PCR

RNA was isolated from $1 \times 10^6$ cells using Trizol reagent (Invitrogen, Burlington, ON, Canada) and first strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer’s instructions. Real time PCR was performed according to manufacturer’s instructions using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and analyzed using the delta-delta CT method. Primer sequences are provided in the Supplementary Methods.

Patient material

Leukemia and healthy BM cells, collected with informed consent and with institutional ethics board approval and stored in our tissue bank, were used to assess expression of EAR-2. The French-American British classification of the AML samples consisted of six AML-M4, seven AMLM4eo, one AML-M3 and one AML-M1; myelodysplastic syndrome (MDS) samples were three RA, six RAEB-1 and three RAEB-2.

Induction and assessment of differentiation

Differentiation was induced in U937 cells by treatment with 10 nM of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (Sigma-Aldrich, St Louis, MO, USA), 1 mM all-trans retinoic acid (atRA) (Sigma-Aldrich) or 1.25% v/v DMSO (Sigma-Aldrich). Differentiation was induced in the 32Dcl3 cell line by washing cells twice with phosphate-buffered saline and incubation with 10 ng/ml granulocyte colony-stimulating factor (G-CSF). Immunostaining for the maturation marker CD11b (eBioscience, San Diego, CA, USA) was performed for 20 min in the dark according to manufacturer’s instructions and cells were analyzed by flow cytometry. Nitroblue tetrazolium reduction test (Sigma-Aldrich) was performed according to the manufacturer’s instructions, with a minimum of 300 cells scored per slide in three different fields of view. Each experimental timepoint was conducted in triplicate.

Culture of hCG-NuMA-RARa transgenic bone marrow

Bone marrow obtained from leukemic hCG-NuMA-RARa transgenic mice was cultured with or without 1 mM of atRA in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin, supplemented with...
10 ng/ml interleukin (IL)-3, 5 ng/ml IL-6, 25 ng/ml c-kit ligand and 10 ng/ml G-CSF. mRNA was collected following 10 days of suspension culture.

**Generation of retroviruses**

Human EAR-2 cDNA (a gift from John Ladias, Harvard University) or Mus NR2F6 cDNA (a gift from Curt D Sigmund, University of Iowa) was subcloned into the pcDNA3.1V5/HIS vector (Invitrogen). V5-tagged EAR-2 was subsequently subcloned into the MMP retrovector such that it lay upstream of an IRES (internal ribosome entry sequence)-GFP cassette. VSV-G pseudotyped retroviral particles were generated by transient transfection of 293GPG cells as described.13

**Antibodies for immunoblotting**

Immunoblotting for human EAR-2 was performed using the PP-N2025-00 (Perseus Proteomics, Tokyo, Japan), or ab12982 (Abcam, Cambridge, MA, USA) antibodies, whereas immunoblotting for mouse EAR-2 was performed using the LS-C40527 (LifeSpan Biosciences, Seattle, WA, USA) antibody.

**Cell cycle analysis and growth kinetics**

Analysis of DNA content was performed by staining cells with PI (Sigma-Aldrich) as described.13 Growth kinetics were assessed by counting cells every 12 h using trypan blue and a Neumeber counter.

**Generation of short-hairpin RNA**

Oligonucleotides targeting human or mouse EAR-2 (sequences are provided in the Supplementary Methods) were synthesized (Sigma-Genosys, Oakville, ON Canada), annealed and cloned into the pSiren vector (Clonetech, Mountain View CA, USA), after which the sequence was verified at The Centre for Applied Genomics (TCAG), Toronto, ON Canada. Virus was prepared by transient transfection of plasmid in the 293GPG cell line as described above.

**Results**

**Early passages of the OCI/AML-4 cell line model the population structure of primary AML**

Primary AML cells are growth factor dependent, clonal and have a low frequency of clonogenic cells. To determine the growth factors required for proliferation in liquid culture, growth was assessed following the removal of individual cytokines from a cytokine supplementation cocktail. OCI/AML-4 cells depend most strongly upon c-kit ligand, Flt-3 ligand and granulocyte-monocyte CSF (GM)-CSF (P<0.01), and less markedly on G-CSF, IL-7 or IL-11 to sustain proliferation for up to 14 days (Figure 1a). Rescue experiments indicate that c-kit ligand and GM-CSF added individually partially restore growth, while simultaneously having an additive effect that was not sufficient to restore growth to the same extent as the entire cocktail.

When observed up to a 3-week time point, OCI/AML-4 cells are dependent on c-kit ligand, GM-CSF (P<0.01) and Flt-3 ligand (P<0.05) (Figure 1b). GM-CSF or c-kit ligand added
alone did not sustain growth in rescue experiments, however, simultaneous culture with GM-CSF and c-kit ligand partially rescued growth. These data confirm that OCI/AML-4 cells are growth factor dependent.

The clonality of early passage OCI/AML-4 cells was analyzed by karyotyping. Of the 20 OCI/AML-4 metaphase cells analyzed all contain the karyotype 46, xx, der (6) add (6) (p25) add (6) (q25), t (11;19) (q23;p13) (Figure 1c). Although it is formally possible that non-detectable events such as point mutations or small deletions account for differences in clonogenicity, these data demonstrate that the heterogeneity in proliferative ability cannot be attributed to the presence of ploidy or translocation variants.

The frequency of clonogenic cells in the OCI/AML-4 cell populations was determined by culturing OCI/AML-4 cell lines at limiting dilutions and scoring colony growth. The frequency of cells able to proliferate continuously for 3 and 6 weeks, determined from the percentage of wells negative for growth at those respective time points, was 1/55 or 1.8% (1.5–3.0%) and 1/154 or 0.64% (0.45–0.91%), respectively (Figure 1d). This heterogeneity in proliferative ability is preserved in subclones of OCI/AML4, which continued to maintain a small portion of cells capable of limited growth (3 weeks) and an even smaller proportion of cells capable of long-term growth (6 weeks) (Table 1). In contrast, OCI/AML-4 that had been passaged continuously for many years (late passage) contained a large proportion of cells with short- and long-term growth ability and hence cannot be organized in a hierarchy based on proliferative ability.

Clonal siblings are faithful reporters of cell fate

Stochastic and hierarchical models lead to mutually exclusive predictions concerning the growth ability of clonal siblings (Figure 2a). In the stochastic model, individual cells have a fixed probability of dividing, independent of their replicative history; a cell’s proliferative outcome is not expected to resemble those of its siblings. In the hierarchical model, by contrast, the proliferative potential of each cell is determined by that cell’s position in the hierarchy, as determined by its replicative history. Clonal siblings, which share identical replicative histories (Figure 2b), should display concordant growth patterns.

We conjectured that OCI/AML-4 clonal siblings would display similar growth patterns, and that this concordance would permit analysis of gene expression in cell subpopulations with strictly defined growth abilities. To confirm this, low-passage OCI/AML-4 cells were grown in microcultures at limiting dilution. From clones consisting of four cells (n = 115 clones), single sister cells were micromanipulated into individual wells (Figure 2b). Growth was monitored several times weekly and the number of cells in each well recorded; cells that divided more than three times were scored positive. In the majority of the samples, either all siblings in a clone grew or none of the siblings in a clone grew (Figure 2c). The outcome strongly favors the hierarchical model over the stochastic model (P<0.0003; Tarone’s test) and establishes that clonal siblings have similar growth propensities and thus reside at similar positions within a proliferative hierarchy.

We wanted to determine the level of accuracy with which the proliferative capacity of a sibling cell destroyed for global RT-PCR would be predicted by the proliferative outcomes
of its sister cells. We hence calculated the fidelity with which three clonal siblings would be able to predict the growth ability of a fourth sibling cell (Table 2) based on the growth probabilities of the clonal siblings in Figure 2c. We established that clonal sibling analysis is a reliable method of predicting the growth potential of a selected cell in the context of AML (97.8% overall accuracy, 96.4% sensitivity and 98.4% specificity), enabling us to associate gene expression profiles with growth potential with a high level of confidence.

**Global gene expression analysis of OCI/AML-4 cells of defined clonogenic potential**

Having established the validity of this approach, we used OCI/AML-4 clonal siblings as reporters of the growth ability of a sibling cell that was sampled for global RT-PCR\textsuperscript{14,15} (Figure 3a). Cells were plated at limiting dilutions. Localized clusters of four cells were identified and micromanipulated such that three of the constituent cells were placed separately into individual microtitre wells containing growth medium and a feeder layer of OP9 cells,\textsuperscript{16} while the fourth cell was lysed and processed for global RT-PCR. The cells in each culture well were counted at 2–3 day intervals until growth stopped. In this manner cDNA was generated from 42 individual OCI/AML-4 cells for which the growth profile of clonal siblings had also been determined (Table 3). The integrity of cDNAs generated by global single cell RT-PCR was validated by hybridization to the L27a housekeeping probe (Figure 3c). Globally amplified cDNA from single OCI/AML-4 cells whose siblings each generated fewer than eight cells (n = 20), and cells whose siblings each generated 8–100 cells (3–6 successive divisions) (n = 17) were respectively pooled and compared by hybridization to cDNA microarrays. Eight genes were expressed in greater abundance in cells with restricted proliferative ability (Table 4) whereas fourteen genes expressed more highly in cells with proliferative ability were identified (Table 5).

**EAR-2 expression is inversely correlated with differentiation**

EAR-2, an orphan nuclear receptor with no previously known roles in hematopoiesis, was found by microarray to be 4.6-fold more highly expressed in cells with proliferative ability. Semi-quantitative PCR on pooled cDNA from cells with and without proliferative ability validated this expression difference (Figure 3d). EAR-2 is a homolog of Drosophila svp, a gene that has a well-characterized role in cell fate decisions of primitive neural cells, and has been reported to interact with the key hematopoietic transcription factor Runx1. As our group has longstanding interest in the roles of nuclear receptors in normal and leukemic hematopoiesis, we decided to pursue EAR-2 in further studies.

We assessed EAR-2 mRNA expression during the induction of leukemia cell differentiation in vitro. We observed that EAR-2 expression declines in human U937 monoblastic leukemia cells induced to differentiate by treatment with a range of chemical agents (Figure 4a). We validated our observations in primary samples using short-term ex vivo cultures of bone marrow from the hCG-NuMA-RARa transgenic mouse, which exhibits promyelocytic leukemia. In these animals myeloid development is arrested at the promyelocytic stage of differentiation and can be induced to mature by treatment with atRA. EAR-2 expression correlated with stage of maturation in these animals: expression of EAR-2 mRNA was higher in hCG-NuMA-RARa cells than in wild-type control (Figure 4b). A marked decline
in EAR-2 expression occurred in hCG-NuMA-RARa cultures treated with atRA, suggesting that EAR-2 expression negatively correlates with differentiation.

To assess the pattern of EAR-2 expression in normal hematopoiesis we used quantitative PCR to measure EAR-2 transcript levels in a graded series of multipotent, oligopotent and unipotent murine hematopoietic cells. Generation of nearly homogeneous samples of cells at various individual stages after commitment to terminal differentiation is beyond the range of current cell-sorting strategies. We overcame this difficulty by acquiring cDNA samples from single cells (isolated from 4- or 8- cell colony starts) that were tetrapotent and pentapotent for the generation of progeny in erythroid, megakaryocyte, macrophage, neutrophil and mast cell lineages. Long-term and intermediate-term repopulating cells (LT-hematopoietic stem cell (HSC) and IT-HSC) were defined as kit$^+$ Sca-1$^+$ lin$^-$$^-$ CD34$^{lo}$ Flt$^-$ Rho$^{lo}$ CD49b$^{lo}$ (LT-HSC) and as kit$^+$ Sca-1$^+$ lin$^-$$^-$ CD34$^{lo}$ Flt$^-$ Rho$^{lo}$ CD49b$^{hi}$ (IT-HSC). EAR-2 transcripts were most abundant in long-term hematopoietic stem cells and became progressively less abundant with differentiation, with the exception of committed megakaryocyte progenitors, in which expression was high (Figure 4c). Next we analyzed expression of NR2F6 in healthy BM (N = 16) and in BM specimens from patients with AML (N = 15), MDS (N = 12) and chronic myelomonocytic leukemia (N = 10). Analysis by quantitative real-time RT-PCR (Figure 4d) reveals that NR2F6 is overexpressed in all three of these disease categories (P$<0.01$ AML, 0.05 chronic myelomonocytic leukemia, 0.01 MDS).

These observations are consistent with EAR-2 having a role in the maintenance of the undifferentiated state of primitive hematopoietic cells. We therefore explored the effects of unregulated expression of EAR-2 on blood cell differentiation next.

**Overexpression of EAR-2 increases proliferation in 32D and U937 cells**

The process of differentiation encompasses a loss of proliferative ability as well as the functional maturation of cells. We hypothesized that the downregulation of EAR-2 is essential to the process of differentiation and that cells in which EAR-2 expression does not decline fail to differentiate. To characterize the effect of forced expression of EAR-2 we transduced two myeloid cell lines, human U937 and murine 32Dcl3, with a retrovirus encoding either EAR-2 or EGFP (Figure 5a). U937 cells overexpressing EAR-2 proliferated at a faster rate than control cells (Figure 5b). Upon induction of differentiation we observed that unregulated expression of EAR-2 abrogates both the growth arrest (Figure 5c) and the shift of cells from S/G$_2$/M to G$_0$/G$_1$ (Figure 5d) that follows atRA-induced differentiation.

**Overexpression of EAR-2 inhibits the differentiation of 32D and U937 cells**

U937 cells are induced to undergo differentiation F characterized by growth arrest, expression of cell surface CD11b and development of oxidative burst capability F when incubated with atRA. We assessed the acquisition of two indices of maturity in U937 cells treated with atRA. In contrast to the control U937-GFP cells, after 72 h exposure to 1 mM atRA U937-EAR-2 cells fail to express CD11b (Figure 6a), and fail to acquire the ability to reduce nitroblue tetrazolium, a functional index of leukocyte maturity (Figures 6b and c). Similarly, cells of the mouse 32Dcl3 cell line can be induced to undergo granulocytic
differentiation when cultured in the presence of G-CSF. Unregulated expression of EAR-2 blocks the induction of cell surface CD11b expression (Figure 6d) and the morphological changes (Figure 6e) that accompany G-CSF-induced differentiation of 32Dc13 cells. We note that expression of CD11b actually decreases when EAR-2 is overexpressed. This is due to the biology of the 32Dc13 line; the cells exhibit limited differentiation while being maintained in IL-3 and then undergo neutrophil maturation when given G-CSF. EAR-2 blocks both phases, allowing the very immature (pre-CD11b) cells to become predominant in culture. Untreated U937 cells do not express CD11b, and hence it is not possible to observe the same phenomenon in these cells using this particular marker. Thus, it appears that downregulation of EAR-2 expression is necessary for differentiation of the U937 and 32Dc13 cell lines to proceed.

Knockdown of EAR-2 induces differentiation of 32D and U937 cells

The results of overexpression studies suggest that downregulation of EAR-2 expression is necessary for hematopoietic differentiation, that is, EAR-2 acts to preserve the undifferentiated state of early hematopoietic cells. However, overexpression of nuclear receptors can result in off-target effects due to, for example, cofactor sequestration. We therefore used short hairpin RNA constructs to silence EAR-2 expression in 32Dc13 (Figure 7a) and U937 cells (Figure 7d). For both cell lines, silencing of EAR-2 expression resulted in spontaneous morphologic differentiation (Figures 7b and e) and upregulation of cell-surface CD11b expression (Figures 7c and f). In U937 cells these manifestations of differentiation were accompanied by induction of apoptosis (Figure 7g).

Discussion

We describe a novel strategy for identifying genes involved in determining clonogenicity of leukemia cells. After establishing that single cells drawn from clones consisting of four sibling cells have similar proliferative capacities (Figure 2c) and that any given sibling in the clone can serve as a biological reporter for their rest of the sibling cells (Table 2), we compared the transcriptional profiles of proliferative and non-proliferative leukemia cells defined by observing the proliferative outcomes of sibling cells in culture. In this way we found that mRNA transcripts of the orphan nuclear receptor EAR-2 were fourfold more abundant in AML cells with growth potential than in cells lacking growth potential (Figure 3d). To validate the finding, we investigated the role of EAR-2 in the terminal differentiation of normal and leukemia cells. In normal mouse hematopoietic tissue we found that EAR-2 expression is highest in long-term repopulating HSCs and declines with differentiation (Figure 4c) and that EAR-2 is highly expressed in AML, MDS and chronic myelomonocytic leukemia. We expressed EAR-2 exogenously in mouse and human hematopoietic cell lines. Unregulated expression of EAR-2 in hematopoietic cell lines blocks induction of differentiation (Figure 6), whereas knockdown of EAR-2 expression results in terminal differentiation and apoptosis (Figure 7).

Our data support the notion that leukemia cells are organized in a hierarchy of growth abilities as suggested by the leukemia stem cell hypothesis. Leukemia cells, and cancer cells in general, are heterogeneous with respect to clonogenicity; we have shown this in OCI/
AML-4 cells using limiting dilution assays (Figure 1d). The heterogeneity in proliferative ability cannot be attributed to the presence of ploidy or translocation variants as we demonstrate that the OCI/AML-4 population is clonal using G-banding (Figure 1c). Furthermore, we show that the heterogeneity present in the parental lines is transferred to subcloned lines (Table 1). Although subclones derived from rare single cells were capable of unlimited growth ability to the point of reestablishing the cell line, the constituent subclones were not distinct homogeneous populations with extensive proliferative ability but rather recapitulated the heterogeneous population structure of the original parental line. This is analogous to the reconstitution of haematopoiesis by a single stem cell and does not favor either the stochastic or the hierarchy model, either of which is consistent with this pattern. However, it does provide further support for our contention that early passages of the OCI/AML4 cell line, despite their heterogeneity, are clonal.

The heterogeneity of AML cell populations can be explained by either a stochastic or a hierarchical model. In the stochastic model, every cell has a fixed probability of division and growth ability is determined by random events; hence, every cell has the potential to be tumorigenic, whereas in the hierarchical ‘cancer stem cell’ model cancer is sustained by rare, self-renewing disease stem cells. Both the stochastic and the hierarchical models provide explanations for the observed heterogeneity in proliferative ability in cancer as both predict an inverse correlation between progenitor frequency and clonal longevity. However, only the leukemia stem cell model predicts that clonal siblings have similar growth abilities. In the stochastic model the growth outcomes of clonal siblings are independent of one another, whereas in the hierarchy model clonal siblings occupy similar positions in the leukemia cell hierarchy, and are expected to have highly correlated proliferative outcomes (Figure 2a). Our data show that clonal siblings have similar growth abilities (Figure 2c), strongly supporting the hierarchical model where growth of the population of leukemia cells is sustained over time by rare leukemia stem cells.

Our study is the first to suggest a role for the orphan nuclear receptor EAR-2 in regulation of the differentiation and clonogenicity of hematopoietic cells. EAR-2 (ref. 19) is a mammalian homolog of the Drosophila gene seven-up, which has a role in neuroblast and retinal cell fate decisions.8,20–23 EAR-2 deficient mice24 are viable and fertile, but show agenesis of the locus coeruleus, a midbrain nucleus that regulates circadian behavior and nociception. Although EAR-2 expression was seen outside the central nervous system, no phenotypic analysis outside the nervous system was reported; in particular, hematopoiesis was not characterized. However, evidence suggestive of a role for EAR-2 in hematopoiesis comes from a report of its interaction in vitro with the key hematopoietic transcription factor Runx1 (ref. 9) and from the observation that EAR-2 antagonizes activation of Th17 CD4 + T cells.25 Importantly, our results are consistent with a role for EAR-2 in normal hematopoiesis as well as in leukemia. EAR-2 is highly expressed in HSC with much lower transcript levels in differentiating cells (Figure 4c); this suggests a function for EAR-2 in hematopoiesis analogous to the role of svp in Drosophila neurogenesis, in which svp expression must be downregulated to permit commitment of neuroblasts to terminal differentiation.

EAR-2 functions as a transcriptional repressor in vitro, inhibiting the transactivating ability of numerous genes.26 We conjecture that EAR-2 functions by repressing genes necessary for
the terminal differentiation of myeloid cells and that expression of EAR-2 needs to be decreased in order for terminal differentiation to proceed. However the target genes of this repression are a matter of speculation. Like many nuclear receptors, EAR-2 heterodimerizes with the retinoid X receptor-a (RXR-a), although the relevance of this interaction in EAR-2 function is unclear. In this regard it is interesting that unregulated expression of EAR-2 is able to impair atRA-induced differentiation of leukemia cells. It is unlikely that interference with retinoid X receptor-a function by EAR-2 accounts entirely for the EAR-2 overexpression phenotype as targeted deletion of retinoid X receptor-a in hematopoietic stem cells results in no major phenotype, although it is possible that this phenomenon contributes. However, the molecular function of EAR-2 is likely to be more complex as the inhibition of terminal differentiation by unregulated expression of EAR-2 is not limited to retinoid induced differentiation; it also inhibited G-CSF-induced differentiation in 32D cells. The hematopoietic transcription factor Runx1 (AML1) is a candidate of considerable interest. Runx1 is essential for the establishment of definitive hematopoiesis, and mutations in the human homolog AML1 are seen commonly in both AML and MDS. The previously reported interaction of EAR-2 with Runx1 suggests a model in which EAR-2 abrogates the differentiation program by recruiting transcriptional corepressors to Runx1 target genes.

Finally, our results identify EAR-2 as a potential therapeutic target in acute leukemia. Knockdown of EAR-2 is sufficient to induce terminal differentiation and apoptosis of two distinct and very different AML cell lines (Figure 7). The dramatic effect of EAR-2 silencing in two vastly dissimilar cell lines speaks to the generalizability of the data and invites further exploration of this avenue. Nuclear receptors are eminently ‘druggable’ Fagonist and antagonist NR ligands are of proven utility in a wide variety of human diseases. The data presented here raise the extremely exciting possibility that natural or synthetic antagonist ligands for EAR-2 can be found that may act as anti-leukemic therapeutics.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Early passages of the OCI/AML-4 cell line maintain cardinal features of patient samples. Early passages of the OCI/AML-4 cell line are growth factor-dependent when growth is assessed following 2 (a) or 3 weeks (b) of culture. Error bars denote the s.e.m. *P<0.05 and **P<0.01. (c) Early passages of the OCI/AML4 were analyzed by G-banding. All 20 metaphases had the karyotype 46, xx, der (6) add (6) (p25) add (6) (q25), t (11;19) (q23;p13). (d) Early passages of the OCI/AML-4 cell line have a low frequency of clonogenic cells that can proliferate to either 3 or 6 weeks, respectively, as determined by culturing at limiting dilutions.
Figure 2.
(a) A hierarchical organization of AML cells would predict that clonal siblings would have similar propensities to grow or not grow; on the other hand, a stochastic organization of AML would predict that each clonal sibling has a fixed probability to grow or to not grow, independent of the growth of other sibling cells. (b) Clonal siblings from low passage cultures of OCI/AML4 cells were generated by allowing a single cell to undergo two consecutive cell divisions. Clonal siblings were then separated and their growth abilities monitored. (c) Clonal siblings in low passage cultures of OCI/AML4 share congruent proliferative potential (n = 115 clones), in contrast with the growth pattern expected for the stochastic model (binomial distribution).
Figure 3.
(a) Clonal siblings were generated, one member of a tetrad of cells is harvested for mRNA, whereas its three clonal siblings serve as reporters of clonogenicity. (b) Ethidium bromide gels of single cell global RT-PCR. Lanes 1–5: cDNA globally amplified from single cells with defined growth abilities. Lanes 6–8: no template control samples. Lane 9: no reverse transcription control. (c) Validation of cDNAs by hybridization to L27a ribosomal probe. Lanes 6–8 are interpreted as primer concatamers as they do not hybridize with mammalian housekeeping probes. (d) RT-PCR amplification of EAR-2 in cDNA from pooled proliferative (P) and non-proliferative (NP) OCI/AML4 cells. NTC, no template control.
Figure 4.
(a) Quantitative (Q)-PCR analysis of EAR-2 mRNA expression in U937 cells induced to differentiate with atRA (triangles), dimethyl sulfoxide (DMSO) (rectangles) or 12-O-tetradecanoylphorbol-13-acetate (TPA) (circles). (b) Q-PCR analysis of EAR-2 mRNA expression in NuMA-RARα transgenic mice or wild-type controls when induced to differentiate with retinoic acid. (c) Q-PCR analysis of EAR-2 mRNA expression in mouse BM progenitors. (First panel) Expression of EAR-2 mRNA relative to GAPDH in quiescent and cycling long-term repopulating HSCs (LT-HSC) and intermediate-term repopulating HSCs (IT-HSC). Cycling samples were obtained from purified LT- and IT-HSC cultured with cytokines until they commenced dividing. (Second through fourth panels) Expression of EAR-2 mRNA in HSCs and committed progenitors: pentapotent progenitor (Penta), committed non-lymphoid progenitor (E Meg Mac), erythroid/megakaryocyte progenitor (E Meg), committed megakaryocyte progenitor (Meg Pro), BFU-E, CFU-E, megakaryocyte (Meg), macrophage/neutrophil progenitor (Mac Neu), committed macrophage progenitor (Mac Pro), committed neutrophil progenitor (Neu Pro), macrophage (Mac) and neutrophil...

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(Neu). All expression levels in panels 2–4 are relative to expression of EAR-2 in E Meg Mac. (d) Q-PCR analysis of EAR-2 mRNA expression in human bone marrow cells from healthy volunteers and from patients with AML, MDS and chronic myelomonocytic leukemia. **P<0.01 *P<0.05.
Figure 5.
(a) EAR-2 protein expression in U937 and 32Dc13 infected with GFP and EAR-2 retroviruses. (b) Growth curves of U937-GFP (circles) and U937-EAR-2 (squares). Data denote the relative increase in cell number relative to day 0 (mean±s.e.m.). (c) Growth of U937-GFP (circles) and U937-EAR-2 (squares) following addition of atRA. Data denote the increase in cell number relative to day 0 (mean±s.e.m.). (d) Cell cycle analysis of U937-GFP and U937-EAR-2. **P<0.01 for difference between GFP and EAR-2 in the presence of atRA.
Figure 6.
(a) CD11b expression induced by atRA treatment in U937-GFP and U937-EAR-2. (b, c) Nitroblue tetrazolium assay for functional maturity in atRA-treated U937-GFP and U937-EAR-2. Data are represented as mean±s.e.m. (d) CD11b expression in 32D-GFP and 32D-EAR-2 grown in G-CSF for 96 h. (e) Morphological maturation in cells from (d) as stained with May–Grunewald Giemsa. Scale bar = 20 mm.
Figure 7.
(a) Relative EAR-2 protein levels as determined by densitometry of immunoblots in 32D cells transduced with three EAR-2 short-hairpin (sh)RNA constructs or scrambled control (scrm). Data are represented as mean±s.d. (b) Morphological maturation of 32D after EAR-2 silencing. (c) Induction of CD11b expression in 32D after EAR-2 silencing. (d) Relative EAR-2 protein expression in U937 transduced with two EAR-2 shRNAs or scrambled control. Data are represented as mean±s.d. (e) Morphological maturation of U937 after EAR-2 silencing. (f) Induction of CD11b expression in U937 after EAR-2 silencing. (g) Induction of apoptosis in U937 after EAR-2 silencing. Scale bar = 20 mm.
### Table 1

Frequency of clonogenic cells in OCI/AML-4 and subclones

|                | Week 3 |          | Week 6 |          |
|----------------|--------|----------|--------|----------|
|                | Frequency | 95% CI    | Frequency | 95% CI    |
| Parental       | 1.80%  | 1.3–2.6% | 0.64%  | 0.45–0.91% |
| Subclone 10C   | 6.10%  | 4.2–8.9% | 3.32%  | 2.3–4.80% |
| Subclone 12E   | 2.50%  | 1.7–3.5% | 0.93%  | 0.66–1.31% |
| Subclone G10   | 2.10%  | 1.4–2.9% | 0.53%  | 0.6–1.20% |
| Subclone G3    | 2.90%  | 2.0–4.1% | 1.12%  | 0.78–1.58% |
| Late passaged  | 83.10% | 63.1–109.5% | 80.90% | 61.4–106.5% |

Abbreviation: 95% CI, confidence interval.
Table 2
Confusion matrix describing the ability of three clonal siblings used as biological reporters to predict the proliferative fate of a fourth clonal sibling consumed in the process of global RT-PCR

| Test predicted outcome | Actual outcome |       |       |       |
|------------------------|----------------|-------|-------|-------|
|                        | Grow           | Did not grow |
| Grow                   | TP = 108       | FP = 5 | PPV, 95.6% |
| Did not grow           | FN = 4         | TN = 304 | NPV, 98.7% |
|                        | Sensitivity, 96.4% | Specificity, 98.4% | Accuracy, 97.8% |

Abbreviations: FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; RT, reverse transcriptase; TN, true negative; TP, true positive.
Table 3

Growth profiles of clones sampled for global cDNA amplification

| Growth potential of sampled clones | Number sampled |
|------------------------------------|----------------|
| 8 cells                            | 20             |
| 9–100 cells                        | 17             |
| 101–10 000 cells                   | 5              |
| 410 000 cells                      | 0              |

Abbreviation: cDNA, complementary DNA.
**Table 4**

Genes expressed more abundantly in AML-4 cells with limited proliferative ability (less than three consecutive cell divisions)

| Accession | Full name                                           | Mean fold increase | s.d. | CV  |
|-----------|-----------------------------------------------------|--------------------|------|-----|
| AA194054  | 28.3 KD protein C21ORF2                              | 4.6                | 1.3  | 0.16|
| AA122236  | F-actin capping protein                              | 4.9                | 1.2  | 0.10|
| AA193614  | Guanylate cyclase                                    | 7                  | 1.1  | 0.07|
| AA018737  | PTB-associated splicing factor                       | 7                  | 1.2  | 0.09|
| H19387    | Maltate oxidoreductase (NAD), mitochondrial precursor| 8.6                | 1.3  | 0.12|
| W61100    | BCL-2                                               | 9.2                | 1.5  | 0.17|
| H59171    | SEC23 homolog                                        | 9.8                | 1.6  | 0.20|
| N42900    | TFIID                                               | 13                 | 1.6  | 0.19|

Abbreviations: AML, acute myelogenous leukemia; s.d., standard deviation; CV, coefficient of variation.
Table 5

Genes expressed more abundantly in AML-4 cells with proliferative ability (3–6 successive divisions)

| Accession | Full name                                                                 | Mean fold increase | s.d. | CV   |
|-----------|---------------------------------------------------------------------------|--------------------|------|------|
| H10327    | RAS-related protein RAP-1B (GTP-binding protein SMG P21B)                 | 4.7                | 1.3  | 0.17 |
| W45621    | Nuclear receptor subfamily 2, group F, member 6 (NR2F6, EAR-2)            | 4.7                | 1.3  | 0.19 |
| N40100    | cAMP-dependent protein kinase type 1-a regulatory chain                   | 4.7                | 1.2  | 0.12 |
| W52609    | Glutaredoxin (Thioltransferase)                                           | 5.2                | 1.2  | 0.13 |
| AA055945  | T-cell surface glycoprotein CD3 delta chain precursor                    | 5.5                | 1.3  | 0.13 |
| H57284    | NEDD5 protein homolog (KIAA0158)                                         | 5.5                | 1.1  | 0.06 |
| W78946    | Electron transfer flavoprotein a-subunit precursor                       | 5.7                | 1.4  | 0.18 |
| W00728    | Guanine nucleotide-binding protein G(I)/G(S)/G(T) b subunit 1             | 5.9                | 1.2  | 0.09 |
| R99156    | Cyclin-dependent kinases regulatory subunit 1 (CKS-1)                     | 6                  | 1.1  | 0.03 |
| W92066    | Activator 1 37 kD subunit (replication factor C 37 kD subunit)            | 7.6                | 1.4  | 0.16 |
| W92260    | RAS-related protein RAB-11A                                              | 8.2                | 1.4  | 0.15 |
| R92367    | Transcription factor ATF-A and ATF-A-delta                               | 8.3                | 1.2  | 0.10 |
| W21228    | Ubiquinol-cytochrome c reductase complex core protein 2 precursor        | 10.3               | 1.3  | 0.13 |
| R34413    | Poly (ADP-ribose) polymerase                                             | 10.7               | 1.3  | 0.12 |

Abbreviations: AML, acute myelogenous leukemia; s.d., standard deviation; CV, coefficient of variation.