The Orphan Nuclear Receptor EAR-2 (NR2F6) is a Leukemia Oncogene and Novel Regulator of Hematopoietic Stem Cell Homeostasis and Differentiation

by

Christine Victoria Ichim

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Medical Biophysics
University of Toronto

© Copyright by Christine Victoria Ichim 2012
The Orphan Nuclear Receptor EAR-2 (NR2F6) is a Leukemia Oncogene and Novel Regulator of Hematopoietic Stem Cell Homeostasis and Differentiation

Christine Victoria Ichim

Doctor of Philosophy

Department of Medical Biophysics
University of Toronto

2012

Abstract

The orphan nuclear receptor EAR-2 (NR2F6) is a gene that I previously found to be expressed at a higher level in clonogenic leukemia single cells than in leukemia cells that can not divide. For this thesis I undertook to perform the first investigations of the roles EAR-2 may play in normal hematopoiesis and in the pathogenesis of acute myelogenous leukemia. Here, I show that EAR-2 is overexpressed in the bone marrow of patients with MDS, AML and CMML compared to healthy controls and that EAR-2 is a gatekeeper to hematopoietic differentiation. Overexpression of EAR-2 prevents the differentiation of cell lines, while knock down induces their spontaneous differentiation. In vitro, primary bone marrow cells that overexpress EAR-2 do not differentiate into granulocytes in suspension culture, but have greatly extended replating capacity in colony assays. In vivo, overexpression of EAR-2 in a chimeric mouse model leads to a condition that resembles myelodysplastic syndrome characterised by hypercellular bone marrow, an increase in blasts, abnormal localization of immature progenitors, morphological dysplasia of the erythroid lineage and a competitive advantage over wild-type cells, that
eventually leads to AML in a subset of the mice. Furthermore, animals that are transplanted with grafts of sorted bone marrow develop a rapidly fatal leukemia that is characterized by pancytopenia, enlargement of the spleen, infiltration of blasts into the spleen, liver and peripheral blood. Interestingly, development of leukemia is preceded by expansion of the stem cell compartment. Overexpression of EAR-2 increases the maintenance of KSL primitive bone marrow cells in \textit{ex vivo} suspension culture, while knockdown of EAR-2 induces rapid differentiation of KSL cells into granulocytes. These data establish that EAR-2 is a novel oncogene that regulates hematopoietic cell differentiation. Furthermore, I show that EAR-2 is also a novel negative regulator of T-cell lymphopoiesis, and demonstrate that down-regulation of EAR-2 is important for the survival, proliferation and differentiation of T-cell progenitors. Overall, this work establishes that expression of EAR-2 is an important determinant of cell fate decisions in the hematopoietic system.
Acknowledgments

Foremost, I wish to thank my parents whom instilled in me reverence for the realm of the intellect through the sacrifices they made to come to a land that espoused intellectual freedom. I thank my father, a philosopher, whom taught me that there is no greater joy than the pursuit of truth, knowledge, and beauty; and my mother, a poet who shared with me her deep appreciation for the magnificence of nature and the beauty inherent in exploration of the unknown.

I thank my mentors past and present for shaping me into the scientist that I am today. I will remain forever grateful to my PhD supervisor Dr. Dick Wells for embarking on this fascinating journey of discovery with me and for his unwavering enthusiasm and support throughout! I thank my supervisory committee Drs. John Dick, Dwayne Barber and Dick Hill for their inspiration and support. I thank Dr. Norman Iscove for introducing me to the fascinating topic of stem cell biology, for teaching me how to critically appraise the literature and to believe in my own ideas even if they go against the grain. I am grateful to Drs. Bonnie Mallard and Bruce Wilkie from the University of Guelph for taking me under their wings as a teenager, and fostering the spark that they saw in me. I thank Dr. Hans Messner and Mark Minden whom have been my role models and a source of inspiration ever since I was a child.

I thank all past and present members of the Wells Lab for the wonderful times we’ve shared. I thank my most brilliant and dedicated collaborator, Džana Dervović for her enthusiasm and undying dedication to the project. I thank all of my friends, family and everyone in the S-wing for their great camaraderie, especially Jacinth Abraham, Rika Okamoto, Ahalya Mahendra, Miriam Mossamba, and Elena Bogdanovic. I thank my aunt and uncle Cevi and Ion Vasilescu for their tireless support. I thank Bhupinder Bains for support, helpful discussions, and just being fabulous. And most of all, I thank the erudite Dr. Fluffles, my darling little bunny, who’s showed me how one chews their way through yet the most complicated of research articles.

This work was funded by a CIHR-Canada Graduate Scholarship, an Adel S. Sedra Award of Excellence, a Dr. Joe Connolly Memorial OSOTF Award, a Government of Ontario/Dr. Dina Gordon Malkin Graduate Scholarship in Science and Technology, and a Frank Fletcher Memorial OSOTF Award.
Dedication

In memory of my mother, Florica Ichim, who battled chronic myelogenous leukemia for 23 years; and to Drs. Minden, Messner, and Lipton, her hope and my inspiration, whom showed me at a very young age the power of research to change lives and impact human health.
# Table of Contents

Acknowledgments.......................................................................................................................... iv  
Dedication....................................................................................................................................... v  
Table of Contents ........................................................................................................................... vi  
List of Tables .................................................................................................................................. xi  
List of Figures ............................................................................................................................... xii  
List of Appendices ....................................................................................................................... xiv  
List of Abbreviations .................................................................................................................... xv  

## 1 Introduction and Background .................................................................................................. 1  
  1.1 Introduction........................................................................................................................... 2  
  1.2 Normal hematopoiesis ......................................................................................................... 3  
    1.2.1 Hematopoiesis is organized as a hierarchy ............................................................. 3  
    1.2.2 Development of T cells ........................................................................................... 6  
  1.3 Neoplastic hematopoiesis .................................................................................................. 11  
    1.3.1 Overview of leukemia ............................................................................................. 11  
    1.3.2 Overview of myelodysplastic syndrome: .............................................................. 12  
  1.4 Cancer as a caricature of normal tissue development: The cancer stem cell ............... 14  
    1.4.1 Overview of the cancer stem cell model ............................................................... 14  
    1.4.2 Targeting the tumour stem cell ............................................................................. 16  
    1.4.3 Cancer stem cells as therapeutic targets ............................................................. 16  
    1.4.4 The central paradox of MDS .............................................................................. 19  
  1.5 The orphan nuclear receptor EAR-2: ................................................................................ 19  
  1.6 Thesis overview ................................................................................................................ 22  

Disclosures and Copyright Acknowledgements .......................................................................... 24  

## 2 Identification of a role for the nuclear receptor EAR-2 in the maintenance of clonogenic status within the leukemia cell hierarchy ................................................................. 25
2.1 Introduction....................................................................................................................... 26

2.2 Materials and methods ...................................................................................................... 27

  2.2.1 Cell lines ............................................................................................................... 27

  2.2.2 Growth factor drop out experiments ..................................................................... 28

  2.2.3 Limiting dilution analysis ..................................................................................... 28

  2.2.4 Subclones .............................................................................................................. 29

  2.2.5 Analysis of growth potential of clonal siblings .................................................... 29

  2.2.6 Single cell global RT-PCR.................................................................................... 29

  2.2.7 Microarray analysis............................................................................................... 30

  2.2.8 Confusion matrix .................................................................................................. 30

  2.2.9 Real-time PCR ...................................................................................................... 31

  2.2.10 Induction and assessment of differentiation ....................................................... 32

  2.2.11 Culture of hCG-NuMA-RARα transgenic bone marrow .................................... 32

  2.2.12 Generation of retroviruses.................................................................................. 32

  2.2.13 Antibodies for immunoblotting........................................................................... 33

  2.2.14 Cell cycle analysis and growth kinetics .............................................................. 33

  2.2.15 Generation of shRNA ......................................................................................... 33

2.3 Results............................................................................................................................... 34

  2.3.1 Early passages of the OCI/AML-4 cell line model the population structure of primary AML .......................................................... 34

  2.3.2 Clonal siblings are faithful reporters of cell fate ................................................ 37

  2.3.3 Global gene expression analysis of OCI/AML-4 cells of defined clonogenic potential ................................................................. 40

  2.3.4 EAR-2 expression is inversely correlated with differentiation......................... 43

  2.3.5 Overexpression of EAR-2 increases proliferation in 32D and U937 cells ........ 46

  2.3.6 Overexpression of EAR-2 inhibits the differentiation of 32D and U937 cells .... 46

  2.3.7 Knockdown of EAR-2 induces differentiation of 32D and U937 cells .......... 49
3 EAR-2 is a novel regulator of hematopoietic differentiation and hematopoietic stem cell homeostasis

3.1 Introduction

3.2 Materials & methods
  3.2.1 Generation of retroviruses
  3.2.2 Antibodies for immunoblotting
  3.2.3 Real-time PCR
  3.2.4 Bone marrow transduction
  3.2.5 Methylcellulose colonies:
  3.2.6 Ex vivo suspension culture
  3.2.7 Hematopoietic stem cell transplants
  3.2.8 Histological sections and cytospins
  3.2.9 Peripheral blood counts
  3.2.10 Analysis of hematopoietic stem cell subsets:
  3.2.11 Generation of shRNA
  3.2.12 Statistical analysis

3.3 Results
  3.3.1 Expression of EAR-2 negatively regulates erythroid and myeloid differentiation of bone marrow cells
  3.3.2 EAR-2 transplant chimeras have perturbed hematopoietic differentiation and dysplastic hematopoiesis
  3.3.3 EAR-2 initiates acute leukemia
  3.3.4 EAR-2 perturbs primitive cellular compartments
  3.3.5 EAR-2 is necessary for the maintenance of hematopoietic stem cells
  3.3.6 EAR-2 functions as a transcriptional repressor in a DNA binding dependent manner

3.4 Discussion
5 Conclusions, Discussion and Future Directions ......................................................... 105

5.1 Thesis review .................................................................................................................. 106
   5.1.1 Review of chapter 2 ............................................................................................ 106
   5.1.2 Review of chapter 3 ............................................................................................ 107
   5.1.3 Review of chapter 4 ............................................................................................ 109

5.2 EAR-2 is a gatekeeper to hematopoietic differentiation: ........................................... 110
   5.2.1 Future experiments: ............................................................................................ 110

5.3 EAR-2 expression impairs T cell development: ............................................................. 118
   5.3.1 Significance of data: ........................................................................................... 118
   5.3.2 Physiological relevance: ..................................................................................... 119
   5.3.3 Future directions: ................................................................................................ 120

5.4 Looking Forward: Identification of an EAR-2 modulator for therapeutic purposes ...... 124
   5.4.1 Development of partial agonists/antagonists: ..................................................... 125

References ............................................................................................................................... 128

Appendices ............................................................................................................................. 159
List of Tables

Table 2.1 Genes expressed more abundantly in AML-4 cells with limited proliferative ability (0-7 cells or less than 3 consecutive cell divisions).......................................................... 37

Table 2.2 Confusion matrix describing the ability of 3 clonal siblings used as biological reporters to predict the proliferative fate of a fourth clonal sibling consumed in the process of global RT-PCR. Abbreviations: TP, true positive; FP, false positive, PPV, positive preditive value; FN, false negative; TN, true negative; NPV, negative predictive value. .............................................. 39

Table 2.3 Genes expressed more abundantly in AML-4 cells with limited proliferative ability (less than 3 consecutive cell divisions)................................................................................. 41

Table 2.4 Genes expressed more abundantly in AML-4 cells with proliferative ability (3-6 successive divisions)............................................................................................................. 42

Table 3.1 Blood Counts of EAR-2 transplant chimeras ............................................................... 66

Table 3.2 Blood counts for animals receiving grafts of 100% sorted cells ................................. 68
List of Figures

Figure 1.1 The hematopoietic hierarchy. ................................................................. 4

Figure 1.2 Overview of T-cell Development ........................................................... 9

Figure 1.3 Tumours as Caricatures of Tissue Renewal........................................... 15

Figure 1.4 Schematic of the functional domains of EAR-2. ..................................... 20

Figure 2.1 Early passages of the OCI/AML-4 cell line maintain cardinal features of patient samples. ..........................................................35

Figure 2.2 OCI/AML4 cells are organized as a hierarchy ......................................... 38

Figure 2.3 Identification of EAR-2 by clonal sibling analysis..................................... 44

Figure 2.4 EAR-2 expression correlates with differentiation...................................... 45

Figure 2.5 Overexpression of EAR-2 increases proliferation..................................... 47

Figure 2.6 Overexpression of EAR-2 inhibits differentiation..................................... 48

Figure 2.7 Knockdown of EAR-2 induces spontaneous differentiation..................... 50

Figure 3.1 EAR-2 affects hematopoietic differentiation ex vivo................................. 63

Figure 3.2 EAR-2 expression inhibits differentiation............................................... 64

Figure 3.3 EAR-2 chimeras develop myeloid dysplasia.......................................... 67

Figure 3.4 EAR-2 overexpression causes leukemia................................................ 69

Figure 3.5: Increased blast leukemia cells in recipients that received bone marrow overexpressing EAR-2.................................................................70

Figure 3.6: EAR-2 overexpression is accompanied by expansion of the stem cell pool and progenitor cell dysregulation in vivo...........................................73
Figure 3.7: EAR-2 regulates maintenance of the undifferentiated state \textit{ex vivo} ...............74

Figure 3.8: Functions as a transcriptional repressor in a DNA binding dependent manner. .................................................................................................................................................................76

Figure 3.9. Overexpression of EAR-2 may regulate decisions at points of bifurcation in the hematopoietic hierarchy. .....................................................................................................................................................79

Figure 4.1 Differentiation of T cells induces decreased EAR-2 expression ..................94

Figure 4.2: EAR-2 expression must decrease for thymocyte development..................95

Figure 4.3. EAR-2 overexpression results in thymic involution. .................................97

Figure 4.4. Severe defects in T cell development observed \textit{in vitro}...............................99

Figure 4.5 Analysis of T cells derived from OP9-DL1 cultures of KSL cells transduced with GFP or Ear-2 retrovirus. .................................................................................................................................100

Figure 5.1. Long term culture of MDS patient samples. ............................................114

Figure 5.2. Tyrosine kinase inhibitors regulate EAR-2 expression. ............................126
List of Appendices

Appendix A: Revisiting immunosurveillance and immunostimulation: Implications for cancer immunotherapy

Appendix B: First among equals: the cancer cell hierarchy.

Appendix C: Progression of myelodysplasia to acute lymphoblastic leukemia: implications for disease biology.

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

Appendix E: Generation of high-titer viral preparations by concentration using successive rounds of ultracentrifugation

Appendix F: NR2F6 and uses thereof (provisional patent)

Appendix G: Kinase Independent Mechanisms of Resistance of Leukemia Stem Cells to Tyrosine Kinase Inhibitors
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>32Dcl3</td>
<td>32D clone 3</td>
</tr>
<tr>
<td>5-FU</td>
<td>5 Fluorouracil</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>AD</td>
<td>Activation domain</td>
</tr>
<tr>
<td>ALIP</td>
<td>Abnormal localization of immature precursors</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelogenous leukemia</td>
</tr>
<tr>
<td>Ang1</td>
<td>Angiopoietin 1</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>B cell leukemia-x long</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>Breakpoint cluster region- Abelson oncogene fusion protein</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMI1</td>
<td>B cell-specific Moloney murine leukemia virus integration site 1</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer binding protein</td>
</tr>
<tr>
<td>CBP</td>
<td>cAMP response element-binding (CREB)-binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation antigen</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Cdx</td>
<td>Caudal-related homeobox gene</td>
</tr>
<tr>
<td>cGY</td>
<td>Centigray</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myelogenous leukemia</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>COUP-TF</td>
<td>Chicken ovalbumin upstream promoter transcription factor</td>
</tr>
<tr>
<td>c-rel</td>
<td>v-rel reticuloendotheliosis viral oncogene homolog (avian)</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CTIP</td>
<td>Coup-TF interacting protein</td>
</tr>
<tr>
<td>CTLA</td>
<td>Cytolytic T lymphocyte-associated antigen</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>dUTP</td>
<td>Deoxyuridine triphosphate</td>
</tr>
<tr>
<td>EAR-2</td>
<td>v-erb A related-2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>ETP</td>
<td>Early thymic progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FLT-3 ligand</td>
<td>Fms-like tyrosine kinase 3</td>
</tr>
<tr>
<td>FN</td>
<td>False negative</td>
</tr>
<tr>
<td>FP</td>
<td>False positive</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>Gfi1</td>
<td>Growth factor independent</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte colony stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte macrophage progenitor</td>
</tr>
<tr>
<td>hCG-NuMA-RARα</td>
<td>human cathepsin G promoter driven nuclear mitotic apparatus - human retinoic acid receptor α fusion protein</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox (gene)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's modified Dulbecco's medium</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>ISP</td>
<td>Immature single positive</td>
</tr>
<tr>
<td>KLCM</td>
<td>c-kit ligand conditioned medium</td>
</tr>
<tr>
<td>KSL</td>
<td>c-kit+, sca-1+, lineage-</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage marker</td>
</tr>
<tr>
<td>Lnk</td>
<td>Lymphocyte adaptor protein, SH2B adaptor protein 3</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemia stem cell</td>
</tr>
<tr>
<td>LSK</td>
<td>Lineage-, sca-1+, c-kit+ cells</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>Long-term hematopoietic stem cell</td>
</tr>
<tr>
<td>Mash</td>
<td>Mammalian Achaete-Scute homolog</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>Meis</td>
<td>Myeloid ecotropic viral integration site</td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte-erythroid progenitor</td>
</tr>
<tr>
<td>Mll</td>
<td>Mixed lineage leukemia (gene)</td>
</tr>
<tr>
<td>MMP</td>
<td>Murine myeloproliferative</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Myb</td>
<td>Protooncogene from the myeloblastosis family of transcription factors</td>
</tr>
<tr>
<td>Myc</td>
<td>Myelocytomatosis oncogene cellular homolog</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>NF-AT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-Ya</td>
<td>Nuclear transcription factor Y subunit alpha</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>Oct4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>OP9-DL1</td>
<td>Osteopetrotic 9 cells transduced with delta-like1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pbx</td>
<td>Pre-B cell leukemia transcription factor 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>Phox</td>
<td>Paired homeobox</td>
</tr>
<tr>
<td>PML-RAR</td>
<td>Promyelocytic leukemia retinoic acid receptor fusion protein</td>
</tr>
<tr>
<td>Psr</td>
<td>Probability of self-renewal</td>
</tr>
<tr>
<td>PU.1</td>
<td>SPI1</td>
</tr>
<tr>
<td>PyY</td>
<td>Pyronine Y</td>
</tr>
<tr>
<td>RA</td>
<td>Refractory anemia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RAEB</td>
<td>Refractory anemia with excess blasts</td>
</tr>
<tr>
<td>RARS</td>
<td>Refractory anemia with ringed sideroblasts</td>
</tr>
<tr>
<td>RCMD</td>
<td>Refractory cytopenia with multilineage dysplasia</td>
</tr>
<tr>
<td>rh</td>
<td>Recombinant human</td>
</tr>
<tr>
<td>RORγT</td>
<td>Retinoid-related orphan receptor γ thymus specific</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RUNX</td>
<td>Runt-related transcription factor</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>Scrm</td>
<td>Scrambled control</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short-hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMAD</td>
<td>Similar to mothers against decapentaplegic</td>
</tr>
<tr>
<td>SNO</td>
<td>Spindle-shaped N-cadherin expressing osteoblast</td>
</tr>
<tr>
<td>SOX</td>
<td>Sry-related HMG box (gene family)</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride-sodium citrate buffer</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>Short-term hematopoietic stem cell</td>
</tr>
<tr>
<td>svp</td>
<td>Seven up</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Th-POK</td>
<td>T-helper inducing POZ-Kruppel like factor</td>
</tr>
<tr>
<td>Tie2</td>
<td>Tyrosine kinase with Ig and EGF (epidermal growth factor) homology domains 2</td>
</tr>
<tr>
<td>TLX</td>
<td>Human homologue of the <em>Drosophila</em> <em>tailless</em> gene</td>
</tr>
<tr>
<td>TN</td>
<td>True negative</td>
</tr>
<tr>
<td>TP</td>
<td>True positive</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>T_{reg}</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSP</td>
<td>Thymic seeding progenitor</td>
</tr>
<tr>
<td>VKH</td>
<td>Vogt-Koyanagi-Harada</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless/Integrated</td>
</tr>
<tr>
<td>Y2H</td>
<td>Yeast 2 hybrid</td>
</tr>
<tr>
<td>ZFX</td>
<td>X-linked zinc finger protein</td>
</tr>
</tbody>
</table>
1 Introduction and Background

1.1 Introduction

“What had that flower to do with being white,
The wayside blue and innocent heal-all?
What brought the kindred spider to that height,
Then steered the white moth thither in the night?

What but design of darkness to appall?--
If design govern in a thing so small.”

Robert Frost

Elucidation of the genes that govern clonogenicity of the cancer stem cell, as well as understanding the roles of said genes in normal physiology is crucial to the development of therapeutics that are specific and effective to the eradication of the cancer stem cells: The cells that drive the disease. Moreover, there is much intellectual appeal in understanding the circuitry of the cancer cell in and of itself. What can be more fascinating than agents whom under normal conditions encode for processes as wholesome as life and growth, whom are hijacked by a force no more sinister than chance, to give rise to a system that is so fit that the very fabric of its being, the fact that it is so efficient, commits the ultimately treason: Killing yet the host which tethers it to life. The intricate make up of the neoplasm, the circuitry that allows for this nascent life-form to thrive; processes so complex and contorted one would be tempted to think it deliberate, yet orchestrated by stochastic processes is as great a work of art as any Shakespearean tragedy.

I have focused my attention on leukemia, a cancer of the blood forming tissue. Hence, I will begin with a discussion of the processes that must be orchestrated with brilliant precision to enable normal functioning of blood production, wherein a single hematopoietic stem cell may give rise to every single type of blood cell in perpetuity. Then, I will describe what happens when a cell indulges on processes yet as benevolent as self-renewal, that in and of itself is the ultimate force of life, but in excess results in clonal dominance, leukemia and death. My
contribution to the field is to introduce a new character to this narrative: The orphan receptor EAR-2, otherwise known as NR2F6. In this work, I show that EAR-2 acts to preserve the undifferentiated state of cancer cells and stem cells and that when expressed in excess in healthy cells results in leukemia characterized by the excess production of stem cells with an impaired production of most blood cell types, as well as abrogation of T cell development.

1.2 Normal hematopoiesis

1.2.1 Hematopoiesis is organized as a hierarchy

Normal hematopoiesis is organized as a hierarchy (Figure 1.1). At the apex of the hierarchy is situated the hematopoietic stem cell (HSC), at the base lies a spectrum of mature blood cells. Between these extremities lies a series of immature blood cells, stratified according to their stage of maturation.

The apical cell in the hierarchy, the HSC, is ultimately the point of inception of hematopoiesis. A single murine HSC is capable of reconstituting the entire hematopoietic system of a lethally irradiated mouse and of maintaining the continuous production of blood cells for the lifetime of that animal. These characteristics, of regeneration and maintenance of long-term hematopoiesis, are the hallmark of HSC. The way by which HSCs manage this great feat is by choosing to divide into one of two cell types. Either the HSC divides into a cell that initiates a protocol of differentiation, ultimately resulting in the production of mature blood cells, or it divides into yet another HSC (a process called self-renewal). Therefore, in order to maintain homeostasis it is vital not only to decide if it is appropriate to divide, but also to balance between divisions of self-renewal and differentiation. In mathematical modelling this decision may be represented as a probability: The probability of self-renewal ($P_{sr}$) (1).

Despite the importance of self-renewal in HSC and cancer biology, the mechanisms governing this function are poorly understood. Progress in this area has been hindered by the scarcity of HSCs within hematopoietic tissue, and by challenges faced in purifying HSCs to the extent necessary for studies of transcription or proteomics. Nonetheless, roles in self-renewal have been identified for several proteins. These include pathways involved in embryonic development (Wnt/β-catenin (2), Notch/Delta-like (3), BMP/SMADs (4)), the hox genes and
Figure 1.1 The hematopoietic hierarchy. All of the blood cells in the body may be derived from a single hematopoietic stem cell. Long-term hematopoietic cells differentiate into short-term hematopoietic cells, that in turn differentiate into cells that eventually bifurcate along the myeloid or the lymphoid lineage, the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) cell respectively. As the common myeloid progenitor cell continues along the path of differentiation, its progeny eventually bifurcates into cells that are able to give rise to either granulocytes and monocytes or to erythroid and myeloid cells, the granulocytic-monocytic progenitor (GMP) cell or the megakaryocyte-erythroid progenitor (MEP) cell respectively. The GMP gives rise to progenitor cells that are unipotent, they are able to differentiate into a single lineage, either that of monocytes or granulocytes; while the MEP also rise to progenitor cells that are unipotent, they are able to differentiate into a single lineage, either that of megakaryocytes or erythrocytes. The common lymphoid progenitor bifurcates its progeny into differentiation into either T cells or B cells.
their partners (Cdx (5), Hoxa9 (6), Hoxa10 (7), Hoxb4 (8), Meis (5), Pbx (5)), and polycomb/trithorax group genes (Bmi1 (9, 10), Mll (11)). In addition, a number of transcription factors involved in blood cell differentiation have also been shown to be necessary for self-renewal (Gata-2 (12), Gfi1 (13), JunB (14), Pu.1 (15), Myb (16), Cbp (17), Myc (18), and Zfx (19)). How these diverse pathways are integrated in vivo is not understood; it has been postulated that epigenetic modifications such as chromatin and histone methylation and acetylation play a key role (20), and that the switch between HSC self-renewal and differentiation is regulated by competition between transcription factor complexes, akin to the interplay among Gata-1, C/EBPα, and Pu.1 that mediates the myeloid/erythroid lineage decision (21, 22).

If a HSC chooses differentiation, it divides into a cell that has committed to a program of maturation (Figure 1.1). The process of maturation begins in this cell by having it commence a number of consecutive cell divisions, each division bringing the progeny closer to maturity. The result is a series of immature intermediates. Since divisions result in exponential expansion of cell numbers, the relative abundances of the mature progenitors are orders of magnitude greater than that of the more primitive progenitors. This is the basis of the hematopoietic hierarchy: HSC are rarer than immature blood intermediates, which are rarer still than the mature blood cells at the base of the hierarchy. Cells are positioned in the hierarchy according to their clonal age, each cell division represents a different stratum of the hierarchy. Cells positioned in the same stratum of the hierarchy have spent similar durations differentiating from the hematopoietic stem cell (HSC). As a result, cells at a given level of the hierarchy have similar biological properties.

Two parameters correlate with the level of differentiation, and hence stratification in this hierarchy: Growth ability and potentiality for lineage divergence. Although a stem cell may divide without exhaustion, this is not the case once a differentiating cell buds off the HSC; a “timer” is initiated that counts down the number of divisions that each cell is able to execute. Thus, the more elevated a progenitor is in the hierarchy the greater its proliferative capacity. This culminates in terminal differentiation, where mature hematopoietic cells cannot divide (of course the exception being lymphocytes which clonally expand upon antigenic stimulation). Rather, mature cells serve their function and die (extinction).
Ultimately, differentiation refers to specialization. Since the HSC is capable of differentiating into a spectrum of blood cell types, through the course of maturation progenitors must commit themselves to the production of only one cell type. This is achieved by progressively restricting the number of different blood cell types that a cell may develop into as the cell proceeds through the differentiation hierarchy. That is, as stem cells differentiate into blood cells that are committed to a single lineage, they transverse from pluripotent cells to multipotent progenitors, to tetrapotent, tripotent, bipotent and finally into those progenitors capable of producing but one single lineage.

Since mature blood cells have a finite life span, they must be produced perpetually. This implies that a continuous number of stem cells are needed, to be ‘consumed’ by this process of differentiation. In order to maintain a bottomless supply of stem cells, a stem cell has the option of self-renewal. This means that it may divide into yet another stem cell, and like the initial stem cell the progeny will also be pluripotent and have extensive—if not unlimited—proliferative ability. The process of self-renewal may be used to maintain a constant number of cells (as stem cells are lost to differentiation) or to expand the stem cell pool in a given system (for example in the recipient of a bone marrow transplant).

1.2.2 Development of T cells

The hematopoietic stem cell is able to form every blood cell lineage. Red blood cells provide oxygen to the cells of the body; platelets mediate blood clotting, while the white blood cells are the body’s immune system: The army that patrols the body to identify and eliminate invading microbes. Historically, hematologists have thought of the process of white blood cell formation in terms of two cognitive camps: Lymphoid and myeloid cell development. For many years, it was thought that bifurcation of progenitor cells along these party lines was the first point of lineage restriction in the cell fate determination of hematopoietic progenitor cells giving rise to the common myeloid progenitors and the common lymphoid progenitors (Figure 1.1). Even leukemias have been classified as belonging to either side of the dichotomy being labeled as either lymphoid or myeloid. However, recent evidence is showing that the divide may not be as distinct as once envisioned, instead HSCs give rise to a series of progenitor cells that gradually restrict their pluripotency (23, 24). Nevertheless, for the most part, myeloid cells constitute the
innate immune system, while lymphoid cells are responsible for mediating the specific immune response. Cells of the innate immune system consist of granulocytes and monocytes, while cells of the specific immune system consists of T lymphocytes and B lymphocytes, so called because T cell development takes place in the thymus while B cell development was first recognized in chickens to occur in the bursa of Fabricius. In both mice and men, B cells develop in the bone marrow.

The innate immune system targets invading pathogens non-specifically either by engulfing them through the process of phagocytosis, or by killing them with noxious agents such as reactive oxygen species. On the other hand, the adaptive immune response is a most marvelous army of cells shared only by us jawed vertebrates. This *magnum opus* of 50 million years of evolution employs a collection of cells so diverse in their area of specialization that each individual cell is able to recognize and eliminate one specific invader. The truly fascinating achievement is that for every possible threat to the body that exists in the pathogenic universe; the specific immune system has just the cell, specifically designed though stochastic means to recognize and eliminate that specific pathogen! How can the body possibly know the diversity of molecules in the pathogenic universe *a priori* so that it can devise such a collection of complementary protective cells? The body accomplishes this through genomic recombination resulting in the generation of random receptor specificities. These receptors are able to recognize both self and non-self (pathogenic invaders). Hence, the repertoire of cells needs to be culled in order to eliminate those cells that would otherwise attack the body’s own self. The place where immature cells go to be educated on how to distinguish friend from foe, to mature into cells that can be deployed to patrol the periphery of the body is a specialized organ called the thymus.

The thymus is a small organ found in the thoracic cavity between the sternum and the heart. The thymus is a bi-lobed organ containing two regions. The outermost region is called the cortex, while in the middle of the thymus is the region called the medulla. The first step in T cell development is the migration of bone marrow cells to the thymus. Hematopoietic stem cells that do not self-renew give rise to progenitor cells with multipotent lineage potential. Some of these cells, conveniently termed thymic seeding progenitors (TSP), enter the thymus in the junction of the thymic cortex and medulla. While it is known that this cell is a primitive cell, with the ability to differentiate into multiple lineages (23), the specifics of what exactly a TSP is and what it may differentiate into remains controversial (24). Nevertheless, this enigmatic bone marrow cell that
is very primitive and not yet committed to a T cell fate enters the thymus and begins to divide in response to Notch signaling (Figure 1.2), giving rise to the early thymic progenitor cell (ETP). From here the course of differentiation is more straightforward: ETPs mature into double-negative (DN) cells, which then mature into double positive (DP) cells, which finally mature into single positive (SP) cells. These terms refer to expression of the markers CD4 and CD8: DN cells do not express either marker, DP cells express both markers and mature T cells express either the CD4 or CD8 marker depending on the type of T cell into which they have chosen to differentiate in to. Of course, the picture is more complex: The DN phase of differentiation may be divided into four sub-stages: DN1 through DN4, so classified based on expression of the markers CD44 and CD25.

There are many fascinating biological events that must take place from the time a bone marrow cell enters the thymus to the time it exits the thymus as a mature cell. First, it is necessary to turn off stem cell genes and commit to the T cell fate. Fascinatingly, the cells that enter the thymus are not committed to the T cell fate by virtue of being in the thymus; this commitment does not actually occur until the late DN2 stage of development (25-27). Next, the immune system must generate a repertoire of cells so diverse that their diversity matches or even supersedes that which is found in the pathogenic universe. This specificity is accomplished by genomic recombination events of the V(D)J genes in the complementary determining region in the T cell receptor, so called because this region is complementary to the antigens of foreign invaders. The first of these recombination events takes place at the DN3 stage of development where the β chain of the T cell receptor is formed through genomic recombination. Since recombination is capricious by nature, the product of this event must be carefully monitored. Hence, the implementation of strict checkpoint that cull those cells that are not up to par. Those cells that did not rearrange a functional T cell receptor (β chain) are banned from proceeding further and sentenced to die by apoptosis. The next recombination event takes place at the DP stage of development, where the α chain of the T cell receptor is formed through genomic recombination. Now with a fully formed T cell receptor the T cell is placed through not one, but two checkpoints. The first test is called positive selection and occurs in the thymic cortex, to
A. T-cell development takes place in the thymus. Multipotent progenitor cells (S) enter the thymus, differentiate and exit as mature T-cells (T). Inside the thymus cells progress through three stages: the double negative (DN) stage, the double positive (DP) stage, and the single positive (SP) stage. Double negative cells can be further subdivided into DN1 through to DN4. 

B. There are many important biological processes that occur in the course of T-cell development. The T-cell receptor consists of an alpha chain and a beta chain. The T-cell beta chain is made through the process of VDJ recombination at the DN3 stage. The ability to form a functional beta-chain is then tested through beta-selection. At the double positive stage, the alpha-chain of the T-cell receptor is formed. This newly formed T-cell receptor then needs to be tested through two separate checkpoints: positive selection and negative selection. In positive selection the ability of the T-cell receptor to function is tested by its ability to bind to MHC. Those cells whose receptors that are able to signal are allowed to live. The second checkpoint called negative selection takes place at the single positive stage. This checkpoint eliminates cells that are autoimmune by having too high an affinity to the self-antigens found in the thymus. Single positive cells then exit the thymus as mature T-cells.
pass this test the T cell must demonstrate that its T cell receptor is able to function. Those with functional receptors are positively selected while the rest of the cells are banished from the repertoire and sentenced to apoptotic death. Those that pass positive selection move then into the thymic medulla where they face a second test: Negative selection. At this roadblock, developing T cells must demonstrate that they are not activated by the cells of the body: Hence, if they bind too tightly to the cells in the thymus then they are deemed to be autoreactive, and hence pose the risk of attacking the body’s own self, thus failing the negative selection checkpoint. As with positive selection, it is only the select few that are able to pass this checkpoint. T cells that have passed both positive and negative selection graduate to the status of mature T lymphocyte and are deployed in the circulation to patrol the body.

Like any military organization, T cells have different ranks and functions. One subtype of T cell functions to orchestrate attacks, while a different subpopulation carries out the attacks. Cytotoxic T cells express the marker CD8. These are effector cells that patrol the body to kill infected cells. On the other hand, T-helper cells express the marker CD4, these are the cells that orchestrate attack against pathogens by secreting the growth factors needed to launch a specific immune response. T helper cells may be further subdivided into Th1 and Th2 type T-helper cells. These cells differ in the growth factors they secrete which support different types of immune effector cells so that the type of attack launched by the immune system is appropriate for the type of pathogen that has invaded the host. The pathogenic universe is generally divided into intracellular pathogens, such as viruses that can only replicate within host cells, and extracellular pathogens such as bacteria. Th1 cells secrete growth factors necessary for the initiation of a cell mediated immune response that is designed to target intracellular pathogens while a Th2 cell secretes growth factors necessary for the initiation of an antibody mediated immune response that generally targets extracellular pathogens. In addition there are another two types of CD4+ T cells that act to orchestrate the body’s immune response: Th17 cells and regulatory T cells. Th17 cells are IL-17 producing cells that are important for bacterial responses and have been associated with a variety of autoimmune and inflammatory diseases including Vogt-Koyanagi-Harada (VKH) disease (28), rheumatoid arthritis (29, 30), asthma (31), psoriasis, inflammatory bowel disease (32, 33), and multiple sclerosis (34, 35). Regulatory T cells (Tregs) are a class of cells that generally express the markers CD4 and CD25 and have high levels of the protein
Foxp3. T\textsubscript{reg} cells function to shut off the immune response by a range of mechanisms such as secretion of the soluble factors TGF-β and IL-10 and direct cell to cell contact via the molecule CTLA-4. All T-helper cell subsets are thought to differentiate in the periphery from the naïve T-helper cell (termed the TH0 cell) based on the growth factors which the cell is exposed to at the time of activation. Which type of T helper cell the TH0 cell chooses to differentiate into is very important to the state of the body, as the immune system may serve as a double-edged sword. Differentiation of a T-helper cell into a subtype that is not protective against the type of pathogen encountered by the body will launch an immune response that not only will fail to protect against the invading pathogen but will also harm the body. The interested reader is kindly referred to the article in Appendix A for a thorough discussion of the duality of the immune system in the particular disease of cancer.

\textbf{1.3 Neoplastic hematopoiesis}

\textbf{1.3.1 Overview of leukemia}

Leukemia is a cancer of the hematopoietic system. It is a clonal proliferative disorder characterized by the accumulation of immature hematopoietic cells in the blood and bone marrow along with concurrent suppression of normal hematopoiesis (36, 37). Leukemia may be classified as belonging to either the myeloid or lymphoid subtypes, depending on whether the leukemia cell population displays lineage markers of the myeloid or lymphoid cell populations. Both lymphoid and myeloid leukemia are then each further classified as belonging to either the chronic or acute subtype. Here, we will discuss one particular type of leukemia: Acute myeloid leukemia (AML).

\textbf{1.3.1.1 Clinical features}

AML is characterized by effacement of normal blood cell production and accumulation of neoplastic cells, known as blasts, in the bone marrow, peripheral blood, and other tissues. As for many other cancers, the incidence of AML rises exponentially with age (38), and thus the incidence of this disease can be expected to rise with the ageing of our population. Despite the many advances made in the understanding of leukemia biology over the past three decades, therapy for AML has remained essentially unchanged for 40 years, and is, in most cases,
debilitating and ineffective – especially in older patients (39). Further progress in improving the efficacy of anti-leukemia therapy hinges upon the identification of specific targets that allow therapy to be focused on the AML stem cell.

1.3.1.2 AML cells have impaired differentiation

The biological characteristic that distinguishes between chronic and acute myelogenous leukemia is the extent to which the leukemia cells can differentiate, whereas chronic myeloid leukemia cells are able to fully differentiate into mature blood cells (at least in the chronic phase of the disease), AML cells are blocked in the ability to mature. However, remnants of the maturation process still do occur. Nevertheless, three lines of experimental evidence suggest that leukemic cells can differentiate aberrantly. First, the leukemia cells present in most AML patients express immunophenotypic markers, enzymes and morphological features characteristic of lineage-committed cells (36, 37). Second, Auer rods, a feature of some AML cells (40), have been observed in the mature neutrophils of AML patients (41-43). Finally, although hematopoiesis during complete remission is typically polyclonal (44), the observation that the mature blood cells of some AML patients are clonal (45-47) suggests that these cells may be derived from the leukemic clone. Collectively, these studies suggest that differentiation in AML is not abrogated, but that leukemia cells undergo vestiges of normal differentiation.

1.3.2 Overview of myelodysplastic syndrome:

Myelodysplastic syndrome (MDS) is a bone marrow failure disorder often thought of as pre-leukemia, however that term is a misnomer as many cases do not in fact progress on to leukemia. MDS is predominantly a disease of middle-age and older adults, very rarely observed in patients less than 50-years old (48). MDS is characterized by inefficient hematopoiesis that paradoxically presents with bone marrow hypercellularity. Clinically, patients with myelodysplastic syndromes have anemia or other cytopenias that often are associated with apoptosis of bone marrow hematopoietic progenitors. Blood smears often contain features of dysplasia, or abnormal blood cell morphology, for examples oval macrocytes, hyposegmented neutrophils (pseudo-Pelger-Huët cells), and hypogranulated neutrophils and platelets. Ironically,
in spite of the inefficient hematopoiesis, the cells in the bone marrow carry a competitive advantage as MDS carries a marked propensity to progress on to acute leukemia (49).

MDS is not a single disease but rather a heterogeneous collection of diseases. Originally MDS was called pseudoaplastic anemia due to the paradoxical presence of excessive bone marrow cellularity in patients that presented with anemia. These diseases were later grouped with refractory anemia because they did not respond to therapy and reclassified as such. In 2001 the World Health Organization (WHO) revised the French-American-British Cooperative Group’s classification of MDS, which was updated again in 2008 to give rise to the current WHO classification which divides MDS into the following eight categories: Refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess blasts, type I (RAEB-I) and RAEB-II, 5q-syndrome, therapy related MDS, and unclassified MDS. RA is defined by the presence of less than 5% blasts, less than 15% ringed sideroblasts (a feature of abnormal accumulation of iron in the mitochondria), and dysplasia in more than 10% in one myeloid lineage. RARS is defined by erythroid dysplasia, less than 5% blasts and 15% or greater sideroblasts. RCMD is defined as cytopenia, dysplasia in more than 10% of cells in two or more lineages, and less than 5% blasts. RAEB-I is defined as having cytopenia, dysplasia, and between 5-9% blasts in the bone marrow or peripheral blood, whereas RAEB-II is similar to RAEB-I but has a greater number of blasts in the bone marrow or peripheral blood, between 10-19%. 5q-syndrome is defined as the presence of the mutation del(5q) in the blood/bone marrow, accompanied by anemia, with less than 5% blasts cells. These patients often have normal or increased hypolobated megakaryocytes. MDS that presents following chemotherapy is classified as treatment related MDS. The final category, unclassified MDS, is defined as cytopenia with less than 5% blasts and dysplasia in one or more myeloid lineages along with cytogenetic abnormalities that are associated with MDS.
1.4 Cancer as a caricature of normal tissue development: The cancer stem cell

1.4.1 Overview of the cancer stem cell model

Heterogeneity is a hallmark of cancer that is apparent both across the spectra of cancer patients and within the neoplasm of an individual patient. Unlike immortalized cell lines, primary cancer cells are heterogeneous with respect to the number of cell divisions that they are able to execute. Most primary cancer cells, irrespective of their primary site, will not proliferate in vitro. In fact, only a tiny minority of cancer cells can execute sufficient divisions to form a colony in semisolid medium (50-53), and a still smaller minority can regenerate a tumour in vivo (54, 55).

According to the cancer stem cell (CSC) model, cancer is organized in a cellular hierarchy with differences in the differentiation status of the constituent cells of the neoplasm comprising the basis of this cellular heterogeneity (Figure 1.3). At the apex of the hierarchy is the CSC, a rare cell with indefinite self-renewal and proliferative ability. The CSC may divide to give rise to another CSC (a process known as self-renewal), or it may initiate a program that culminates in differentiated cancer cells that can no longer undergo mitosis. The process of cancer cell differentiation gives rise to an intermediate population of cells, descendants of the CSC that have lost self-renewal capacity and that are able to proliferate for a finite number of cell divisions. Once these cells have executed their allotted number of cell divisions they enter a terminal post-mitotic state. This forms the basis of the cellular hierarchy: Rare CSCs with indefinite proliferative ability irreversibly differentiate into non-stem cancer cells with limited proliferative ability; these cells in turn ultimately differentiate to give rise to a much larger population of cells that can no longer proliferate. It is important to specify that while these cells have differentiated, in the sense that they can no longer undergo mitosis, they remain morphologically and functionally immature in comparison to normally differentiated cells of the tissue from which the cancer arose. Furthermore, it is also important to note that even though CSCs are the cells situated at the apex of the cellular hierarchy, recent evidence using retrovirally marked human leukemia cells suggests that CSCs may not be a homogenous population. That is the self-renewal and repopulating capacity of the CSCs may vary even within the leukemia stem cell compartment (56). Evidence for the existence of such a cancer cell hierarchy has been
Figure 1.3 Tumours as Caricatures of Tissue Renewal. The notion that cancer might be a problem of developmental biology, in which the cellular organization of the cancerous tissue resembles the organization of normal developing tissue was proposed by G. Barry Pierce, Robert Shikes and Louis M. Fink: “Our concept postulates that a tumour is an aberration of tissue renewal that results in a caricature of the parental tissue.”
reported for several malignancies including acute myelogenous leukemia (AML), brain cancer, prostate cancer and breast cancer (57-59).

1.4.2 Targeting the tumour stem cell

The stem cell model has enormous implications for the study and treatment of cancer. The fundamental premise of the cancer stem cell model is that the only cells in the neoplasm that are immortal are the very rare CSCs. Since these are the only cells capable of unlimited cell divisions, they are the cells responsible for the long-term maintenance of neoplasm, and are the only cells capable of re-establishing a neoplasm at sites of metastasis or following induction of remission. Therefore, eradication of the cancer stem cells is necessary for cure. The notion that the cancer stem cell must be eradicated to achieve cure is supported by the observation that while that majority of patients with chronic myelogenous leukemia (CML) that are treated with imatinib achieve a hematological remission (60, 61), imatinib does not cure CML. The induction of remission but the inability to achieve cure is consistent with a drug that eradicates the bulk of the leukemia cell population—the differentiating leukemia cells—but that does not target the leukemia stem cells (Appendix G). Indeed, the persistence of primitive BCR-ABL positive hematopoietic cells has been observed in CML patients that have achieved a complete remission using imatinib (62, 63). Further evidence suggesting that imatinib does not eradicate the leukemia stem cells of CML comes from the work of Michor et al who used measurements of the level of BCR-ABL expression in 169 CML patients treated with imatinib to model the in vivo kinetics of the reduction of various leukemic sub-populations during therapy (64). They observed that while imatinib targets the leukemia progenitor cell population, this agent does not reduce the number of leukemia stem cells. These data suggest that imatinib does not eradicate the leukemia stem cell in CML which is why patients that respond to imatinib are not cured. To cure CML it is necessary to eliminate the residual CML stem cells that re-establish the disease.

1.4.3 Cancer stem cells as therapeutic targets

The hierarchical organization of AML implies that eradication of the leukemic stem cell compartment is necessary and sufficient for cure. One obvious therapeutic objective is to use molecular knowledge to develop methods of inhibiting self-renewal or inducing differentiation
of the leukemia stem cells (LSC). Differentiation therapy would hence be more precisely defined as therapy that increases the probability of LSC divisions giving rise to daughter cells that are committed to terminal differentiation; that is, therapy that decreases the probability of self-renewal ($P_{sr}$) of the LSC. The $P_{sr}$ is not ‘hard-wired’, that is, it is not an intrinsic and immutable characteristic of stem cells, but instead is subject to regulation in response to extrinsic factors. Under steady state conditions the $P_{sr}$ theoretically should be 0.5 (1); however, maintenance of homeostasis may under certain circumstances require a temporary increase in the $P_{sr}$. For example, following hematopoietic depletion induced by radiation or chemotherapy repopulation of the hematopoietic system is achieved by stem cell expansion. In order to achieve stem cell expansion the $P_{sr}$ increases, as observed in early experiments that detected a $P_{sr}$ of 0.6 during bone marrow engraftment of irradiated recipients (65, 66). By analogy, it is logical to expect the $P_{sr}$ of LSCs to be, at least in part, regulated by cell-extrinsic factors.

It has been postulated that hematopoietic stem cells reside within a niche, a specialized milieu that prevents their differentiation and is conducive to HSC maintenance and self-renewal (67). The situation of hematopoietic cells within the bone marrow is not random, but rather is organized along a cellular gradient: Hematopoietic stem cells and primitive hematopoietic cells reside close to the endosteal surface of trabacular regions of long bones (68-70). Whereas, more mature cells predominantly reside in the central bone marrow (71). Recent observations suggest that osteoblasts, in particular the spindle-shaped N-cadherin expressing osteoblasts (SNO), are important participants of the HSC niche. Adhesion of HSCs to osteoblasts, mediated in part by the interaction of Ang-1 with Tie2, was shown to be important in the maintenance of HSC quiescence and self-renewal (72). A correlation between the number of osteoblasts and HSCs has been determined \textit{in vivo}. By conditionally targeting the bone morphogenetic protein receptor 1A, Zhang et al (73) observed an increase in both the number of osteoblasts and the number of long-term repopulating HSCs. Furthermore, overexpression of the parathyroid hormone receptor \textit{in vivo} also resulted in an expansion of both osteoblasts as well as HSCs (68). These studies suggest that indeed the microenvironment is important for maintenance of normal HSC self-renewal. Although, little is known about the niche-requirements of AML stem cells, determination of the specific alterations that occur to the bone marrow niche during leukemogenesis will provide important insight into the regulation of leukemia stem cell self-renewal. By exploiting this feature and resetting downward the $P_{sr}$, differentiation therapy could
enhance significantly the efficacy of standard antileukemic therapy as LSC clonal survival is exquisitely sensitive to small changes in $P_{sr}$.

Furthermore, it is reasonable to conjecture that LSC are also regulated by cell intrinsic factors. In this thesis I will discuss a novel gene that is necessary for the maintenance of stem cell homeostasis, EAR-2 (NR2F6). Knowledge of the molecular mechanisms governing self-renewal could make it possible to generate therapeutic agents that specifically target the parameter of self-renewal. This is currently an extremely active area of research, with roles in the regulation of self-renewal recently being ascribed to Notch/Wnt/β-catenin (2, 74-76), Bmi-1 (9, 77, 78), HoxB4 (79), Lnk (80), Gfi-1 (13, 81), STAT5 (82), runx1 (83), NF-Ya (84), PU.1 (15) and ZFX (19). Nevertheless, a challenge remains in obtaining specificity for leukemia cells. If leukemia arises in a normal hematopoietic stem cell, then it is likely that the genes regulating self-renewal in normal cells will also be the genes regulating self-renewal in the leukemia stem cells. It is likely that such a drug will be most successful in combination therapy. Of special interest would be the combination of such a drug not with chemotherapy, *per se*, but with drugs designed to target features that are unique to leukemia stem cells. One of these differences may be that leukemia cells self-renew more often than normal stem cells. Thus if a specific inhibitor of self-renewal were pulsed, it would be more likely to induce differentiation of leukemia rather than normal cells. This is based on the assumption that the specific inhibitor of self-renewal would affect cells that are in the process of deciding between self-renewal and differentiation. Furthermore, leukemia cells with extended clonal longevity have been reported to express some antigens that are not expressed on human bone marrow stem cells (CD123, CD90 and CD117 (85-87)). Specificity towards the leukemic clone could possibly be attained by conjugating a drug to an antibody that is expressed on leukemia but not normal stem cells.

One distinguishing feature of the LSC that could serve as a target for therapy may be the $P_{sr}$ itself. The $P_{sr}$ of healthy bone marrow stem cells under steady state conditions is 0.5, and it has been postulated that one of the methods through which leukemia stem cells achieve clonal dominance is by having a $P_{sr}$ greater than 0.5—a characteristic of the LSC that could be exploited therapeutically. Assuming that molecular processes distinct from steady state kinetics are required for an augmentation in the $P_{sr}$, elucidation and targeting of these molecular processes might allow for a more selective targeting of the leukemia stem cell.
1.4.4 The central paradox of MDS

The central paradox of MDS biology resides in the observation that the MDS clone, which is characterized by reduced numbers of mature progeny and by maturing progenitors that exhibit impaired clonogenicity (88) and a high rate of apoptosis (89), nonetheless comes to dominate the bone marrow at the expense of residual normal hematopoiesis and thereby causes disease. Consideration of MDS as a disease of hematopoietic stem cells suggests a resolution to this paradox: The MDS clone, despite the defects seen in its differentiating progeny, out-competes normal hematopoiesis because of a selective advantage at the stem cell level. The competitive advantage of the MDS stem cell hence consists in an increased capacity for self-renewal. A key implication of this hypothesis is that identification of therapeutically tractable regulators of HSC self-renewal could lead to effective new therapies for MDS. To that end, our lab is interested in identifying genes that regulate self-renewal in MDS and AML, and hence we sought to identify genes that govern the regulation of clonogenicity of leukemia single cells. This led to the identification of previously unsuspected roles in HSC homeostasis and MDS/AML pathogenesis for the orphan nuclear receptor EAR-2.

1.5 The orphan nuclear receptor EAR-2:

EAR-2, also known as NR2F6, is an orphan nuclear receptor that was cloned in a search for homologues of the retroviral oncogene v-erbA using low stringency hybridization (90). The EAR-2 protein consists of four domains (Figure 1.4). The N-terminal contains a regulatory domain that is conserved amongst nuclear receptors. This regulatory domain is involved in transactivation and contains the activation function-1 helix. EAR-2 contains a DNA binding domain (DBD) consisting of two zinc fingers. The hinge region domain connects the DBD with the ligand binding domain (LBD). EAR-2 is an orphan nuclear receptor which means that the ligand for EAR-2, if it exists, is not known. The LBD is presumed to bind to the hypothesized EAR-2 ligand and be regulated in a ligand-dependent manner. Nevertheless, some nuclear receptors are true orphans in the sense that their ligand binding domains are unable to bind
Figure 1.4 Schematic of the functional domains of EAR-2. The EAR-2 protein may be divided into four parts, the N-terminal transactivation domain, the DNA-binding domain, the hinge region, and the ligand binding domain. The EAR-2 DNA binding domain is found between residues 57 to 122. Herein lie two zinc fingers. The P-box is highlighted with pink, whereas the D box is highlighted with teal. For the sake of clarity some residues have been depicted by an asterisk.
ligand. Other functions of the LBD include receptor dimerization and transactivation as a result of the activation function-2 helix.

EAR-2 is a member of the chicken ovalbumin upstream promoter (COUP) family of nuclear receptors. The COUPs function \textit{in vitro} as transcriptional repressors, antagonizing the activation ability of a wide range of nuclear receptors that play prominent roles in differentiation. Accordingly, aberrant expression of COUP-TFI inhibits retinoid-induced epithelial and neuronal differentiation \textit{in vitro} (91-93). The roles of COUP-TFI and COUP-TFII in mammalian development have been studied by targeted deletion in the mouse. COUP-TFI deficient mice exhibit numerous defects in axonal development, including failure of development of the nucleus of the 9th cranial nerve (94). COUP-TFII deletion causes widespread defects in angiogenesis and cardiac development, leading to embryonic lethality in mid-gestation (94, 95). \textit{Seven-up (svp)}, the \textit{Drosophila} COUP family homologue, is also important in embryonic development; with null mutations of seven-up being embryonic lethal (96). \textit{svp} is involved in decisions of cell fate determination during the development of the photoreceptors in the ommatidium of the eye (97) and regulates proliferation during the development of the Malpighian tubules by regulating the expression of cell cycle regulators (98).

In contrast to the related proteins COUP-TFI and COUP-TFII, the function of EAR-2 has not been well characterized. EAR-2 functions as a transcriptional repressor \textit{in vitro}, inhibiting the transactivating ability of numerous genes including the thyroid hormone receptor (99). Like many nuclear receptors, EAR-2 heterodimerizes with the retinoid X receptor-\(\alpha\) (RXR-\(\alpha\)) (100), although the relevance of this interaction in EAR-2 function is unclear.

Targeted disruption of the EAR-2 locus gives rise to animals that are viable and fertile (101), but show agenesis of the locus coeruleus, a midbrain nucleus that regulates circadian behaviour and nociception. \textit{In situ} mRNA hybridization in EAR-2 \textit{-/-} animals places EAR-2 downstream of Mash1 and upstream of Phox2a and Phox2b in the specification of the locus coeruleus. Although EAR-2 expression is seen outside the central nervous system, there was no description of any phenotypic analysis outside the nervous system; in particular, hematopoiesis is not characterized.
Evidence highly suggestive of a role for EAR-2 in hematopoiesis comes from studies of its interaction with the key hematopoietic transcription factor RUNX1 (also known as AML1). Targeted deletion of RUNX1, a component of the core binding factor complex, results in abrogation of definitive hematopoiesis and embryonic lethality (102, 103) and RUNX1 rearrangements result from several commonly seen chromosome translocations in acute leukemia (104). EAR-2 interacts physically with RUNX1 and represses its transcriptional activating ability in the murine myeloblast cell line 32Dcl3 (105). EAR-2 is down regulated in 32Dc13 cells induced to differentiate with G-CSF, and forced expression of the EAR-2 protein blocks 32Dc13 differentiation (105).

1.6 Thesis overview

In this thesis I sought to determine the function of EAR-2, a gene that I found to be differentially expressed in clonogenic leukemia cells versus leukemia cells with negligent proliferative ability. For my M.Sc. I conducted microarray experiments on single cells of the OCI-AML4 cell line (described in Chapter 2) in which I observed that EAR-2 is expressed four fold greater in clonogenic cells than in cells that lacked growth ability. For my PhD I first set out to validate the role of EAR-2 in regulating the clonogenicity of leukemia cells (Chapter 2): I observed that expression of EAR-2 decreased upon induction of differentiation in the human myelomonocytic cell line, U937. I also showed the clinical relevance of EAR-2 by demonstrating that patients from a variety of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) subtypes had greater expression of EAR-2 in their bone marrow than healthy controls. I then showed that expression of EAR-2 is necessary and sufficient for maintenance of the undifferentiated state by showing that overexpression of EAR-2 inhibits the differentiation of U937 and 32Dcl3 cells; while a decrease of EAR-2 expression attained using shRNA is sufficient for induction of differentiation. I then turned my attention to deciphering the role of EAR-2 in primary healthy bone marrow (Chapter 3). In vitro I observed that EAR-2 overexpression inhibited the differentiation of erythromyeloid cells, while in vivo I observed the overexpression of EAR-2 in chimeric models caused a myelodysplastic like syndrome while transplantation of EAR-2 sorted grafts caused a rapidly lethal leukemia characterized by pancytopenia and expansion of the stem cell fraction. Finally, I observed that EAR-2
overexpression blocked the differentiation of T lymphocytes (Chapter 4) in both the thymus and periphery. In competitive bone marrow transplantation models, thymocytes overexpressing EAR-2 demonstrated a stark disadvantage at repopulation of T cells. Bone marrow transplantation of sorted grafts revealed that unabated expression of EAR-2 drastically decreased thymic cellularity, and increased apoptosis in the thymic medulla. It is likely that at least part of this phenomenon is a cell intrinsic effect as opposed to a problem in the homing of progenitor cells to the thymus as in vitro OP9-DL1 cultures showed an immediate dramatic reduction in cellularity as well as increased apoptosis at the DN to DP transition phase. Overall, these data show that the orphan nuclear receptor EAR-2 is an important regulator of leukemia cell differentiation, hematopoietic stem cell homeostasis, and T cell development.
Disclosures and Copyright Acknowledgements

Part of Chapter 1 was published in *Leukemia and Lymphoma* as follows: Ichim CV, and Wells RA. First among equals: the cancer cell hierarchy. *Leuk Lymphoma* 2006 Oct; 47(10): 2017-2027.

Chapter 2 was published in *Leukemia* as follows: Ichim CV, Atkins HL, Iscove NN, Wells RA. Identification of a role for the nuclear receptor EAR-2 in the maintenance of clonogenic status within the leukemia cell hierarchy. *Leukemia* 2011 Nov; 25(11): 1687-1696.

Chapter 3 has been submitted to *The Journal of Experimental Medicine* as follows: Ichim, C.V., Chesney, A., Reis, M., and Wells, R.A. EAR-2 is a novel regulator of hematopoietic differentiation and hematopoietic stem cell homeostasis. *The Journal of Experimental Medicine (submitted).*

Chapter 4 has been submitted to *Experimental Hematology* as follows: Ichim, C.V., Dervovic D., Zuniga-Pflucker J.C. and Wells R.A. The orphan nuclear receptor EAR-2 (NR2F6) is a novel negative regulator of T-cell development. *Experimental Hematology(submitted).*

Figures 2.1, 2.2, 2.3A,B,C; as well as table 2.1, 2.2, 2.3 and 2.4 were generated during my Master of Science.

Figures 4.4 and 4.5 were conducted in collaboration with Džana D. Dervović
2 Identification of a role for the nuclear receptor EAR-2 in the maintenance of clonogenic status within the leukemia cell hierarchy

Chapter 2 was published as follows:


Figures 2.1, 2.2, 2.3A,B,C; as well as table 2.1, 2.2, 2.3 and 2.4 were generated during my Master of Science.
2.1 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 (106). The majority of blasts are not clonogenic, while only a tiny fraction can proliferate extensively. The leukemia stem cell (LSC) model (107) explains this heterogeneity by proposing that the population of leukemia cells is composed of stem and non-stem leukemia cells. Once a LSC 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 (108).

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, since such analyzes 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 (109-111) 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 (112) 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 homologue of Drosophila svp, a gene that has a well-characterized role in cell fate decisions of primitive neural cells (113). EAR-2 has previously been shown to interact \textit{in vitro} with the key hematopoietic transcription factor Runx1/AML1 (114). 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.

2.2 Materials and methods

2.2.1 Cell lines

Early passages of the OCI/AML-4 cell lines were obtained from Dr. M.D. Minden, University Health Network, Toronto, Canada. U937 and 32Dcl3 cells were purchased from ATCC (Manassas, VA). The 293GPG retroviral packaging cell line was a gift of Richard Mulligan, Harvard University. Culture conditions and methods for growth factor dropout experiments are described: Early passages of the OCI/AML-4 cell lines (obtained from Dr. M.D. Minden, University Health Network, Toronto, Canada) were cultured on an irradiated OP9 feeder layer in IMDM with $\alpha$-thioglycerol, 5% v/v fetal bovine serum (FBS) and supplemented with recombinant human (rh)G-CSF (50ng/mL), rhIL-11 (30ng/mL), rhFlt3 ligand (30ng/mL), rhGM-CSF (30ng/mL), rhIL-6 (1ng/mL), 3% v/v conditioned medium of Chinese hamster ovary (CHO) cells transfected with a vector expressing murine c-kit ligand (KL-CM) (D. Donaldson, Genetics Institute, Cambridge, MA), insulin (10µg/mL), transferrin (5µg/mL), and 0.5% v/v bovine serum albumin (BSA). Cultures were passaged twice weekly to maintain a density of 2 x 10$^5$ cells/mL. To limit selection for highly proliferative subclones through continuous passaging, cultures were discarded after approximately three months of passage. U937 cells were purchased
from ATCC and grown in RPMI supplemented with 10% FBS. 32Dc13 cells were purchased from ATCC and grown in RPMI with 1ng/mL of rmIL-3. The 293GPG retroviral packaging cell line (a gift of Richard Mulligan, Harvard University) was grown in DMEM medium supplemented with 10% FBS, tetracycline (1 mg/mL), G418 (0.3mg/mL) and puromycin (2 mg/mL).

2.2.2 Growth factor drop out experiments

Growth factor requirements of the OCI/AML-4 cell line was established at either 14 days (Figure 2.1a) or 23 days (Figure 2.1B) by assessing proliferative ability following growth factor drop-out. All experimental groups were cultured without a feeder layer in Iscove’s modified Dulbecco’s medium plus 5ug/mL transferrin, 0.5% bovine serum albumin, 10ug/mL insulin, monothioglycerol and 5% fetal bovine serum. Positive controls (the first bar in the graph) were additionally supplemented with recombinant human (rh)G-CSF, murine c-kit ligand, rhIL-7, rhIL-11, rhFlt-3 ligand, rhIL-6 or rhGM-CSF. From this starting point, growth factors were individually deleted. Cells were seeded at a density of 30 cells per well in 100 µL of appropriate medium and scored for growth after either 14 days (A) or 23 days (B). Viable collections containing a minimum of 120 cells were scored positive. Each experimental group contained 48 replicates.

2.2.3 Limiting dilution analysis

The relative number of clonogenic cells in the OCI/AML-4 cell populations was inferred by culturing cells at limiting dilutions and scoring colony growth. Cells were seeded at limiting dilutions in liquid cultures on top of a feeder layer, OP-9 and each well was monitored for growth weekly under a light microscope. The frequency of cells able to continuously proliferate for 3 and 6 weeks, respectively, was determined from the percentage of wells negative for growth at those respective time points using Poisson statistics (115).
2.2.4 Subclones

Subclones were generated from early passages of OCI-AML4 cells by plating cells at limiting dilution in culture conditions described. While 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 (CI) 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/iscoveland/homebrew.html (116).

2.2.5 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 hours (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. One hundred and fifteen OCI/AML-4 clones were analyzed.

2.2.6 Single cell global RT-PCR

Clusters of four cells were identified and three clonal siblings were plated in individual wells under standard culture conditions, while the fourth cell was taken for global amplification of its mRNA. Global single cell RT-PCR was performed as previously described (112). The integrity of cDNAs generated by the global single cell RT-PCR was validated by hybridization to the L27 housekeeping probe (Figure 2.3c).
2.2.7 Microarray analysis

Labeling of cDNA was conducted using aminoallyl-dUTP as described (112). Labeled targets were hybridized to 1.7K cDNA microarrays obtained from the University Health Network Microarray Center (http://www.microarray.ca). Microarrays were processed as described (112). The primary analytic approach is described: Microarray scans were inspected to ensure the quality of individual hybridization spots. Spots that displayed defects which would lead to miscalculation of the intensity ratio were flagged and excluded from further analysis. The fluorescence intensity for each fluorophore was set as the median intensity of the pixels enclosed in the perimeter of a spot, minus the median intensity of the background of that hybridization spot. Hybridization spots were normalized regionally. A lower threshold was established for each microarray that would exclude 90% of spots containing saline sodium citrate (SSC) buffer, PCR buffer or Arabidopsis cDNA. Genes determined to be differentially expressed satisfied two conditions. First, the normalized intensity ratio of the gene had to have a coefficient of variation (standard deviation/mean) less than 0.20 over a minimum of three replicates. Second, the normalized intensity ratio had to be a minimum of three standard deviations (SD) greater or less than 1.0.

2.2.8 Confusion matrix

Our analysis included only those samples where three or more of the cells showed similar cell fates since we decided that a stipulation of the assay is that cDNA would only be kept if the three other clonal siblings all displayed the same biological outcome. True positives (TP) were determined by multiplying the number of cells per clone by the number of clones in which all four sibling cells grew. True negatives (TN) were determined by multiplying the number of cells per clone by the number of clones in which all 4 sibling cells failed to grow. False positives (FP) were determined from the number of clones in which all but one cell failed to grow, divided by four since there was only a one in four chance the cell which failed to grow was selected for PCR. False negatives (FN) were determined from the number of clones in which all but one cell grew, divided by four since there was only a one in four chance the cell that grew would have been selected for PCR. Using the data generated in Figure 2.2c, 112 clones of four cells were
analyzed giving a potential 417 tests (numbers do not add up to 448 because clones with mixed fate had only a one in four chance of theoretically being selected for analysis).

2.2.9 Real-time PCR

RNA was isolated from 1 x10^6 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) and analyzed using the delta-delta CT method. Primer sequences are as follows:

Human EAR-2:

Fwd: 5’-TCTCCAGCTGTCTTCTCATGC-3’
Revs: 5’-CCAGTTGAAGGTACTCCCCG-3’

Human GAPDH:

Fwd: 5’-GGCCTCCAAGGAGTAAGACC -3’
Revs: 5’-AGGGGTCTACATGGCAACTG-3’.

3’ end Mus EAR-2:

Fwd: 5’-CCTGGCAGACCTTCA ACAG -3’
Revs: 5’-GATCCTCCTGGCCCATAGT -3’

3’ end Mus L32:

Fwd: 5’-GCCATCAGACCTTCA ACAG -3’
Revs: 5’-AAACATGCACACAAGCCATC -3’
2.2.10 Induction and assessment of differentiation

Differentiation was induced in U937 cells by treatment with 10nM of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma-Aldrich, St. Louis, MO), 1µM all-trans retinoic acid (ATRA) (Sigma-Aldrich), or 1.25% v/v DMSO (Sigma-Aldrich) respectively. Differentiation was induced in the 32Dcl3 cell line by washing cells twice with PBS and incubation with 10 ng/mL G-CSF. Immunostaining for the maturation marker CD11b (eBioscience, San Diego, CA) was performed for twenty minutes in the dark according to manufacturer’s instructions and cells were analyzed by flow cytometry. Nitroblue tetrazolium (NBT) 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 time point was conducted in triplicate.

2.2.11 Culture of hCG-NuMA-RARα transgenic bone marrow

Bone marrow obtained from hCG-NuMA-RARα transgenic mice (117) was cultured with or without 1µM of ATRA in DMEM containing 10% FBS, 2 mM L-Glutamine, and 1% penicillin/streptomycin, supplemented with 10 ng/mL 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.

2.2.12 Generation of retroviruses

Human EAR-2 cDNA (a gift from John Ladias, Harvard University) or Mus EAR-2 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 (118).
2.2.13 Antibodies for immunoblotting

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

2.2.14 Cell cycle analysis and growth kinetics

Analysis of DNA content was performed by staining cells with PI (Sigma-Aldrich) as described (118). Growth kinetics were assessed by counting cells every 12 hours using trypan blue and a Neumeber Counter.

2.2.15 Generation of shRNA

Oligonucleotides targeting human or mouse EAR-2 were synthesized (Sigma-Genosys, Oakville, ON Canada), annealed and cloned into the pSiren vector (Clonetech, Mountain View CA), after which 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.

Sense shRNA hairpin sequences were as follows:

**mus shEAR-2.1**

5’- GAT CCG CAT TAC GGC GTG TTC ACC TTC AAG AGA GGT GAA CAC GCC GTA ATG CTT TTT TCT AGA G 3’

**mus shEAR-2.2**

5’ –GAT CCG CAA CCG TGA CTG GAT TAA GTT CTC TAA TCT GAC AGT CAC GGT TGT TTT TTC TAG AG-3’
mus shEAR-2.3

5’- GAT CCG TGT CCG AGC TGA TTG CGC ATT CAA GAG ATG CGC AAT CAG CTC GGA CAT TTT TTC TAG AG-3’

human shEAR-2.1

5’-GAT CCG CAT TAC GGT GTC TTC ACC TTC AAG AGA GGT GAA GAC ACC GTA ATG CTT TTT TCT AGA G-3’

human shEAR-2.2

5’-GAT CCG CCT CTG GAC ACG TAA CCT ATT CAA GAG ATA GGT TAC GTG TCC AGA GGT TTT TTC TAG AG-3’

2.3 Results

2.3.1 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 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 2.1a). 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 three-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 2.1b). GM-CSF or c-kit ligand
Figure 2.1 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 SEM. * p<0.05 and ** p<0.01. (c) Early passages of the OCI/AML-4 were analysed 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.
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 2.1c). These data suggest that the cell line is clonal and that differences in the proliferative ability of individual cells are not due to genetic differences, or to the presence of multiple clones.

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 2.1d). This heterogeneity in proliferative ability is preserved in subclones of OCI/1AML4, 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 2.1). 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.
### Table 2.1 Genes expressed more abundantly in AML-4 cells with limited proliferative ability (0-7 cells or less than 3 consecutive cell divisions)

<table>
<thead>
<tr>
<th>Clonal Sibling</th>
<th>Week 3</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>1.80%</td>
<td>0.64%</td>
</tr>
<tr>
<td>Subclone 10C</td>
<td>6.10%</td>
<td>3.32%</td>
</tr>
<tr>
<td>Subclone 12E</td>
<td>2.50%</td>
<td>0.93%</td>
</tr>
<tr>
<td>Subclone G10</td>
<td>2.10%</td>
<td>0.53%</td>
</tr>
<tr>
<td>Subclone G3</td>
<td>2.90%</td>
<td>1.12%</td>
</tr>
<tr>
<td>Late Passaged</td>
<td>83.10%</td>
<td>80.90%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frequency</th>
<th>95% CI</th>
<th>Frequency</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3-2.6%</td>
<td>0.64% 0.45-0.91%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.2-8.9%</td>
<td>3.32% 2.3-4.80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.7-3.5%</td>
<td>0.93% 0.66-1.31%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4-2.9%</td>
<td>0.53% 0.6-1.20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0-4.1%</td>
<td>1.12% 0.78-1.58%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>63.1-109.5%</td>
<td>80.90% 61.4-106.5%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 2.3.2 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 2.2a). 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 2.2b), 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
Figure 2.2  OCI/AML4 cells are organized as a hierarchy. (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 stochastical 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).
microcultures at limiting dilution. From clones consisting of 4 cells (n=115 clones), single sister cells were micromanipulated into individual wells (Figure 2.2b). 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 2.2c). 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 2.2) based on the growth probabilities of the clonal siblings in Figure 2.2c. 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.

<table>
<thead>
<tr>
<th>Test predicted outcome</th>
<th>Actual outcome</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grow</td>
<td>Did not grow</td>
<td></td>
</tr>
<tr>
<td>Grow</td>
<td>TP= 108</td>
<td>FP=5</td>
<td></td>
</tr>
<tr>
<td>Did not grow</td>
<td>FN= 4</td>
<td>TN=304</td>
<td>PPV, 95.6%</td>
</tr>
<tr>
<td></td>
<td>Sensitivity,</td>
<td>Specificity,</td>
<td>Accuracy, 97.8%</td>
</tr>
<tr>
<td></td>
<td>96.4%</td>
<td>98.4%</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 Confusion matrix describing the ability of 3 clonal siblings used as biological reporters to predict the proliferative fate of a fourth clonal sibling consumed in the process of global RT-PCR. Abbreviations: TP, true positive; FP, false positive, PPV, positive predictive value; FN, false negative; TN, true negative; NPV, negative predictive value.
2.3.3 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 (119, 120) (Figure 2.3a). 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 microtiter wells containing growth medium and a feeder layer of OP9 cells (121), 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. The integrity of cDNAs generated by global single cell RT-PCR was validated by hybridization to the L27a housekeeping probe (Figure 2.3c). Globally amplified cDNA from single OCI/AML-4 cells whose siblings each generated fewer than 8 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 2.3) while fourteen genes expressed higher in cells with proliferative ability were identified (Table 2.4).
<table>
<thead>
<tr>
<th>Accession</th>
<th>Full name</th>
<th>Mean Fold Increase</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA194054</td>
<td>28.3 KD protein C21ORF2</td>
<td>4.6</td>
<td>1.3</td>
<td>0.16</td>
</tr>
<tr>
<td>AA122236</td>
<td>F-actin capping protein</td>
<td>4.9</td>
<td>1.2</td>
<td>0.10</td>
</tr>
<tr>
<td>AA193614</td>
<td>Guanylate cyclase</td>
<td>7</td>
<td>1.1</td>
<td>0.07</td>
</tr>
<tr>
<td>AA018737</td>
<td>PTB-associated splicing factor</td>
<td>7</td>
<td>1.2</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Maltate oxidoreductase (NAD), mitochondrial precursor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H19387</td>
<td></td>
<td>8.6</td>
<td>1.3</td>
<td>0.12</td>
</tr>
<tr>
<td>W61100</td>
<td>BCL-2</td>
<td>9.2</td>
<td>1.5</td>
<td>0.17</td>
</tr>
<tr>
<td>H59171</td>
<td>SEC23 homolog</td>
<td>9.8</td>
<td>1.6</td>
<td>0.20</td>
</tr>
<tr>
<td>N42900</td>
<td>TFIID</td>
<td>13</td>
<td>1.6</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Table 2.3 Genes expressed more abundantly in AML-4 cells with limited proliferative ability (less than 3 consecutive cell divisions)
<table>
<thead>
<tr>
<th>Accession</th>
<th>Full name</th>
<th>Mean Fold Increase</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>H10327</td>
<td>RAS-related protein RAP-1B</td>
<td>4.7</td>
<td>1.3</td>
<td>0.17</td>
</tr>
<tr>
<td>W45621</td>
<td>V-erb A related protein EAR-2 (NR2F6)</td>
<td>4.7</td>
<td>1.3</td>
<td>0.19</td>
</tr>
<tr>
<td>W52609</td>
<td>Glutaredoxin (Thioltransferase)</td>
<td>5.2</td>
<td>1.2</td>
<td>0.13</td>
</tr>
<tr>
<td>AA055945</td>
<td>T cell surface glycoprotein CD3 delta chain precursor</td>
<td>5.5</td>
<td>1.3</td>
<td>0.13</td>
</tr>
<tr>
<td>H57284</td>
<td>NEDD5 protein homolog (KIAA0158)</td>
<td>5.5</td>
<td>1.1</td>
<td>0.06</td>
</tr>
<tr>
<td>W78946</td>
<td>Electron transfer flavoprotein alpha-subunit precursor</td>
<td>5.7</td>
<td>1.4</td>
<td>0.18</td>
</tr>
<tr>
<td>W00728</td>
<td>Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1</td>
<td>5.9</td>
<td>1.2</td>
<td>0.09</td>
</tr>
<tr>
<td>R99156</td>
<td>Cyclin-dependent kinases regulatory subunit 1 (CKS-1)</td>
<td>6</td>
<td>1.1</td>
<td>0.03</td>
</tr>
<tr>
<td>W92066</td>
<td>Activator 1 37 kD subunit</td>
<td>7.6</td>
<td>1.4</td>
<td>0.16</td>
</tr>
<tr>
<td>W92260</td>
<td>RAS-related protein RAB-11A</td>
<td>8.2</td>
<td>1.4</td>
<td>0.15</td>
</tr>
<tr>
<td>R92367</td>
<td>Transcription factor ATF-A and ATF-A-delta.</td>
<td>8.3</td>
<td>1.2</td>
<td>0.10</td>
</tr>
<tr>
<td>W21228</td>
<td>Ubiquinol-cytochrome C reductase complex core protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R34413</td>
<td>Poly (ADP-ribose) polymerase</td>
<td>10.7</td>
<td>1.3</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 2.4 Genes expressed more abundantly in AML-4 cells with proliferative ability (3-6 successive divisions)
2.3.4 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 2.3d). EAR-2 is a homologue 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. Since 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 2.4a). We validated our observations in primary samples using short-term *ex vivo* cultures of bone marrow from the hCG-NuMA-RARα 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 all trans retinoic acid (ATRA). EAR-2 expression correlated with stage of maturation in these animals: Expression of EAR-2 mRNA was higher in hCG-NuMA-RARα cells than in wild-type control (Figure 2.4b). A marked decline in EAR-2 expression occurred in hCG-NuMA-RARα 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 q-PCR to measure EAR-2 transcript levels in a graded series of multipotent, oligopotent, and unipotent murine hematopoietic cells (122-124). 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 2.4c). 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.
Figure 2.3 Identification of EAR-2 by clonal sibling analysis. (a) Clonal siblings were generated, one member of a tetrad of cells is harvested for mRNA, while its three clonal siblings serve as reporters of clonogenicity. (b) Ethidium bromide gels of single cell global RT-PCR. Lanes1-5: 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 nonproliferative (NP) OCI/AML4 cells. NTC: no template control.
Figure 2.4 EAR-2 expression correlates with differentiation. (a) Q-PCR analysis of EAR-2 mRNA expression in U937 cells induced to differentiate with atRA (triangles), DMSO (rectangles) or 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 (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.
2.3.5 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 2.5a). U937 cells overexpressing EAR-2 proliferated at a faster rate than control cells (Figure 2.5b). Upon induction of differentiation, we observed that unregulated expression of EAR-2 abrogates both the growth arrest (Figure 2.5c) and the shift of cells from S/G2/M to G0/G1 (Figure 2.5d) that follows ATRA-induced differentiation.

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

U937 cells are induced to undergo differentiation – characterized by growth arrest, expression of cell surface CD11b, and development of oxidative burst capability – 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 hours exposure to 1 mM ATRA U937-EAR-2 cells fail to express CD11b (Figure 2.6a), and fail to acquire the ability to reduce nitroblue tetrazolium (NBT), a functional index of leukocyte maturity (Figure 2.6b, c). Similarly, cells of the mouse 32Dcl3 cell line can be induced to undergo granulocytic differentiation when cultured in the presence of granulocyte colony stimulating factor (G-CSF). Unregulated expression of EAR-2 blocks the induction of cell surface CD11b expression (Figure 2.6d) and the morphological changes (Figure 2.6e) that accompany G-CSF induced differentiation of 32Dcl3 cells. We note that expression of CD11b actually decreases when EAR-2 is overexpressed. This is due to the biology of the 32Dcl3 line – the cells exhibit limited differentiation while being maintained in IL3 and then undergo neutrophil maturation when given G-CSF. EAR-2 blocks
Figure 2.5 Overexpression of EAR-2 increases proliferation. (a) EAR-2 protein expression in U937 and 32Dcl3 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 ± SEM). (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 ± SEM). (d, e) Cell cycle analysis of U937-GFP and U937-EAR-2.
Figure 2.6 Overexpression of EAR-2 inhibits differentiation. (a) CD11b expression induced by atRA treatment in U937-GFP and U937-EAR-2. (b, c) NBT assay for functional maturity in atRA-treated U937-GFP and U937-EAR-2. Data are represented as mean ± SEM. (d) CD11b expression in 32D-GFP and 32D-EAR-2 grown in G-CSF for 96h. (e) Morphological maturation in cells from (d) as stained with May-Grunewald Giemsa. Scale bar = 20μm.
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 32Dcl3 cell lines to proceed.

2.3.7 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, i.e. EAR-2 acts to preserve the undifferentiated state of early hematopoietic cells. However, overexpression of nuclear receptors can result in off-target effects due e.g. to cofactor sequestration. We therefore used short hairpin RNA constructs to silence EAR-2 expression in 32Dcl3 (Figure 2.7a) and U937 cells (Figure 2.7d). For both cell lines silencing of EAR-2 expression resulted in spontaneous morphologic differentiation (Figure 2.7b and 2.7e) and upregulation of cell-surface CD11b expression (Figure 2.7c and 2.7f). In U937 cells these manifestations of differentiation were accompanied by induction of apoptosis (Figure 2.7g).

2.4 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 2.2c) and that any given sibling in the clone can serve as a biological reporter for their rest of the sibling cells (Table 2.2) 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 four-fold more abundant in AML cells with growth potential than in cells lacking growth potential (Figure 2.3d). To validate the
Figure 2.7 Knockdown of EAR-2 induces spontaneous differentiation. (a) Relative EAR-2 protein levels as determined by densitometry of immunoblots in 32D cells transduced with three EAR-2 shRNA constructs or scrambled control (scrm). Data are represented as mean ± SD. (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 ± SD. (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 = 20µm.
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 2.4c). 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 2.6), while knockdown of EAR-2 expression results in terminal differentiation and apoptosis (Figure 2.7).

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 2.1d). 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 2.1c). Furthermore, we show that the heterogeneity present in the parental lines is transferred to subcloned lines (Table 2.1). 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.

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, while 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 since 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, while in the hierarchy model clonal siblings occupy similar positions in the leukemia cell hierarchy, and are expected to have highly correlated proliferative outcomes (Figure 2.2a). Our data show that clonal siblings have similar growth abilities (Figure 2.2c), 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 (125) is a mammalian homologue of the *Drosophila* gene *seven-up*, which plays a role in neuroblast and retinal cell fate decisions (113, 126-129). EAR-2 deficient mice (130) are viable and fertile, but show agenesis of the locus coeruleus, a midbrain nucleus that regulates circadian behaviour 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 (114) and from the observation that EAR-2 antagonizes activation of Th17 CD4+ T cells (131). 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 2.4c); 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 (132). 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-α (RXR-α) (133), 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 RXRα function by EAR-2 accounts entirely for the EAR-2 overexpression phenotype since targeted deletion of RXRα 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 since 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 (134), and mutations in the human homologue AML1 are seen commonly in both AML and MDS (135, 136). 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 2.7). 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” – agonist 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.
3 EAR-2 is a novel regulator of hematopoietic differentiation and hematopoietic stem cell homeostasis

Chapter 3 has been submitted to The Journal of Experimental Medicine as follows:

3.1 Introduction

The cancer stem cell (CSC) model proposes that each cancer consists of a small population of cells capable of unlimited growth and self-renewal, known as CSCs, and a much larger population of cells, descendants of the CSCs, that have lost self-renewal capacity and are undergoing terminal differentiation. Evidence supporting this model has been reported for several malignancies including acute myelogenous leukemia (AML) (109), brain cancer (59, 137), colon (138), ovary (139) and breast cancer (140). The CSC model has important implications for cancer therapy; eradication of CSCs, the cells responsible for maintenance of the neoplasm, would be necessary to achieve cure (141). Thus, identification of genes or proteins involved in the origin or maintenance of CSCs is of tremendous interest. Oncogenes have important cellular functions in normal cells, hence, an understanding of the function of molecules that regulate the clonogenicity of leukemia cells in normal hematopoietic cells is essential to the development of therapies that target the leukemia stem cell.

Hematopoiesis is orchestrated through a series of cell fate decisions, commencing with the decision of the hematopoietic stem cell to choose between self-renewal and entering a program of differentiation in which pluripotent ability is progressively restricted en route to the maturation of a progenitor cell that is committed to a single hematopoietic lineage. The developmental cascade essentially consists of a series of cell fate decisions at points of lineage bifurcation, as the cell must decide which developmental fork to pursue. The molecular events that govern these decisions are of great interest.

Discovery of genes that code for proliferative ability of CSCs remains technically challenging due in large part to the difficulty of prospectively isolating homogeneous populations of human CSCs. We previously reported using a novel strategy that compares the transcriptional profiles of proliferative and non-proliferative leukemia single cells that mRNA transcripts of the orphan nuclear receptor EAR-2 are more abundant in AML cells with growth potential than in cells lacking growth potential (142). In normal mouse hematopoietic tissue EAR-2 expression is highest in long-term repopulating HSCs, declining with differentiation,
while in human specimens expression of EAR-2 is increased in the BM of patients with AML and myelodysplastic syndrome (MDS) compared to normal BM. Conversely, silencing of EAR-2 expression in AML cell lines results in terminal differentiation and apoptosis.

EAR-2 is an orphan nuclear receptor that was cloned in a search for homologues of the retroviral oncogene v-erbA using low stringency hybridization (125). EAR-2 is a mammalian homologue of the Drosophila gene seven-up, which plays a role in neuroblast and retinal cell fate decision, but the function of EAR-2 in mammalian development has not been well characterized. EAR-2 deficient mice are viable and fertile, but show agenesis of the locus coeruleus, a region of the midbrain that regulates circadian behaviour and nociception (130). In situ mRNA hybridization in EAR-2−/− animals places EAR-2 downstream of Mash1 and upstream of Phox2a and Phox2b in the specification of the locus coeruleus. Although EAR-2 expression is seen outside the central nervous system, this report contains no description of any phenotypic analysis outside the nervous system; in particular, hematopoiesis was not characterized. A later report (131) showed that EAR-2 antagonizes activation of Th17 CD4+ T cells and that EAR-2-null mice have hyperreactive lymphocytes and are hypersusceptible to Th17-dependent experimental autoimmune encephalomyelitis.

Indirect evidence suggestive of a role for EAR-2 in hematopoiesis comes from studies of its interaction with the key hematopoietic transcription factor Runx1. Targeted deletion of Runx1, a component of the core binding factor complex, results in abrogation of definitive hematopoiesis and embryonic lethality (134, 143) and Runx1 point mutations and rearrangements are seen commonly in MDS (136) and acute leukemia (144). EAR-2 interacts physically with Runx1 and represses its transcriptional activating ability in the murine myeloblast cell line 32Dcl3 (114). EAR-2 is overexpressed in samples from patients with MDS and AML (142) as well as colon cancer (145).

We postulated that EAR-2 is a key regulator of hematopoietic cell fate decisions in the developmental hierarchy of hematopoietic differentiation. We surmised that overexpression of EAR-2 in vivo would inhibit hematopoietic differentiation, and would result in leukemia. We used a retroviral construct to express EAR-2 exogenously in normal murine bone marrow cells. Unregulated expression of EAR-2 in bone marrow cells resulted in impairment of differentiation and extended proliferative capacity in vitro, while in vivo EAR-2 caused expansion of the stem
cell population, followed by the development of a preleukemic state resembling MDS that culminated in AML. The effects of EAR-2 on differentiation appear to be mediated by regulation of gene expression in a DNA-binding dependent fashion and at least in part by histone acetylation. We observed a decrease in expression of HOX genes and increase in expression of the self-renewal gene ZFX. Our study is the first to establish that the orphan nuclear receptor EAR-2 is a regulator of hematopoietic cell differentiation, controlling lineage commitment and hematopoietic stem cell homeostasis, and that unregulated expression of EAR-2 \textit{in vivo} results in AML.

### 3.2 Materials & methods

#### 3.2.1 Generation of retroviruses

The 293GPG retroviral packaging cell line (a gift of Richard Mulligan, Harvard University) was grown in DMEM medium supplemented with 10% FBS, tetracycline (1 mg/mL), G418 (0.3mg/mL) and puromycin (2 mg/mL). Human EAR-2 cDNA (a kind gift from John Ladias, Harvard University) or \textit{Mus} EAR-2 cDNA (a kind 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 murine myeloproliferative (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 (146).

#### 3.2.2 Antibodies for immunoblotting

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

RNA was isolated from 1 x10^6 cells using Trizol reagent (Invitrogen) and first strand cDNA was synthesized using SuperScript reverse transcriptase (Qiagen) 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 ) and analyzed using the delta-delta CT method (147).

3.2.4 Bone marrow transduction

Using the retroviral constructs described above, we forced expression of EAR-2 in primary murine BM cells and monitored the effects on differentiation using colony assays. Donor 12-week old C57Bl/6 mice were given 5 fluorouracil, 150 μg/g body mass, by intraperitoneal injection and humanely sacrificed ninety-six hours later. Bone marrow was collected from femurs and tibiae and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with fetal bovine serum (5%), c-Kit ligand conditioned medium (3%), Flt-3 ligand (30 ng/mL), and TPO (30 ng/mL), conditions that minimize differentiation but initiate cycling of long-term repopulating cells. After 24 hours of culture, the cells were infected with MMP-GFP or MMP-EAR-2 retroviral supernatant at a multiplicity of infection (MOI) of 100. Forty-eight hours after retroviral infection GFP-positive cells were collected by fluorescence activated cell sorting (FACS). We have shown that our transduction procedure preserves long-term multilineage potential by observing multilineage hematopoiesis at 12 months (data not shown).

3.2.5 Methylcellulose colonies:

Bone marrow cells were collected from C57Bl/6 mice 72 hour after intraperitoneal injection of 5-fluorouracil and were infected ex vivo either with retrovirus to overexpress EAR-2 or lentiviral particles to knock the gene down. GFP-positive cells were purified by fluorescence activated cell sorting (FACS) and cultured in methylcellulose medium containing hematopoietic growth factors that favored multilineage differentiation.
Following bone marrow transduction with MMP-GFP or MMP-EAR-2 GFP positive cells were collected by FACS and plated in methylcellulose medium (Methocult GF 3434, Stem Cell Technologies). Colony formation was evaluated after 10-14 days; clusters containing more than 30 cells were scored as a colony. Secondary colony formation was tested by harvesting entire primary colony cultures, washing the cells two times with PBS, and plating 10,000 cells in methylcellulose a second time. Secondary colonies were enumerated 12-14 days following secondary plating.

We confirmed using fluorescent microscopy that all colonies continued to maintain transgene expression.

### 3.2.6 Ex vivo suspension culture

Following transduction of mouse bone marrow with MMP-GFP or MMP-EAR-2, cells were placed unsorted into IMDM with 5% FBS, 10% v/v IL-3 conditioned medium from WEHI-3 cells, 1ng/mL IL-6 and 3% v/v c-kit ligand conditioned medium. Following ten days of culture the cells were washed twice with PBS, stained with either fluorescently labeled c-kit or with fluorescently labeled CD11b and GR-1, and analyzed by flow cytometry.

### 3.2.7 Hematopoietic stem cell transplants

We generated bone marrow transplant recipients that received either chimeric EAR-2 or GFP transduced grafts or grafts that contained 100% bone marrow cells sorted for expression of GFP (and hence the transgene). To generate recipients transplanted with bone marrow grafts containing a mixture of transduced and wild-type cells 5FU-primed C57Bl/6 bone marrow cells were transduced with either MMP-GFP or MMP-EAR-2 as described above. Cells were then sorted by FACS. Grafts contained a mixture of transduced (GFP or EAR-2; 2.5x10⁴ cells) and mock-transduced (7.5x10⁴ cells) donor cells. Primary chimeric transplants were performed as described. In some experiments chimeric transplant recipients were harvest at 4-6 weeks post transplant for analysis, and bone marrow was transplanted into another lethally irradiated mouse.
by tail-vein injection. Secondary recipients of chimeric bone marrow were harvested at either early time points 4-6 weeks or at late time points 12-16 weeks. To generate recipients transplanted with bone marrow grafts containing 100% transduced bone marrow cells 5FU-primed C57Bl/6 bone marrow cells were transduced with either MMP-GFP or MMP-EAR-2 as described above. Cells were then sorted by FACS and introduced into recipient mice by tail vein injection at a dosage of between $4 \times 10^4$ and $1 \times 10^5$ cells per recipient. All recipients of a given cohort received the same graft size. Recipient C57Bl/6 mice were irradiated in a Cs-137 small animal irradiator with 900 cGy, a dose we previously established to be lethal. For the competitive transplant experiment primary grafts were prepared as described above except the transduced bone marrow was not sorted rather the percentage of marked cells was determined based on expression of GFP using flow cytometry.

3.2.8 Histological sections and cytospins

Immediately following sacrifice of animals tissues were rinsed in PBS and fixed for 24 hours in buffered formalin before being given to the Sunnybrook Research Institute Histology facility for paraffin embedding, slicing and staining with hematoxylin and eosin. Bone tissues were decalcified following fixation before further processing. Cytospins were prepared by centrifuging single celled suspensions onto glass slides using a Shandon cytocentrifuge. Cytospins were air dried, and fixed in methanol before staining with May-Gruwald and Giemsa stains. Cytospins were coverslipped following treatment with a toluene-based synthetic resin mounting medium.

3.2.9 Peripheral blood counts

Blood from bone marrow transplant recipients was obtained at 4 weeks post-transplant by saphenous vein puncture. Alternatively, moribund animals were bled by cardiac puncture just prior to death. To give matched data, a GFP control animal was analyzed with every EAR-2 moribund animal analyzed. Blood was collected in a heparinized capillary tube and hematological parameters were acquired on a Hemavet analyzer.
3.2.10 Analysis of hematopoietic stem cell subsets:

For analysis of c-kit+, sca-1+, lineage- (KSL) cells, red blood cell depleted bone marrow cells were stained with a cocktail containing biotin CD3, biotin CD45R/B220 (RA3-6B2), biotin CD11b (M1/70), biotin erythroid marker (TER-119), biotin Ly-6G (RB6-8C5), c-kit APC, sca-1 PE-Cy7 and either CD34 PE or CD49b PE (all eBioscience) in the dark. Bone marrow was washed once and incubated with streptavidin PE-Cy5 for 20 minutes in the dark. Bone marrow was washed twice and analyzed using flow cytometry on a Becton Dickinson LSR II. All samples analyzed were gated based on FSC/SSC and GFP+ cells. The population of KSL cells is highly enriched for hematopoietic stem cell activity. This population was analyzed and further subdivided based on the expression of the CD34 and CD49b antigen.

3.2.11 Generation of shRNA

Oligonucleotides targeting human or mouse EAR-2 were synthesized (Sigma), annealed and cloned into the pSiren vector (Clontech), after which sequence was verified (The Centre for Applied Genomics, Toronto). Virus was prepared by transient transfection of plasmid in the 293GPG cell line as described above. Knockdown was confirmed by western blot for these vectors in cell lines and by qPCR in bone marrow cells (data not shown and (142)).

3.2.12 Statistical analysis

Student’s t-tests and one-way ANOVA followed by the Tukey Post-Hoc Test was used to assess statistical significance for normally distributed data. The Mann-Whitney U test or analysis of variance by Kruskal-Wallis test paired with Dunn’s test was used for non-normally distributed data.
3.3 Results

3.3.1 Expression of EAR-2 negatively regulates erythroid and myeloid differentiation of bone marrow cells

We hypothesized that downregulation of EAR-2 is a key step in the process of hematopoietic differentiation, and that unregulated expression of EAR-2 would block differentiation. We tested this hypothesis by manipulating EAR-2 expression in normal murine bone marrow cells in vitro. We first sought to characterize the effect of forced expression of EAR-2 by transducing bone marrow cells with a retrovirus encoding EAR-2. EAR-2-transduced bone marrow cells yielded 42.9% fewer myeloid and 34.6% fewer erythroid colonies in comparison to GFP controls (Fig. 3.1 A). Colonies derived from EAR-2 transduced bone marrow cells were also significantly smaller than controls, showing a 49.4% decrease in the average number of cells per colony (Fig. 3.1 B). EAR-2-overexpressing bone marrow cells maintained in suspension culture containing myeloid growth factors retained an immature immunophenotype, maintaining cell surface expression of CD117 while failing to activate expression of CD11b and Gr-1 (Fig. 3.1 H and I).

Next, we assessed the effect of EAR-2 expression on clonogenicity of hematopoietic cells by means of a replating assay. Surprisingly, while GFP transduced hematopoietic cells exhausted their clonogenic potential after two rounds of replating, serial replating of EAR-2 transduced bone marrow revealed extended clonogenic ability (Fig. 3.1 C). These observations are consistent with the notion that constitutive expression of EAR-2 inhibits differentiation and enhances clonogenicity.

We then assessed the effects of silencing of EAR-2 expression on colony formation in vitro. While silencing of EAR-2 did not significantly reduce the number of colony forming units (Fig. 3.1 D), it did significantly increase the colony size (Fig. 3.1 E, Fig. 3.1 F and Fig. 3.2 B). Next, we tested whether silencing of EAR-2 reduced the clonogenicity of bone marrow cells. In replating experiments we observed a significant decrease in secondary colonies in cells in which EAR-2 expression was silenced (Fig. 1G). Taken together, these results show that exogenous expression
Figure 3.1 EAR-2 affects hematopoietic differentiation *ex vivo*. A) Overexpression of EAR-2 decreases the number of colonies and B) colony size but C) increases the replating capacity. D) Knock down of EAR-2 does not affect colony number but drastically increases colony size (E) under multilineage conditions and (F) erythroid specific condition. G) Knockdown of EAR-2 decreases secondary replating ability. H,I) Differentiation in suspension culture.
Figure 3.2 EAR-2 expression inhibits differentiation. A) EAR-2 overexpression inhibits the growth of progenitor cells in methylcellulose culture in mice of both the C57BL/6 and Balb/c strain. B) Colonies from murine bone marrow in which EAR-2 was knocked down in were significantly larger, and contained more cells. In fact they were so large that the medium turned acidic. C, D) EAR-2 overexpression reduces the number & size of CFU-S colonies. The photo shows spleens taken from animals sacrificed at day 12, injected with an inoculum of 100K cells. Note, this is consistent with the notion that EAR-2 inhibits differentiation and preserves HSC function as a CFU-S cell needs to differentiate in order to form a CFU-S colony.
of EAR-2 blocks myeloid maturation and augments clonogenicity of hematopoietic cells, and support the idea that EAR-2 is a negative regulator of hematopoietic differentiation in vitro.

3.3.2 EAR-2 transplant chimeras have perturbed hematopoietic differentiation and dysplastic hematopoiesis

To investigate the effects of exogenous expression of EAR-2 on hematopoiesis in vivo, we performed adoptive transfer of EAR-2-transduced hematopoietic cells into lethally irradiated syngeneic recipients in a competitive repopulation model. Early engraftment was evaluated in 16 recipients at 4 weeks post-BMT. With the exception of 2 recipients in which engraftment failed, we observed an increase in the proportion of GFP+ bone marrow cells in EAR-2 recipients (Fig. 3.3 A) but not in GFP controls, suggesting that EAR-2-transduced cells had a competitive advantage over non-transduced cells in repopulation. Correspondingly, EAR-2-transduced cells recovered from recipients had a striking increase in replating ability relative to GFP-transduced cells (Fig. 3.3 B) and there was an increase in overall bone marrow cellularity in the EAR-2 chimeras, a difference that was more marked at 12 weeks post-BMT (Fig. 3.3 C).

To assess the role of EAR-2 on hematopoietic lineage specification and differentiation in vivo we analyzed the composition of recipient bone marrow using flow cytometry. We observed a significant increase in the proportion of B cells (B220+ cells) (Fig. 3.3 D and E), as well as a significant decrease in the proportion of granulocytes (CD11b+, GR-1+ cells) in the GFP portion of bone marrow in recipients that overexpressed EAR-2 (Fig. 3.3 D, E and F).

Histological examination showed bone marrow hypercellularity and distortion of splenic architecture, with expansion of myeloid cells in the germinal centre regions (Fig. 3.3 G). The cytomorphological appearance of bone marrow cells was abnormal, with numerous dysplastic erythroid precursors (Fig. 3.3 H). Accordingly, we observed anemia in EAR-2 BMT recipients 12 weeks post-transplant (Table 3.1). In contrast to GFP controls, where clusters of primitive cells were found only in paratrabecular locations, EAR-2 BMT recipients exhibited such clusters also in the intertrabecular region (Fig. 3.3 I) – this phenomenon, known as abnormal localization of immature precursors (ALIP), is seen in myelodysplastic syndrome, where it is a predictor of
aggressive disease behavior (148). Consistent with this EAR-2 recipients also had a higher percentage of morphologically primitive cells (myeloblasts and promyelocytes) compared to controls (Fig. 3.3 J) and some recipients, after a variable latency, developed acute leukemia (Fig. 3.3 K and L). These data are consistent with a model in which EAR-2 perturbs hematopoietic differentiation of various lineages.

We then performed secondary transplantation in which \(1 \times 10^6\) unfractionated bone marrow cells were transferred 12 weeks after initial transplantation from primary recipients into a second lethally irradiated host. All secondary recipients (two independent experiments, \(n=5\); from 3 separate primary recipient donors and \(n=10\), from 10 separate primary recipient donors) developed acute leukemia (Fig. 3.3 M) and became moribund within 4 weeks of transplant. These data confirm that the leukemia induced by EAR-2 overexpression is transplantable, and therefore contains leukemia stem cells.

<table>
<thead>
<tr>
<th>4 weeks post-BMT</th>
<th>12 weeks post-BMT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hgb (g/L)</strong></td>
<td><strong>PLT (10^9/L)</strong></td>
</tr>
<tr>
<td><strong>GFP (n=7)</strong></td>
<td><strong>EAR-2 (n=8)</strong></td>
</tr>
<tr>
<td>135.0 (125-144.5)</td>
<td>134.6 (132.8-141.7)</td>
</tr>
<tr>
<td>507.4 (334-567)</td>
<td>517.5 (478-562.3)</td>
</tr>
<tr>
<td>3.9 (1.9-5.3)</td>
<td>3.1 (2.3-3.6)</td>
</tr>
</tbody>
</table>

**Table 3.1 Blood Counts of EAR-2 transplant chimeras**

### 3.3.3 EAR-2 initiates acute leukemia

Lethally irradiated C57Bl/6 mice transplanted with bone marrow graft sorted for expression of the EAR-2 transgene developed a rapidly progressing leukemia. In an initial cohort of 12 recipients (6 for each experimental group) we observed ill health early in the recipients of EAR-2-transduced grafts. Whereas the six GFP recipients remained healthy throughout the
Figure 3.3 EAR-2 chimeras develop myeloid dysplasia. A) Overexpression of EAR-2 gives a competitive advantage to bone marrow cells as assessed by competitive repopulation. B) Overexpression of EAR-2 increases the serial replating ability of bone marrow cells. C) Overexpression of EAR-2 increased bone marrow cellularity at 12-weeks post transplant. D, E) Flow cytometry was used to analyse the cellular composition of bone marrow from chimeric animals. Overexpression of EAR-2 increased the proportion of B220+ B-cells, while decreasing CD11b+/GR-1+ granulocytes. F) A flow cytometry dot plot of expression of CD11b+/GR-1+ in representative animals showing that overexpression of EAR-2 drastically decreases granulocyte numbers. G) Histological sections from representative animals showing that overexpression of EAR-2 in transplant chimeras increases both the bone marrow cellularity and the white pulp region of the spleen. H) Erythroid dysplasia observed in cytopsins of bone marrow from EAR-2 overexpressing transplant chimeras. I) Histological sections of bone from EAR-2 transplant chimeras shows abnormal localization of immature precursors (ALIP). J) Transplant chimeras that overexpress EAR-2 have an increase in the percentage of immature blast cells in their bone marrow. K) Survival curve showing that EAR-2 transplant chimeras die of leukemia. L) EAR-2 transplant chimeras that die of leukemia show infiltration of the bone marrow (upper left), spleen (upper right), and liver (lower right) by immature blast cells that are shown in the bone marrow cytopsin (lower left). M) Primary EAR-2 transplant chimeras develop a rapidly progressing leukemia upon secondary transplantation of their bone marrow cells. The immunophenotype of this secondary leukemia was GFP+, CD45lo, CD11b-/GR-1-, c-kitlo, B220-. 
experiment, four of six EAR-2 recipients became moribund within five weeks of transplantation (Fig. 3.4A). The two remaining recipients in the EAR-2 cohort showed no signs of ill health, and were found to have <1% engraftment by GFP-expressing cells when sacrificed 16 weeks post-transplant (not shown). All four sick animals had profound reduction of peripheral blood cell counts (Table 3.2), particularly anemia, which was evident on gross inspection of the blood and the liver in EAR-2 overexpressing animals (Fig. 3.4F and 3.4G). Nevertheless, these animals exhibited bone marrow hypercellularity in comparison to matched controls, with infiltration of the marrow cavity by primitive cells (Fig. 3.4 B and Fig. 3.5). Infiltration by myeloid cells was also seen in the spleen and liver of EAR-2 recipients (Fig. 3.4 C). In EAR-2 recipients, there was enlargement of the spleen with effacement of the normal architecture with infiltration by primitive myeloid cells, in contrast to the small lymphoid cells that comprised the majority of cellularity in the GFP spleens, in which normal follicular architecture was maintained, with well-demarcated white and red pulp (middle lower and upper panels, respectively). Comparison of stained cytospin preparations of bone marrow cells (Fig. 3.4 E) showed an increase in primitive cells in EAR-2 recipients compared to GFP recipients. At higher magnification, these cells were large and had the appearance of blasts with high nucleus:cytoplasm ratio and hypogranular basophilic cytoplasm. Cells of this type were also found in low numbers in the peripheral blood of EAR-2 recipients, but not in GFP recipients. These features are diagnostic of acute leukemia, and establish that EAR-2 is a leukemia oncogene *in vivo*. This experiment was repeated a total of three independent times with similar results.

<table>
<thead>
<tr>
<th></th>
<th>GFP (n=8)</th>
<th>EAR-2 (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb (g/L)</td>
<td>100.9 (81.7-122.3)</td>
<td>55.7 (27.4 - 80.2)</td>
</tr>
<tr>
<td>PLT (10^9/L)</td>
<td>264.4 (167.3-351.8)</td>
<td>77.7 (17.9 - 146.5)</td>
</tr>
<tr>
<td>WBC (10^9/L)</td>
<td>4.8 (2.4-5.8)</td>
<td>2.6 (0.4 - 4.0)</td>
</tr>
</tbody>
</table>

Table 3.2 Blood counts for animals receiving grafts of 100% sorted cells
Figure 3.4 EAR-2 overexpression causes leukemia. A) Animals transplanted with bone marrow grafts in which every cell overexpressed EAR-2 died rapidly of leukemia. B) Bone marrow cellularity of animals transplanted with bone marrow that overexpressed EAR-2 was greatly increased. The morphology of the bone marrow cells is shown (far right panel). C) Histological sections from animals transplanted with bone marrow that overexpresses EAR-2 shows infiltration of the spleen and liver by leukemia blast cells. The morphology of the cells infiltrating the spleen is also shown (middle panel). D) Accordingly, animals transplanted with bone marrow that overexpresses EAR-2 had an increase in the size of their spleens. E) The cellular morphology of EAR-2 leukemia blast cells is depicted here. The image on the right shows bone marrow cells. The image on the left shows infiltration of the peripheral blood by leukemia blast cells. F) Animals transplanted with bone marrow that overexpresses EAR-2 were so anemic that their blood lacked the deep red pigment observed in the control animals (right panel), their livers (middle panel) were also more pale than controls. Nevertheless, these animals had a dramatically increased spleen size.
Figure 3.5: Increased blast leukemia cells in recipients that received bone marrow overexpressing EAR-2. Low magnification view of cytospins of bone marrow cell suspensions from bone marrow transplant recipients of GFP (left) or EAR-2 (lower right) transduced grafts.
3.3.4 EAR-2 perturbs primitive cellular compartments

In these recipients and in a second cohort of 17 recipients (10 EAR-2, 7 GFP), we next performed further characterization of hematopoietic cells by flow cytometry 3-5 weeks after transplantation. Analysis of bone marrow and spleen cells gated on GFP-expressing cells revealed the presence in all EAR-2 recipients of a unique population of cells with high forward scatter and variable side scatter (Fig. 3.6 A). The GFP+ population in EAR-2 recipients showed strikingly higher percentages of c-kit+ cells (median 24.3% in 11 EAR-2 recipients, vs. 7.9% in 7 GFP recipients; p=0.0006, Mann-Whitney U test) (Fig. 3.6 A) and lineage marker negative cells (median 18.7% in 11 EAR-2 recipients, vs. 9.0% in 7 GFP recipients; p=0.0006, Mann-Whitney U test) (Fig. 3.6 B). Primitive cells of the c-kit+, SCA-1+, lineage- (KSL) phenotype were present at similar frequencies in the GFP+ and GFP+ populations in the BM of GFP recipients, and in the GFP- population in EAR-2 recipients; however, we observed a significantly higher KSL frequency in the GFP+ population of EAR-2 recipients (p<0.01; Dunn’s test) (Fig. 3.6 C). Thus, exogenous expression of EAR-2 increases the frequency KSL cells in a cell-autonomous fashion.

The KSL population is itself heterogeneous, containing committed progenitors, short-term repopulation HSCs, and long-term repopulating HSCs. Although functional assays are necessary to distinguish definitively among these cell types, cell surface expression of other markers can provide some information. For example, it has been reported that, in steady state hematopoiesis, long-term repopulating HSCs are contained within the CD49b+KSL and CD34-KSL populations. We therefore examined these populations in recipients of GFP and EAR-2 transduced BM (Fig. 3.6 D). We observed an increase in frequencies of both CD49b+KSL (1.86% of GFP-expressing cells in EAR-2 recipients vs. 0.25% in GFP recipients; n=4) and CD34-KSL (0.89% of GFP-expressing cells in EAR-2 recipients vs. 0.14% in GFP recipients; n=4). Although these analyzes were performed at 4 weeks post-BMT and therefore do not represent steady-state hematopoiesis, they suggest that exogenous expression of EAR-2 increases the frequency of primitive cells with the immunophenotype of long-term repopulating HSCs. Consistent with this, bone marrow cells from EAR-2 recipients exhibited increased clonogenicity.
in replating assays (Figure 3.3 B). We further went on to analyze the distribution of bone marrow cells in progenitor cell subsets observing an accumulation of megakaryocyte-erythroid progenitor cells (MEPs) and a decrease in common myeloid progenitor cells (CMPs) in the bone marrow of animals transplanted with EAR-2 transduced bone marrow (Fig. 3.6 E), as well as an increase in common lymphoid progenitor cells (CLP) (Fig. 3.6 F).

Taken together, these data show that exogenous expression of EAR-2 in hematopoietic cells in vivo results in a cell-autonomous increase in KSL cells, particularly in the most primitive KSL subpopulation, increased clonogenicity, impaired differentiation, and ultimately acute leukemia.

3.3.5 EAR-2 is necessary for the maintenance of hematopoietic stem cells

We sought to observe the effects of EAR-2 on hematopoietic stem cells by analysing the effects of overexpression or silencing of EAR-2 on maintenance of KSL cells in 5-FU treated bone marrow cells. EAR-2-transduced bone marrow cells contained significantly more KSL cells after 4 days of ex vivo suspension culture (post-transduction) than cells expressing the GFP control vector (36.8% ± 6.3% compared to 23.2% ± 4.4% respectively (Fig. 3.7 A). Similarly, silencing of EAR-2 resulted in a drastic reduction in KSL cells after six days of ex vivo suspension culture (post transduction) (Fig. 3.7 B) and caused a dramatic decline in lineage negative cells (Fig 3.7 C) and concurrent increase in cells expressing the myeloid markers CD11b and Gr-1 surface antigens suggesting that they have differentiated into cells of the neutrophil lineage (Fig 3.7 D). This observation was confirmed by examination of cytomorphology of the bone marrow cultures, which showed that cultures in which EAR-2 was silenced were comprised predominantly of mature neutrophils (Fig 3.7 E). These data are consistent with our hypothesis that EAR-2 is a negative regulator of differentiation and that expression of EAR-2 is necessary for maintenance of the hematopoietic stem cell state.
Figure 3.6: EAR-2 overexpression is accompanied by expansion of the stem cell pool and progenitor cell dysregulation in vivo. A) Animals transplanted with bone marrow that overexpresses EAR-2 showed an increase in bone marrow cells that expressed the stem cell associated antigen c-kit, while B) showing a decrease in bone marrow cells expressing antigens associated with mature blood lineages. C) These animals had a remarkable increase in the size of their KSL cell compartment, a heterogeneous cell population enriched for hematopoietic stem cells. D) Analysis of the KSL cell compartment from animals transplanted with bone marrow that overexpresses EAR-2 showed an increase in cells that have an immunophenotype associated with long-term hematopoietic cells. E) Analysis of the progenitor cell compartment in the bone marrow of transplant recipients showed that overexpression of EAR-2 decreases CMP cells, increases the number of MEP cells, and F) increases the number of CLP cells.
Figure 3.7: EAR-2 regulates maintenance of the undifferentiated state ex vivo. A) Bone marrow from five animals preconditioned with 5-FU was collected and divided into two aliquots, one of which was infected with EAR-2-IRES-GFP and the other with GFP. The percentage of GFP positive KSL cells was determined relative to the total number of transduced (GFP positive) cells four days post infection. (p=0.0032, two-tailed T-test). B) Knockdown of EAR-2 in 5-FU treated bone marrow cells resulted in a depletion of KSL cells in ex vivo culture. Bone marrow from three animals preconditioned with 5-FU was collected and divided into two aliquots, one of which was infected with shEAR-2 and the other with the scrambled control, both constructs contained a GFP cassette. The percentage of GFP positive KSL cells was determined relative to the total number of transduced (GFP positive) cells six days post infection. (p= 0.0265, two-tailed T-test). C) Knockdown of EAR-2 in 5-FU treated bone marrow cells resulted in a rapid depletion of lineage negative cells. The samples described in Fig 3.5 B were analysed for expression of lineage antigens. Representative of six independent experiments. D) Knockdown of EAR-2 in 5-FU treated bone marrow cells resulted in rapid differentiation into the granulocytic lineage. The samples described in Fig 3.5 B were analysed for expression of myeloid antigens GR-1 and CD11b. E) Morphological confirmation that knockdown of EAR-2 in 5-FU treated bone marrow cells resulted in rapid differentiation into the granulocytic lineage. Cytospins of the cultures described in Fig 3.5 B were generated. In contrast to the culture transduced with the scramble control that contains many undifferentiated blast cells, the culture transduced with shEAR-2 contains many mature granulocytes.
3.3.6 EAR-2 functions as a transcriptional repressor in a DNA binding dependent manner

Nuclear receptors may possess DNA binding dependent as well as DNA binding independent function. To examine whether the ability of EAR-2 to inhibit differentiation is DNA-binding dependent we used constructs of EAR-2 with mutations that disrupt the DNA binding domain, specifically in the P-box (M1) and D-box (M2) of the zinc finger domains. We observed that mutation of either the P-box or the D-box abrogated the ability of EAR-2 to inhibit differentiation of 32Dcl3 cells (Fig. 3.8 A) suggesting that this phenotype is dependent on the DNA-binding ability of EAR-2 and establishing that this effect is not simply a squelching phenomenon resulting from sequestration of nuclear receptor cofactors by EAR-2.

It has been reported that EAR-2 exerts its regulatory effects primarily as a transcriptional repressor (149). The repressor activity of nuclear receptors is mediated by recruitment of corepressors with histone deacetylase (HDAC) activity; we therefore evaluated the importance of this mechanism in the effects of EAR-2 on hematopoiesis. 32Dcl3-EAR-2 cells were incubated with the non-specific histone deacetylase inhibitor sodium butyrate prior to treatment with G-CSF. Whereas non-treated 32Dcl3-EAR-2 cells failed to differentiate in response to G-CSF, sodium butyrate pretreated cells showed recovery of G-CSF induced differentiation as indicated by cell surface CD11b expression (Fig. 3.8 B). Thus, HDAC-mediated transcriptional repression may account for at least part of the mechanism by which EAR-2 impairs hematopoietic differentiation.

Given that EAR-2 function is consistent at least partially with that of a transcriptional repressor, we wished to identify changes in gene expression associated with expression of EAR-2 in early hematopoietic cells. Analysis of expression of select candidate genes associated with HSCs in mice (n=11) revealed reduced expression of HOX-A10 and HOX-A9 in bone marrow KSL cells that overexpress EAR-2, while expression of ZFX is significantly increased (Fig. 3.8 C). While increased expression of HOXA9 is associated with proliferation of a specific type of leukemia, knock-down of HOXA9 increases self-renewal and prevents differentiation (150).
Figure 3.8: Functions as a transcriptional repressor in a DNA binding dependent manner.

A) EAR-2 inhibition of differentiation is dependent on DNA binding and B) the recruitment of histone deacetylases given that sodium butyrate rescues the EAR-2 induced block in differentiation. C) Expression of genes involved in hematopoiesis were assessed both in cell lines and in D) KSL cells from animals at 3 weeks post-transplant (n=10 animals).
Furthermore, downregulation of HOX-A9 is observed in several types of cancer (151-162). ZFX is associated with maintenance of self-renewal ability in embryonic and hematopoietic stem cells (19). These observations lead to a model in which EAR-2 represses hematopoietic differentiation by acting as a DNA-specific transcriptional repressor and altering the Hox program in the HSC.

3.4 Discussion

We have previously observed that EAR-2 expression correlates with the clonogenic status of leukemia cell lines and demonstrated that EAR-2 is overexpressed in AML and MDS patient bone marrow samples (142). Nevertheless, the role of EAR-2 has never been studied neither in hematopoiesis nor in leukemogenesis. Understanding the physiological function of genes that regulate leukemia cell clonogenicity is essential to making clinical advances in the treatment of these diseases.

Using *in vitro* progenitor culture and adoptive transfer techniques, we found that unregulated expression of EAR-2 in bone marrow cells impairs differentiation and extends clonogenicity, resulting in expansion of the stem cell compartment and ultimately in acute leukemia. Conversely, silencing of EAR-2 expression promotes hematopoietic differentiation, leading to depletion of stem cells. These effects of EAR-2 on hematopoiesis are mediated via its actions as a DNA-binding transcriptional repressor, which cause perturbation of the self-renewal gene expression signature. These data are consistent with a role for EAR-2 in regulation of hematopoietic cell differentiation and HSC homeostasis, and demonstrate that unregulated expression of EAR-2 is oncogenic.

3.4.1 Blocked hematopoietic differentiation

In bone marrow that was infected and cultured *ex vivo* (Fig. 3.1), overexpression of EAR-2 decreased colony number while knockdown increased colony size but not colony numbers, consistent with the notion that EAR-2 functions to limit hematopoietic differentiation. Since
overexpression prevents cells from differentiating it is expected that fewer colonies would be observed since progenitor cells simply would not differentiate into colonies. On the other hand, knockdown of EAR-2 is expected to promote differentiation. Hematopoietic differentiation occurs through a series of cell divisions and hence requires time in order for the differences to be visualized. This difference may not be resolved in colony forming assays given that these assays measure the number of progenitor cells in a snapshot of time.

While overexpression of EAR-2 inhibited differentiation \textit{in vivo} as demonstrated by an increase in KSL cells and pancytopenia, and extended replating ability \textit{ex vivo}, consideration of the nuances of hematopoietic maturation suggests that EAR-2 does not block differentiation indiscriminately but may in fact be involved in cell fate decisions at points of lineage bifurcation (Fig. 3.9). At the level of the KSL cell, EAR-2 encourages an increase in KSL cells at the expense of cells committing to differentiation. Analysis of progenitor cell populations showed both an accumulation of CLP and a decrease in CMP in EAR-2 animals compared to control. This could be the reason that there is an increase in B-lymphocytes, and a decrease in granulocytes. Further down the commitment hierarchy, there is an increase in the number of MEP progenitor cells. It is likely that cells are partially blocked at this stage, explaining the decrease in mature red blood cells. It is likely that the blockade is partial since we observe an excess of megakaryocytes in bone marrow histological sections. Overall, these data suggest that the level of expression of EAR-2 has a profound role in the fine tuning of hematopoietic differentiation. The data suggest that EAR-2 does not act by preventing a program of terminal differentiation \textit{per se} but rather by regulating decisions of cell fate throughout the hematopoietic hierarchy. This is consistent with our previous report showing that expression of EAR-2 in highly purified progenitor cell populations is expressed in a binary fashion in cells downstream of points of hierarchical bifurcation (142). A role for EAR-2 in cell fate determination is not surprising given that EAR-2 (125) is a mammalian homologue of the Drosophila gene \textit{seven-up}, which plays a role in neuroblast and retinal cell fate decisions (113, 126-129). Precisely how EAR-2 regulates each specific hematopoietic fate remains to be determined, our observation that EAR-2 is able to modify expression of HOX genes, determinants of cell fate, is consistent with this observation.
Figure 3.9. Overexpression of EAR-2 may regulate decisions at points of bifurcation in the hematopoietic hierarchy. At the level of the KSL cell I have shown that EAR-2 regulates the decision between KSL cell expansion (Figure 3.4)/maintenance (Figure 3.5) and differentiation (Figure 3.5). The next point of bifurcation that a primitive cell would encounter is the decision to progress down the CMP versus CLP fork. Evidence that EAR-2 might regulate this decision point comes from analysis of progenitor cells which shows that EAR-2 overexpression is associated with a decrease in CMP as well as an increase in CLPs (Figure 3.4 E and F). Furthermore, chimeric animals have a dearth of myeloid cells but a gross increase in their numbers of B-lymphocytes (Figure 3.3 E and F). Along the CLP fork, overexpression of EAR-2 increases the number of B-lymphocytes (Figure 3.3 E) while abrogating T-cell development (Chapter 4). Along the CMP fork, overexpression of EAR-2 increases the numbers of MEPs (Figure 3.4 E), this is corroborated by the decrease in granulocytes observed upon overexpression of EAR-2, and the increase in megakaryocytes that we observe in histological sections of bone marrow from EAR-2 overexpressing animals. While we have not been able to observe any differences in the differentiation of monocytes versus granulocytes, we have observed that EAR-2 inhibits erythropoiesis (Figure 3.1 F and 3.3 F) while encouraging the differentiation into megakaryocytes.
3.4.2 KSL cell expansion

A role for EAR-2 in decisions of cell fate would be consistent with the exciting possibility that EAR-2 may regulate such decisions as hematopoietic stem cell self-renewal versus differentiation. This possibility is consistent with our observations that EAR-2 regulates hematopoietic stem cell homeostasis wherein we observed that overexpression of EAR-2 induces KSL cell accumulation in vivo and enables HSC maintenance ex vivo, while knockdown impedes maintenance and induces differentiation. While we have shown that EAR-2 perturbs HSC homeostasis, cellular and molecular mechanism by which exogenous expression of EAR-2 leads to expansion of the stem cell pool and ultimately to AML remain to be elucidated. One possibility is that the accumulation of KSL cells suggests a block in the ability of primitive cells to progress down a pathway of differentiation, alternatively EAR-2 may increase the HSC probability of self-renewal. These two options of course are not mutually exclusive. We conjecture that both a bias of HSC divisions toward self-renewal, an impediment toward ability to differentiate and an increase in the fraction of HSCs actively cycling must occur. This notion is consistent with our observation that in KSL cells EAR-2 increased expression of ZFX, a molecule necessary for stem cell self-renewal in HSCs and pluripotent embryonic stem cells (19) that is overexpressed in numerous cancer subtypes (163-165).

While sequentially we observed expansion of KSL cells before the development of leukemia, the precise relationship between KSL cell expansion and leukemogenesis has yet to be determined. One possibility is that the entire pool of expanded KSL cells are in fact pre-leukemia cells giving rise to a polyclonal disease, on the other end of the spectrum is the possibility that expansion of the KSL cell population is independent of leukemogenesis. The truth may lie somewhere in between, where expansion of KSL cells provides a population of cells with a clonal advantage, a population that is already expanded in number and hence represents an increase in targets available to take a second hit. The observation that the immunophenotype of the leukemia is sca1− does not necessarily suggest that the leukemia cell population is distinct from the KSL cells. While we have shown that the leukemia is indeed transplantable suggesting the presence of leukemia stem cells we have not determined the immunophenotype of the leukemia initiating cell. Given the dearth of leukemia initiating cells in most populations it is
certainly possible that the immunophenotype of the leukemia stem cells is distinct from the immunophenotype of the general leukemia cell population that we observe, hence not precluding the possibility that expansion of the KSL cell population predeceases leukemogenesis.

3.4.3 One hit or two?

Interestingly, we have observed that in each cohort one or two EAR-2 transplanted animals do not develop leukemia. These animals, though lethally irradiated and transplanted with grafts of 100% transduced bone marrow cells become negative for both the transgene and GFP expression, unlike GFP control animals. These observations would be consistent with a model in which EAR-2 induces expansion of HSCs, perhaps by increasing HSC cycling, that is followed by stem cell exhaustion, unless a second hit occurs that induces leukemic transformation. Previously, we have shown that EAR-2 is expressed highly in both long- and short-term hematopoietic stem cells, and that expression increases upon stem cell cycling (142). The KSL cell compartment is a highly heterogeneous population consisting of stem and progenitor cells. Even the HSCs within the population comprises of cells heterogeneous in their ability to self-renew, and capacity for long- and short- term engraftment. Elucidating the effect of EAR-2 in defined hematopoietic stem cell subpopulations will be important in deciphering precisely how EAR-2 expression regulates HSC activity.

We observed that EAR-2 transplant chimeras also develop leukemia following secondary transplantation. We conjectured that the reason secondary transplantation induces the rapid development of leukemia is that reconstitution during transplantation induces a temporary increase in the rate of stem cell divisions in order to achieve HSC expansion. Therefore, the increased rate of HSC divisions allows for perturbations in HSC dynamics to be resolved in a shorter period of time than had primary recipients been observed for extended periods of time. To test this hypothesis, we observed EAR-2 transplant chimeras over a period of 29 weeks, observing the spontaneous development of leukemia in a portion of the recipients. These animals were transplanted with a graft consisting of 10% transduced cells, the low percentage of transduced cells may explain the long latency required for the development of leukemia.
Nevertheless, the long duration of the latency phase preceding the development of AML in EAR-2 transplant chimeras suggests that a second “hit” is required to initiate AML in these animals.

### 3.4.4 Mechanism

In the absence of ligand, nuclear receptors act as transcriptional repressors by recruiting histone deacetylase (HDAC) co-repressor complexes to specific target genes, and EAR-2 functions \textit{in vitro} in this way (Zhu et al.; 2000). Our observations that the effects of EAR-2 on myeloid differentiation are dependent on DNA binding and are abrogated by HDAC inhibition are consistent with this mechanism, suggesting a model in which EAR-2 represses the transcriptional program necessary for lineage commitment.

The reduced expression of the homeobox genes HOXA9 and HOXA10 in EAR-2-transduced KSL cells may seem counterintuitive, since bone marrow overexpression of either gene results in acute leukemia in adoptive transfer experiments (7, 166, 167). However, the relationship of the HOX gene program to malignancy is complex. Although HOXA gene upregulation has been reported (168-170), epigenetic silencing of both HOXA9 (93, 151-162) and HOXA10 (152, 171, 172) also occurs in a wide variety of cancers, where it is associated with poorly-differentiated tumour phenotype (152, 172, 173). In addition, targeted deletion of HOXA9 results in impaired differentiation of hematopoietic progenitors in the mouse (150) and the progression of myeloid leukemia from chronic phase to blast crisis is accompanied by silencing of HOXA10 expression (174, 175) amongst other changes. Thus, repression of HOXA9 and HOXA10 is seen in malignancy and is associated with impairment of differentiation, fully consistent with the EAR-2-induced leukemia phenotype.

Furthermore, we observed greater expression of the stem cell associated gene ZFX in KSL cells transduced with EAR-2 compared to GFP. ZFX expression is necessary for stem cell self-renewal in HSCs and pluripotent embryonic stem cells (19), and ZFX is overexpressed in numerous cancer subtypes (163-165). While the observation that EAR-2 increases expression of ZFX may be counterintuitive given our observation that HDAC inhibitors rescue the EAR-2 overexpression phenotype, however, our observations with HDAC inhibitors merely studied the ability of EAR-2 to inhibit differentiation, it remains to be determined if KSL cell expansion is
dependent on the ability of EAR-2 to repress transcription. It is possible that KSL cell expansion is HDAC-independent. Indeed, while COUP-TF family members are predominantly transcriptional repressor, they are not so exclusively (176). COUP family transcription factors can activate transcription in both a DNA-binding dependent (177, 178) and independent fashion (179-182). Of course, it is also possible that ZFX is not a direct transcriptional target of EAR-2 but rather a secondary target.

3.4.5 Knockdown

At first glance the dramatic phenotype we observe upon perturbation of EAR-2 expression may appear at odds with the phenotype of EAR-2 deficient animals described in the literature which does not examine hematopoiesis or report a difference therein (101, 183), nevertheless this is not the case. We observed that knockdown of EAR-2 increases the size of bone marrow colonies, while it is difficult to extrapolate these findings to animals it would be reasonable to assume that animals with targeted deletion of EAR-2 would be expected to exhibit hematopoietic differentiation. Whatever predilection toward differentiation that EAR-2/- bone marrow cells might have would produce a phenotype that is difficult to distinguish from control animals. We also observed that EAR-2 knockdown induces rapid depletion of KSL cells ex vivo suggesting that EAR-2 expression in HSCs is necessary for stem cell maintenance. Hence, we would expect the phenotype of an animal with EAR-2 targeted deletion to exhibit hematopoietic differentiation but to contain HSCs with compromised repopulation ability or compromised ability to respond to stress or injury. To address this point the functional abilities of HSCs from animals with targeted deletions of EAR-2 will need to be examined.

3.4.6 MDS mouse model

Progress in understanding MDS has been hampered by the lack of suitable cell lines or animal models for this disease. A mouse model that accurately recapitulates the essential qualities of MDS – stem cell competitive advantage, dysplastic hematopoiesis, peripheral blood cytopenias, and progression to acute leukemia – would be tremendously valuable for investigations of the
pathological mechanisms of these qualities and for preclinical testing of new MDS therapies. The EAR-2 transplant chimera has several qualities that recommend it as a model of MDS. The first of these is prima facie plausibility – since EAR-2 is commonly overexpressed in MDS and AML, it is not unexpected that its overexpression might lead to MDS in the mouse. Second, the essential elements of MDS – dysplasia, bone marrow hypercellularity, and progression to acute leukemia – are faithfully recapitulated in the model. Third, in contrast to germline transgenic mouse models, our model has mixed hematopoietic chimerism and therefore reflects the competitive aspect of myelodysplastic hematopoiesis, in which normal and MDS HSCs co-exist. Finally, the long latency preceding the development of leukemia in our model provides opportunity for the study both of the biology of progression of MDS, and of therapeutic interventions.

3.4.7 Future directions:

Our observation that EAR-2 is involved in hematopoiesis and that overexpression causes leukemia brings up the interesting possibility that EAR-2 may be involved in the pathophysiology of other hematopoietic conditions, such as non-myeloid leukemias, lymphomas and chronic leukemia. Our data suggest that EAR-2 influences stem cell homeostasis and prevents differentiation. It is possible that the mechanisms by which EAR-2 regulates differentiation is redundant in other organ systems. For example, the recent report that EAR-2 is overexpressed in colon cancer (145) suggests that EAR-2 may be involved in decisions of cell fate and cancer cell differentiation for tissues other than blood.

Our results implicate EAR-2 as a player not only in self-renewal of normal HSCs but also as a leukemia oncogene. Nuclear receptors are eminently “druggable” – agonist and antagonist NR ligands are of proven utility in a wide variety of human diseases. We have shown previously that silencing of EAR-2 expression by shRNA in AML cell lines causes differentiation and apoptosis. We show that expression of EAR-2 is sufficient to initiate myeloid leukemia. These data raise the extremely exciting possibility that natural or synthetic antagonist ligands for EAR-2 can be found that may act as anti-leukemic therapeutics. The observation that animals with targeted deletions of EAR-2 are viable and fertile suggests that while such a drug would possess
minimal toxicity even though it would target both normal and leukemia stem cells. Conversely, it may be possible to identify agonist EAR-2 ligands that promote HSC self-renewal in a controlled fashion – such ligands would be of enormous utility in therapeutic expansion of HSCs for autologous or allogeneic transplantation.
4 The Orphan Nuclear Receptor EAR-2 (NR2F6) Is a Novel Negative Regulator of T Cell Development

Chapter 4 has been submitted to Experimental Hematology as follows:


Figures 4.4 and 4.5 were conducted in collaboration with Džana D. Dervović
4.1 Abstract

EAR-2 (NR2F6) is an orphan nuclear receptor belonging to the COUP-TF family of transcriptional regulators; one of three mammalian homologues of the *Drosophila seven-up* gene, which plays a role in neuroblast and retinal cell fate decisions. Ear-2 is highly expressed in hematopoietic stem cells (HSCs), with expression being necessary and sufficient to block the differentiation of leukemia cells. However, the role of Ear-2 in T cell development has not been fully established. Since both EAR-2 and the T cell regulator RORγt (Rorc) antagonize retinoid and thyroid hormone receptor signaling, we investigated whether EAR-2 expression may have a role in T cell development. Here, we describe a novel role for the orphan nuclear receptor EAR-2 in regulating T cell development. Specifically, in comparison to the WT cells, overexpression of EAR-2 (EAR-2++) in a competitive bone marrow (BM) transplantation assay resulted in limited T cell development *in vivo*. Furthermore, mice that received grafts consisting of 100% EAR-2++ BM cells demonstrated a more than 10-fold decrease in their thymic size and cellularity relative to controls. Thymic cortices from EAR-2++ mice had decreased lymphocyte cellularity; while medullas had a starry-sky appearance, indicative of apoptosis. *In vitro*, differentiation of EAR2-transduced murine BM-HSCs in OP9-DL1 cultures showed a reduction in the number of cells generated from EAR-2++ BM-HSCs and a block at the differentiation from DN4 to DP and SP cells, as a consequence of increased apoptosis. Based on these findings we conclude that EAR-2++ cells were able to migrate to, but not fully repopulate, the thymus due to cell intrinsic defects in the proliferation of DN cells followed by their inadequate differentiation from the DP to SP stage of T cell development accompanied by apoptosis. Thus, downregulation of EAR-2 is indispensable for the proliferation, survival and differentiation of developing T cells.
4.2 Introduction

The orphan nuclear receptor v-erb-A related-2 (EAR-2, NR2F6) is a mammalian homologue of the *Drosophila* gene *seven-up* (*svp*). We have previously established that EAR-2 functions as a gatekeeper of the undifferentiated state, preventing the differentiation of acute myeloid leukemia cells into mature blood cells thereby maintaining their clonogenicity (142). The expression of EAR-2 is highest in hematopoietic stem cells (HSCs) and declines drastically upon normal hematopoietic differentiation, while overexpression of EAR-2 blocks differentiation of leukemia cell lines. This role for EAR-2 in the regulation of differentiation is consistent with the function of the *Drosophila* homologue *svp*, which plays a role in cell fate decisions in neurological development (113). The mammalian homologues of EAR-2 also play important roles in differentiation and cell fate decisions. Specifically, COUP-TFI is important in neuronal development (95, 184, 185), while COUP-TFII is involved in determination of cell identity for venous-arterial cells (186), coronary vessels (187), lymphatic endothelial cells (188-190), Leydig cells (191), trophoblast giant cells (192), adipocytes (193-195), and neuronal cells (196-198). EAR-2 null mice exhibit agenesis of the locus coeruleus (130), a midbrain nucleus that regulates circadian behavior and nociception.

A role of EAR-2 in lymphopoiesis has not been established. Although targeted deletion of EAR-2 does not result in aberration of lymphocyte development, EAR-2-null mice have hyper-reactive Th17 CD4+ T lymphocytes and are hyper-susceptible to Th17-dependent experimental autoimmune encephalomyelitis (131).

In addition, EAR-2 functions as a transcriptional repressor *in vitro* by directly inhibiting the transactivating ability of numerous genes including the thyroid hormone receptor (132). Like many nuclear receptors, EAR-2 heterodimerizes with the retinoid X receptor-α (RXR-α) (133), and can inhibit retinoid signaling *in vitro* as demonstrated by blocking differentiation induced by all trans-retinoic acids (142). No interaction has been shown between EAR-2 and the known Th17 master-regulator RORγt (Rorc) (199). Interestingly, like EAR-2, RORγt is also an orphan nuclear receptor that represses both retinoid and thyroid hormone receptor signaling (200).
Overexpression studies have demonstrated that a decrease in expression of RORγt is necessary for T cell development to proceed (201). Furthermore, retinoid signaling has been associated with protection of immature T cells from activation-induced cell death, a type of apoptosis, during T cell development (202-206); hence it is conceivable that inhibition of retinoid signaling may adversely affect T cell development. Given that EAR-2 regulates both retinoid signaling and thyroid hormone receptor signaling, it is possible that overexpression of EAR-2 may have a role similar to RORγt, expression of which needs to be decreased for T cell development to ensue.

Here we describe for the first time a role for EAR-2 in the specification of lymphoid cells. Similar to RORγt, overexpression of EAR-2 abrogates the developmental program necessary for T cell lymphopoiesis. We show that EAR-2 expression is lower in thymocytes than in HSCs; and that in adoptive transfer experiments, early progenitor cells engineered to express EAR-2 at a high level (EAR-2++) fail to repopulate the thymus. Moreover, in vitro differentiation of EAR-2++ HSCs in an OP9-DL1 cell system results in greatly reduced numbers of cells relative to controls, indicating that this phenotype is cell intrinsic. Overall, our data demonstrate that EAR-2 is a novel regulator of T cell development necessary for the proliferation and survival of developing T cells.
4.3 Materials and methods

4.3.1 Animals

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and stored in a specific pathogen-free facility at Sunnybrook Research Institute. All work was done in accordance with the Sunnybrook Research Institute Animal Care and Use Guidelines.

4.3.2 Bone marrow transduction

Twelve-week-old C57BL/6 donor mice were treated with 5 fluorouracil [150 µg/g body mass] by intraperitoneal injection. Ninety-six hours later, mice were sacrificed and bone marrow collected. Cells were then stimulated for twenty-four hours prior to infection with MMP-GFP or MMP- EAR-2-IRES-GFP retroviral supernatants generated as described (146, 207). Forty-eight hours later, GFP-positive cells were collected by fluorescence activated cell sorting (FACS).

4.3.3 Bone marrow transplantation

Chimeric mice were generated by injection of lethally irradiated animals with grafts containing a mixture of transduced (GFP or EAR-2; 2.5x10⁴ cells) and mock-transduced (7.5x10⁴ cells) donor cells. Thymi, bone marrow and spleens from recipients of chimeric bone marrow cells were harvested at either early (4-6 weeks) or late (12-16 weeks) time points. Animals transplanted with 100% transduced cells were generated by transplanting lethally irradiated animals with bone marrow grafts containing between 4x10⁴ and 1x10⁵ transduced bone marrow cells. The same graft size was administered to all recipients in each transplantation experiment.
4.3.4 Histological sections

Thymi were fixed for 24 hours in buffered formalin (Sigma-Aldrich, St Louis, MO) followed by paraffin embedding, slicing, and staining with hematoxylin and eosin by the Sunnybrook Research Institute histology core facility.

4.3.5 Cell lines

OP9-DL1 cells were generated from the OP9 bone marrow stromal cell line and maintained as previously described (208).

4.3.6 Flow cytometry

Biotin-, FITC-, PE-, PE-Cy5-, PE-Cy7-, APC-, APC-Cy7-mAbs were purchased from BD Biosciences or eBioscience. The following conjugated antibodies were used: anti-B220 (RA3-6B2), anti-CD3e (145-2C11), anti-CD4 (GK1.5, L3T4), anti-CD8a (LY-2, Lyt-2, 53-6.7), anti-CD11b (M1/70), anti-CD16/CD32 (2.4G2), anti-CD19 (1D3), anti-CD25 (7D4), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD117 (2B8), anti-TCRβ (H57-597), Sca-1 (E13-161.7) and TER119 (TER-119). Cells were stained by standard staining techniques and analyzed on a FACSCalibur or LSRII flow cytometer (BD Biosciences). Data files were analyzed with Flow-Jo (Tree Star). Dead cells were excluded from all data by forward- and side- scatter, and 4’6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (Molecular Probes). Cell sorting was performed with a FACSDiVa or a FACSARia cell sorters (BD Biosciences). Annexin V-FITC staining was performed according to the manufacturers’ recommended procedure (BD Pharmingen), and the percentage of cell death was calculated by adding percentages of Annexin V+, Annexin V+ PI+ and PI+ cells. Purity was typically greater than 98% for all populations as determined by post-sort analysis.
4.3.7 OP9-DL-1 cultures and analysis of T cell subsets

Bone marrow infected with either EAR-2 or GFP was sorted for transduced cells (GFP+) with the c-kit+, sca-1+, lineage- (KSL) immunophenotype. Transduced BM-HSCs cells were plated on OP9-DL1 cells in a 6-well plate in triplicate and maintained in conditions previously described (209, 210).

4.3.8 Quantitative PCR

T cell subsets were sorted directly into Trizol (Invitrogen, Carlsbad, CA). Total mRNA was isolated as per the manufacturer’s instructions. mRNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed on an ABI light cycler using SYBR® Green Master Mixes (Invitrogen, Carlsbad, CA). Calculations were completed using relative quantification method, where the samples were normalized to β-actin expression.

Primer sequences for EAR-2 are as follows

Fwd: 5’-CCTGGCAGACCTTCA ACAG -3’ and Rev: 5’-GATCCTCCTGGCCCATAGT -3’.
4.4 Results

4.4.1 EAR-2 expression decreases upon hematopoietic stem cell differentiation into immature T cells

EAR-2 expression is heterogeneous throughout the hematopoietic hierarchy, with its expression being highest in long-term repopulating HSCs and generally declining with the differentiation of progenitor cells (207). Likewise, expression of EAR-2 mRNA declined sharply from the KSL (c-kit+, sca-1+, lineage−) hematopoietic stem cell stage, to the immature double negative (DN) 1 T cell stage (Figure 4.1). Expression of EAR-2 amongst immature T cell subsets (DN1-DN4) did not differ significantly; however, expression of EAR-2 decreased sharply from the double positive (DP) cells to CD8+ single positive (SP) cells (Figure 4.1).

4.4.2 EAR-2 expression places developing thymocytes at a competitive disadvantage

The effects of EAR-2 on lymphopoiesis were assessed \textit{in vivo} by competitive transplantation of EAR-2 or GFP transduced bone marrow cells (n=43). Competitive repopulation of lethally irradiated murine hosts with GFP transduced bone marrow cells resulted in successful engraftment and T cell development, with GFP+ T cells present in the thymus and periphery at rates comparable to those of transduced cells in the original graft (Figure 4.2A). In contrast, six weeks post-transplant, the proportion of CD3+ cells derived from EAR-2 transduced bone marrow cells was greatly diminished relative to the controls. Complete abrogation of CD3+ cells derived from EAR-2 transduced T cells was observed in both the thymus (Figure 4.2B) and periphery (Figure 4.2C, 4.2D, and 4.2E) at 12 weeks post-transplant. A lack of mature T cells was observed in all recipients of EAR-2 transduced bone marrow. We determined this using both expression of the mature cell surface markers CD3, as well as the markers CD4 and CD8 in some recipients (data not shown), to confirm that these mice lack mature T cells and that the observed phenomenon is not attributed to a direct effect of EAR-2 on expression of the CD3 gene.
Figure 4.1 Differentiation of T cells induces decreased EAR-2 expression. Shown is expression of EAR-2 relative to the L32 housekeeping gene in stem cells versus T cell subsets in the thymus. Data shown are the mean and SD of 3 independent animals. “Stem cells” denote hematopoietic stem cell cDNA.
Figure 4.2: EAR-2 expression must decrease for thymocyte development. Competitive bone marrow transplants were conducted with EAR-2 retrovirally transduced bone marrow cells or GFP control. (A) Bone marrow that overexpresses EAR-2 does not engraft the thymus. Shown are representative animals from a chimerical bone marrow transplantation experiment. While both GFP and EAR-2++ animals engrafted well in the bone marrow (18.9% and 40.0% respectively), only the GFP control animals demonstrated engraftment in the thymus as well (22.0% engraftment for GFP versus 0.4% engraftment for EAR-2++). (B) Pooled data showing engraftment in the thymus relative to the engraftment in the bone marrow as depicted in (A) is shown from three independent experiments, n=22 at 4 weeks and n=7 at week 12. ** denotes P<<0.001. (C) EAR-2 overexpressing cells do not contribute to peripheral T cells in the spleen or peripheral blood. Chimerical bone marrow transplantation experiments were conducted. Representative animals from the GFP and EAR-2++ cohorts show that while the GFP negative competitor cells were able to generate CD3+ cells in both the spleen and peripheral blood, only cells transduced with the GFP vector and not the EAR-2 vector were able to generates CD3+ T cells. Data representative of three independent experiments are shown. (D,E) Pooled data from the three independent competitive bone marrow transplantation experiments described in (C) are show. The x-axis lists first whether the animal belongs to the GFP control or EAR-2++ cohort, followed by whether the GFP negative competitors or whether the GFP positive transduced fraction is represented. The percentage of either GFP positive or negative CD3+ T cells are shown as a percentage of all the GFP positive or negative cells respectively in either (D) the peripheral blood or (E) the spleen. We observe that while competitor cells from both the GFP (GFP GFP-) and EAR-2++ (EAR-2 GFP-) cohort were able to generate CD3+ T cells in both (D) the spleen and (E) peripheral blood, only cells transduced with the GFP vector (GFP GFP+) and not EAR-2 transduced cells (GFP EAR-2+) vector were able to generates CD3+ T cells. Bars represent the mean ± SEM, n=20 at 6-8 weeks. ** denotes P<<0.001. (E) EAR-2 overexpressing cells do not contribute to peripheral T cells in the spleen. Pooled data from two independent experiments are shown, bars represent the mean ± SEM, n=35. ** denotes P<<0.001.
4.4.3 Effacement of thymic cortex and apoptosis in medulla of EAR-2++ animals

In a second series of bone marrow transplants, cells transduced with EAR-2 or GFP were purified by fluorescence-activated cell sorting and transferred by tail vein injection into lethally irradiated recipients. Animals transplanted with EAR-2 transduced bone marrow demonstrated a striking decrease in thymus size (Figure 4.3A) and lower cellularity (Figure 4.3B). As observed in competitive transplants, these recipients demonstrated a dramatic reduction in the proportion of CD3+ cells in the thymus (Figure 4.3C), and CD3+ cells in the spleen, lymph nodes and peripheral blood (data not shown). Histological examination revealed effacement of the thymic cortex with a dramatic decrease in cortical lymphocytes (Figure 4.3D and 4.3F). The medulla of EAR-2 transplant recipients showed a starry sky appearance at low power (Figure 4.3F), and tangible body macrophages with intra-cytoplasmic apoptotic bodies at high power (Figure 4.3E), indicating apoptosis. These data demonstrate that unregulated expression of EAR-2 blocks repopulation of the thymus after lethal irradiation in vivo.

4.4.4 In vitro experiments identify a cell intrinsic defect in T cell development

To examine further the development of T cells from EAR-2++ hematopoietic progenitors, FACS-sorted EAR-2 or GFP transduced KSL cells were differentiated into T cells in OP9-DL1 cultures. A reduction in the number of cells generated from EAR-2 BM-HSCs was observed as early as day 4 and was more distinct at day 19 (Figure 4.4A). The reduction in cell number was also accompanied by a reduction in the proportion of cells that expressed the EAR-2 transgene (Figure 4.4B) confirming that expression of EAR-2 greatly impairs T cell development in this in vitro system.

The decrease in cell number at early time points, as early as day 4 of culture, could not be attributed to apoptosis or a block in differentiation (Figure 4.5) suggesting that expression of
**Figure 4.3. EAR-2 overexpression results in thymic involution.** Animals transplanted with sorted grafts containing 100% EAR-2 transduced bone marrow cells that were sacrificed 3-4 weeks post transplant showed a dramatic reduction in (A) thymic size and (B) cellularity. (C) In addition, the majority of thymocytes recovered from the EAR-2 cohort did not express the EAR-2-GFP transgene. (D) Histological sections revealed an abnormal thymus (E) tinglible body macrophages in the medulla were observed, indicative of apoptosis (F) the thymic cortex showed decreased cellularity while the medulla showed a “starry sky” appearance in animals transduced with grafts that overexpressed EAR-2. Pooled data from two independent experiments are shown; n=6 EAR-2 and n=6 GFP for experiment one; n=10 EAR-2 and n=7 GFP for experiment two.
EAR-2 inhibits the proliferation of cells committed to a T cell fate. However, a block in the transition from the DN4 to double positive (DP) and single positive (SP) stage at days 24 and 28 of culture (Figure 4.4C), accompanied by an increase in apoptosis (Figure 4.4D and 4.4E) was observed.

These data suggest that the reduced proliferation of T cell progenitors and decreased differentiation of DPs to SPs observed upon sustained EAR-2 expression is cell intrinsic. Consequently, T cell progenitors fail to repopulate the thymus effectively and thus are unable to maintain normal cell numbers.
Figure 4.4. Severe defects in T cell development observed in vitro. Hematopoietic stem cells (KSL cells) were induced to differentiate on OP9-DL1 cells. (A) A 59.1% decrease in cell number was observed at day 4, increasing to 97.4% decrease at day 19. Cumulative cell numbers are shown. (B) Expression of GFP by cells in the cultures was assessed by flow cytometry at day 19. In the control culture, 90.6% of cells expressed GFP and 9.4% were GFP-negative – the increase in percentage of GFP-negative cells represents outgrowth of untransduced cells and/or loss of transgene expression by transduced cells. In the EAR-2++ culture on day 19, only 18.1% of cells expressed GFP, indicating impaired growth or survival of cells expressing the transgene. (C) Analysis of the late stages of T cell development was performed by flow cytometry for expression of CD4 and CD8 in GFP-gated cells at days 24 and 28 of culture. Control cultures showed the expected progression from DN through DP to CD8 SP stages, with 25.2% DP cells at day 24 and 21.5% SP CD8 cells at day 28. In contrast, development of EAR-2++ cells was impaired, with 10.7% DP cells and 11.7% SP CD8 cells at days 24 and 28, respectively. (D,E) Apoptosis and cell death were assessed in the cultures by flow cytometry for annexin V and propidium iodide. EAR-2++ cultures exhibited an increase in cell death on day 24 of culture, coinciding temporally with the observed block in appearance of DP and SP cells.
Figure 4.5 Analysis of T cells derived from OP9-DL1 cultures of KSL cells transduced with GFP or EAR-2 retrovirus. Cultures were analyzed by flow cytometry four and eight days after being established. No differences between the GFP and Ear-2 cultures are seen in apoptosis (A and D), differentiation (B and E), or proportion of B cells (C and F).
4.5 Discussion

EAR-2 is an orphan nuclear receptor that we have previously shown to be involved in the regulation of differentiation in myeloid leukemias. It is expressed highly in HSCs and its expression decreases as cells mature into progenitor and mature blood cells\(^{(142)}\). Similar to our previous observations in myeloid cells, we observed in this study that expression of EAR-2 decreases upon HSC differentiation into T cells.

This decrease in the expression of EAR-2 is necessary for early thymic progenitor cells to progress towards a T cell lineage. Accordingly, in adoptive transfer experiments, enforced expression of EAR-2 prevented T cell progenitors from repopulating the thymus. *In vitro* experiments confirmed that EAR-2 expressing cells have severe cell-intrinsic defects in T cell development.

It is known that thymic seeding progenitors enter the thymus at the corticomedullary junction. They then undergo a highly regulated process of differentiation and proliferation in the thymic cortex, including the differentiation from DN to DP cells. Subsequently, DP cells undergo the process of positive and negative selection in the cortex and the medulla respectively. Following positive selection, DP cells downregulate either CD4 or CD8 to become SP cells. Newly selected SPs migrate into medulla where they undergo the process of negative selection. Positive and negative selection ensures that auto-reactive clones are deleted from the T cell repertoire and only functional, self-restricted cells are selected and exported to the periphery.

Further examination of the thymus histology in this study revealed a reduction in the cellularity of the thymic cortex, while in the medulla the decrease in cell number was associated with an increase in apoptosis. Our observation of the hypocellular thymic cortex is consistent with the dramatic decrease in cellularity in our OP9-DL1 cultures, suggesting that EAR-2 is necessary for proliferation and survival of early developing T cells. Furthermore, the observation of apoptotic bodies in the medulla suggests that EAR-2 downregulation is necessary for the survival of cells undergoing negative selection and that a decrease in EAR-2 expression is necessary for protection against apoptosis. It is worth noting that the increased apoptosis in the medulla may be attributed not only to deletion of auto-reactive clones, but also self-tolerant clones from the repertoire.
Furthermore, a decrease in EAR-2 expression is critical to T cell development, both in vitro and in vivo. This is demonstrated by the ability of BM-HSCs when cultured with OP9-DL1 cells to develop into all DN subsets, as well as DP and SP cells, albeit at dramatically reduced numbers, suggesting that EAR-2 does not affect differentiation per se, but other parameters such as survival and proliferation. Although we have demonstrated that this defect is observed both in vitro and in vivo, suggesting that it is a cell intrinsic phenomenon, we have not excluded the possibility that EAR-2 affects the migration of early progenitor cells to the thymus, though we do not believe that this is the case as the decrease in cell number observed in vitro is able to explain the observed decrease in cellularity in vivo.

Mechanistically, our data suggests that EAR-2 regulates the transcription program of thymocyte development. EAR-2 functions predominantly as a transcriptional repressor, inhibiting the transcriptional function of the thymic hormone receptor (99), retinoid signaling (100), and the transactivating ability of genes such as Runx1(105). We previously reported that EAR-2 inhibits retinoic acid induced differentiation (142), suggesting that like other COUP-TFs, EAR-2 is able to inhibit retinoic acid signaling. Another distantly related orphan nuclear receptor that inhibits retinoic acid signaling is RORγt, a molecule whose precise regulation is necessary for T cell development (201, 211, 212). It is instructive to compare the effects of EAR-2 on T cell development to those of RORγt. Akin to overexpression of EAR-2, overexpression of RORγt results in severely reduced thymic cellularity attributed to inhibition of thymocyte proliferation, and a reduction in the number of mature T cells in the periphery (201). In addition, blocked thymocyte development was observed at the transition from DN to DP stage, similar to the stage at which we show an increase in apoptosis. Furthermore, RORγt transgenic animals show decreased CD8 SP cells, while we show a decrease in EAR-2 expression in CD8 SP cells. Given the similarities between the phenotype of EAR-2 overexpression and overexpression of the T cell master regulator RORγt, it is conceivable that the phenotype in both cases can be attributed to regulation of similar transcriptional targets.

Both EAR-2 and RORγt appear to have a role in the regulation of retinoid signaling. Nuclear receptors have long been known to influence the regulation of cell death in T cell development, the best known example being glucocorticoid-induced thymic atrophy, although glucocorticoids may also inhibit cell death in developing thymocytes (206, 213-215). Retinoic
acids are metabolites of vitamin A, that bind to receptors from the same family of molecules as the receptor that bind glucocorticoids; also, retinoic acids have a significant role in shaping decisions of cell death in T cells, serving both pro-apoptotic (213, 216, 217) and anti-apoptotic roles (203, 204, 206). The importance of retinoid signaling in immunity is highlighted by the well-established observations of immune deficiencies in both humans and animal models of vitamin A deficiency (202, 218), with oral administration of vitamin A increasing thymic weight and cellularity in animals (219). More specifically, retinoic acids inhibit activation induced cell death in thymocytes by inhibiting FasL production (203-206), and have been used to inhibit activation induced cell death \textit{ex vivo} in T cells infected with HIV (220). Knockout of RXRα impairs the DN to DP transition, which is seen also with RORγt overexpression as well as in our EAR-2 model.

Since both EAR-2 overexpression and RORγt transgenic models show a decrease in thymocyte proliferation that could not be accounted for entirely by increased apoptosis, it is possible that these molecules may share other downstream transcriptional targets. Nevertheless, while these molecules are similar, the fact that they do not share identical roles in T cell development and activation is demonstrated by their contrasting knockout phenotypes. The RORγt knockout mouse exhibits a delay in the transition from the immature single positive (ISP; CD4+/CD8lo) to DP stage of thymocyte development accompanied by apoptosis at the DP stage caused by failure of expression of Bcl-XL (211, 212). This results in a small thymus (221, 222) with marked reduction in numbers of DP and SP thymocytes, but an increased proportion of ISP cells. Furthermore, differentiation into Th17 cells was impaired (223). In contrast, no appreciable differences were reported in lymphopoiesis in the EAR-2 knockout mouse. EAR-2 antagonizes activation of Th17 CD4+ T cells and EAR-2-null mice have hyper-reactive lymphocytes and are hyper-susceptible to Th17-dependent experimental autoimmune encephalomyelitis (131). This effect of EAR-2 is mediated directly by interference with DNA binding by the NF-AT:AP-1 transcription factor complex, and is dependent on EAR-2 phosphorylation at residue Ser-83.

While the dramatic effects that we observed upon overexpression of EAR-2 may at first glance appear at odds with the lack of phenotype reported in the knockout animal, we conjecture that in lymphoid progenitors EAR-2 functions as a transcriptional repressor preventing the activation of pathways necessary for T cell survival, proliferation and possibly differentiation.
Hence, in the absence of EAR-2, repression of those pathways responsible for differentiation would not occur, therefore resulting in the observed phenotype. It will be interesting to elucidate these EAR-2 governed molecular pathways.

Of course, there are implicit limitations to the overexpression model system, and hence one must evaluate the notion of whether the effects of overexpression of EAR-2 on thymocyte development can be attributed to the phenomenon of squelching, wherein the observations described can be attributed to non-specific effects due to binding of EAR-2 to cofactors of other transcription factors, rather than via regulation of transcription of specific target genes by EAR-2. We feel that this is an unlikely scenario because of the specificity of the observed effects to the T cell lineage: overexpression of EAR-2 in the animals described herein does not impede the proliferation or development of B cells nor does it decrease the numbers of KSL cells (data not shown). Not only are the observations we described specific to the T cell lineage but they are also specific to precise phenomena in T cell development.

Taken together, these data identify the orphan nuclear receptor EAR-2 as a novel negative regulator of T cell lymphopoiesis that acts at multiple steps in the developmental cascade, and demonstrates that down-regulation of EAR-2 is necessary for the survival and proliferation of T cell progenitors.
5 Conclusions, Discussion and Future Directions
5.1 Thesis review

5.1.1 Review of chapter 2

EAR-2 (NR2F6) is an orphan nuclear receptor that I found to be differentially expressed in clonogenic versus non-clonogenic single cells from early passages of an AML cell line during my M.Sc. For my PhD, I began by validating that the role of EAR-2 is to serve as a gatekeeper to the differentiated state (Chapter 2). I began by establishing the clinical relevance of this gene, showing that EAR-2 is overexpressed in a wide spectrum of leukemia and preleukemia subtypes. Leukemia is characterized by an inability to differentiate, amongst other features, certain cell lines are amenable to induction of differentiation, maturing into blood cells upon treatment with a variety of chemical agents. I showed that expression of EAR-2 decreases when U937 cells are induced to differentiate irrespective of the chemical stimuli used to induce differentiation. I also showed this in primary mouse bone marrow samples from animals that had acute promyelocytic leukemia (NuMA-RARα transgenic mouse), expression of EAR-2 decreased when bone marrow from these animals was induced to differentiate into granulocytic blood cells following culture with retinoic acid. Furthermore, I looked at expression of EAR-2 in highly purified immature blood cells as they underwent differentiation into mature cells, observing that EAR-2 was expressed very highly in hematopoietic stem cells but decreased drastically as the cells underwent differentiation.

The correlation between EAR-2 expression and the undifferentiated status of both leukemia cells and healthy hematopoietic stem cells is very interesting because it implies that EAR-2 may have a causative role in preventing the differentiation of leukemia cells. Therefore, I decided to test this hypothesis directly by modifying the expression of EAR-2 by overexpressing the gene and as well as silencing it. I observed that overexpression of EAR-2 abrogates the ability to differentiate of two distinct leukemia cell lines, the human myelomonocytic leukemia cell line U937 and the mouse multilineage hematopoietic cell line 32D. I showed this in a number of ways including analysis of surface antigens using flow cytometry, cellular morphology of MGG stained cytospins, as well as the NBT functional assay that measures the ability of mature blood cells to release reactive oxygen species. Furthermore, I went on to show
that overexpression of EAR-2 increases the rate of cell divisions and is sufficient to prevent the cell cycle arrest that mature blood cells undergo when they terminally differentiate. On the other hand, when I silenced expression of EAR-2 using shRNA the leukemia cell lines spontaneously differentiated into mature blood cells in culture. Again, I showed this in multiple ways including analysis of surface antigens using flow cytometry, cellular morphology of stained cells. I also showed this using multiple shRNA constructs. Overall, this work establishes the clinical relevance of EAR-2 overexpression, and shows that EAR-2 is a novel negative regulator of leukemia cell differentiation.

5.1.2 Review of chapter 3

I sought to establish a physiological role for this molecule in normal hematopoietic tissue and to investigate its ability to initiate leukemia (Chapter 3). Understanding the physiological function of genes that regulate leukemia cell clonogenicity is essential to making clinical advances in the treatment of these diseases. To determine the role of EAR-2 in normal blood cell development I both overexpressed and silenced this gene in mouse bone marrow, but first I had to optimize the procedure for introducing genetic material into mouse bone marrow cells as these cells are difficult to transduce. I thus developed a way of increasing my viral titres which was amenable for use with both retroviral and lentiviral vectors (Appendix E). For the silencing experiments I devised high-titre stocks of both retroviral and lentiviral shRNA vectors deciding to proceed with the lentiviral vectors. I sought to observe if altering expression of EAR-2 would have an effect on the ability of bone marrow to become mature blood cells in culture. In normal tissue as well I showed that expression of EAR-2 acts as a gatekeeper to the differentiated state. Overexpression of EAR-2 prevented the differentiation of bone marrow cells in methycellulose culture showing a reduction in the both the number and size of colonies. While silencing of EAR-2 had no effect on colony number but greatly increased colony size, suggesting that EAR-2 knockdown promotes differentiation. I corroborated the overexpression studies in suspension culture, and showed that not only does EAR-2 overexpression prevent differentiation, but it also prevents the loss of growth ability associated with differentiation by showing that overexpression of EAR-2 extends serial replating ability.
I then turned my focus to see what EAR-2 does to blood cell development inside animals. I first generated a series of animals using bone marrow transplantation where only a portion of their bone marrow cells overexpressed EAR-2, while the rest of their bone marrow comprised unaltered, normal bone marrow cells. In these animals I observed that overexpression of EAR-2 gave bone marrow cells a competitive advantage, so that over time the number of cells that overexpressed EAR-2 increased inside the animal compared to the cells that expressed normal levels of EAR-2. I also showed that cells that overexpressed EAR-2 had a reduced capacity to differentiate into granulocytic cells, but had increased differentiation into the B cell lineage. These animals developed a disease similar to myelodysplastic syndrome characterized by dysplasia of the red blood cell lineage, an increase in the number of cells in the bone marrow, an increase in the number of white blood cells in the spleen, abnormal localization of their immature blood cells in the bone marrow, as well as a propensity to develop acute leukemia. Onset of leukemia could be accelerated by performing a second transplant, in that case, all animals developed leukemia and died within weeks.

I generated yet another series of animals using bone marrow transplantation, in these animals every single bone marrow cell overexpressed EAR-2. The majority of these animals died of leukemia within 3-5 weeks. I showed that they had leukemia by analyzing sections of their bone, liver and spleen observing that these organs were infiltrated by leukemia cells. As well I looked at their blood counts, where I noticed a dearth of red blood cells, white blood cells and platelets. Interestingly, when I analysed the composition of the bone marrow of these animals at time point before the development of leukemia I noticed perturbations in their stem cell and progenitor cell compartments. Overexpression of EAR-2 greatly increased the stem cell compartment, and altered the progenitor cell compartment in such a way that would suggest that EAR-2 expression serves as a gene that guides lineage bifurcation. The observation that animals that overexpressed EAR-2 in their bone marrow had expanded stem cell compartments suggested that EAR-2 might have a direct effect on stem cells and their decision to choose between remaining a stem cell or engaging in differentiation. When stem cells are placed in culture, over time, they lose the ability to retain their stem cell ability, thus, I tested whether the expression of EAR-2 could influence the maintenance of stem cells in culture. When I overexpressed EAR-2 in bone marrow cells I was able to slow the decline of stem cells in culture, whereas when I
silenced EAR-2 I hastened the decline of stem cells, converting the cultures into mature blood cells.

I wanted to investigate mechanistically how EAR-2 is able to influence blood cell development. EAR-2 is a member of a family of proteins that regulates the expression of other genes. It may do this either by directly binding to DNA or by tethering to other molecules that in turn bind to DNA, and then recruiting proteins that either repress or activate gene expression. To discriminate between the former two scenarios I tested the function of EAR-2 proteins that are mutated in their ability to bind DNA, showing that DNA binding ability is critical for the ability of EAR-2 to inhibit differentiation. I then tested whether EAR-2 functions by recruiting a class of proteins involved in gene repression, showing that inhibition of histone deacetylases also inhibits the function of EAR-2, with respect to its ability to block differentiation. Finally, I wanted to determine which specific genes EAR-2 might be regulating, so looked at the expression of a panel of stem cell genes in bone marrow enriched for stem cells, showing that EAR-2 decreases expression of HOX genes and increases expression of the stem cell gene ZFX.

Collectively, these data show that expression of EAR-2 needs to decrease in order for hematopoiesis to proceed as unabated expression of EAR-2 leads to leukemia that is characterized by impaired hematopoietic differentiation and an expansion of the stem cell compartment.

5.1.3 Review of chapter 4

In addition, I observed an unexpected role for EAR-2 in preventing the generation of T cells (Chapter 4). To study the role of EAR-2 in T cell development I began by looking at expression of EAR-2 at various stages of T cell maturation, observing that EAR-2 mRNA is expressed very highly in stem cells and that expression decreases rapidly when cells enter the thymus and commence differentiation toward a T cell fate. Mice that overexpressed EAR-2 in only a portion of their bone marrow cells were indeed able to generate T cells, but surprisingly, they were not able to generate T cells from those cells that overexpressed EAR-2. This suggested that expression of EAR-2 antagonized T cell development. I wondered if perhaps cells that overexpressed EAR-2 were just less competitive than normal cells and were merely being
crowded out, so I looked at T cell development in animals whose entire bone marrow comprised of cells that overexpressed EAR-2. These animals as well had very few T cells, in addition they had a very small thymus, the organ in which T cell development takes place. The numbers of immature T cells in the thymus of these animals was drastically reduced compared to controls. Hence, I asked whether EAR-2 actually prevents the development of T cells or whether it merely prevents cells from entering the thymus, a step that is necessary for the formation of T cells. Along with my collaborator we looked to see if bone marrow cells that overexpressed EAR-2 were able to form T cells in culture, indeed they were not. We observed an immediate decrease in the ability of EAR-2 overexpressing cells to proliferate in culture as well as a block in progression from the DN to the DP stage of maturation that was accompanied by an increase in cell death. These data establish that EAR-2 is a novel negative regulator of T cell development whose expression must decrease in order for the generation of T cells. Given that T cell development is thought to be more immunology than hematology, I will first discuss it separately from the general hematopoiesis phenotype.

5.2 EAR-2 is a gatekeeper to hematopoietic differentiation:

5.2.1 Future experiments:

For my doctoral project I have shown that EAR-2 expression is able to impede the differentiation of leukemia cell lines as well as primary bone marrow cells. Additional future experiments include:

5.2.1.1 Does EAR-2 act to control lineage bifurcation?

To directly address the question of whether EAR-2 expression controls lineage bifurcation bone marrow cells that either overexpress or have silenced EAR-2, along with their respective vector controls, could be sorted into highly purified progenitor cell subsets and have their differentiation potential assessed in vitro under conditions that favour the growth of multiple cellular lineages. I would predict that overexpression of EAR-2 would divert HSCs toward a lymphoid lineage.
(Figure 3.4, E we observe a decrease in CMP); CLP cells towards a B cell lineage; CMP cells toward an MEP lineage; and MEP cells toward a megakaryocytic lineage.

5.2.1.2 What effect does EAR-2 have on quiescent stem cells:

The effect of EAR-2 overexpression in the most primitive bone marrow stem cell compartment, has not been addressed, specifically the question of whether EAR-2 overexpression preserves, enhances or perturbs quiescence in stem cells. Expression of EAR-2 was very high in hematopoietic stem cells that were induced to cycle, suggesting that EAR-2 overexpression might result in augmented exit of cells from the quiescent state and entry into the cell cycle. This can be tested experimentally by observing the $S$ and $G_2/M$ phases of the cell cycle in pulse labeling BrdU experiments in vivo in KSL cell populations. This effect may be hidden in the analysis of the entire KSL population, which consists predominantly of committed hematopoietic progenitors; it will still give us important information about the kinetics of the KSL cell population. In any case, this uncertainty could be resolved by Pyronin Y (PyY) staining. Pyronin Y specifically and quantitatively stains double stranded RNA, accurately reflecting the $G_0$ phase (224). We will use this reagent to measure the quiescent fraction of cells in the KSL cell population as described (225), anticipating a reduced quiescent (PyY$^-$) fraction and, correspondingly, an increased actively cycling (BrdU$^+$) fraction in the presence of EAR-2 overexpression. If this is the case then we will expect to show increased sensitivity of EAR-2 transduced cells to myelotoxic assault using the drug 5FU. Cycling cells are killed by this agent whereas quiescent cells are not. However, because we hypothesize that a greater fraction of EAR-2 transduced HSCs are actively cycling, we anticipate also a more rapid recovery, although this of course is dependant on the dose of 5FU used. To assess initial sensitivity to the myelotoxic stress, mice can be sacrificed 72 hours post-5FU and the size of the various stem cell populations assessed. Alternatively, it is also possible to conduct this experiment without sacrificing the animals by examining recovery of red blood cells, platelets, and white blood cells (226). The second part of the experiment can assess recovery of the marrow following myelotoxic stress by looking at survival.
5.2.1.3 How does EAR-2 perturb stem cell homeostasis? Identification of the target cell of transformation:

While we have observed an expansion of the KSL cell compartment, precisely how EAR-2 perturbs HSCs is not completely understood. Specifically, we have not shown that the increase in stem cells is the result of increased HSC self-renewal. Therefore, a key question is does EAR-2 increase self-renewal? And if so, does EAR-2 have a direct effect on the self-renewal machinery, activating it de novo? Or is self-renewal intrinsic to the cell population and EAR-2 acts by disrupting homeostasis?

Leukemia stem cells (leukemia initiating cells) can arise, depending upon the nature of the transforming event either from transformation of hematopoietic stem cells or early hematopoietic progenitors (227). The latter case identifies a category of oncogenes with the capacity to reactivate self-renewal, and so may give important insight into the pathogenetic mechanism of an oncogene. For example, in some models mutations must occur in HSCs to be able to initiate transformation. Deficiency of Jun B in LT-HSCs but not more differentiated cells will lead to a transplantable myeloproliferative disorder (228). One possibility is that EAR-2 causes leukemia by transforming an HSC. Our previous studies have shown that HSC homeostasis is deregulated with an increase in the number of HSCs. However, it is also possible and equally interesting that EAR-2 might cause leukemia by bestowing the capacity to self-renew to a committed progenitor cell. Krivstov et al showed that some leukemia oncogenes such as MLL-AF9 are able to convert granulocyte-macrophage progenitors to leukemia initiating cells by re-expression of a subset of HSC genes (229).

To determine which hematopoietic cell population is the one that initiates leukemia in animals that overexpress EAR-2, cells from defined hematopoietic subpopulations isolated based on immunophenotype will be transduced with EAR-2 retrovirus and transplanted into lethally irradiated recipients. Briefly, bone marrow will be infected with EAR-2 or control vectors respectively, and sorted cells into highly purified bone marrow subpopulations, specifically: LT-HSCs, ST-HSC, CLPs, CMPs, GMPs, and MEPs as described (230, 231). These cells can be cultured in methycellulose medium that favours multilineage differentiation (Methocult 3434) to
observe the range of colonies produced in order to validate the identities of the purified subsets and to assess their clonal longevity in serial passaging experiments, asking whether the various populations are amenable to transformation *in vitro*. In tandem, the BM subset can be injected into lethally irradiated recipient animals along with a dose of whole bone marrow cells that will be used for radioprotection using different isoforms of CD45 to discriminate between donor cells (Ly5.1), support cells (Ly5.1 x Ly5.2) and recipient cells (Ly5.2). The endpoint of this study will be development of leukemia determined by overall survival and post-mortem examination of BM recipients.

5.2.1.4 Does silencing of EAR-2 induce differentiation in human AML and MDS?

The question of whether EAR-2 knockdown is able to induce differentiation of human leukemia and preleukemia cells is very intriguing. I was able to optimize conditions so that I could test this question: I first devised a method of culturing primary MDS patient samples, I then optimized creation of viral stocks of sufficient titer to infect primary bone marrow cells (Appendix E) as infection of primary cells, especially human bone marrow, is very difficult. I was able to create high titer retroviral and lentiviral stocks.

While primary cells are difficult to culture I have optimized conditions that have allowed for the maintenance of MDS cells in culture for a period of greater than 4 months. Figure 5.1 shows a cytospin of cells from an MDS patient that had been maintained for 4 months in culture (growing at an exponential rate) using the conditions proposed here. Progenitor cells and blasts will be enriched for by negative selection using RosetteSep concurrent with Ficoll-Paque gradient centrifugation. Mononuclear cells will be cultured on a monolayer of irradiated OP9 cells in IMDM medium supplemented with 30ng/mL TPO, 30ng/mL rhIL-11, 30ng/mL rhFlt-3 ligand, 50ng/mL rhG-CSF, 30ng/mL rhGM-CSF, 30ng/mL rh SCF, 10ug/mL insulin, 5ug/mL transferrin, 0.5% BSA Fraction V, 5% FBS; herein referred to as MDS cell medium. Finally, cultures will be maintained in a nitrogen incubator under hypoxic conditions (2% oxygen).

In the future, patient samples will be infected with either shEAR hairpin1, shEAR hairpin2 or the scrambled control respectively as previously described (232) and select for
Figure 5.1. Long term culture of MDS patient samples. A cytospin from bone marrow from a patient with myelodysplastic syndrome that was cultured in vitro for more than four months.
transduced cells with puromycin. Cells will be cultured and assessed for maturation twice a week until terminal differentiation occurred. Flow cytometry will be used to look for maintenance of the primitive phenotype based on expression of CD34, CD38, and CD117. Maturation will be assessed by expression of CD41 and CD61 for megakaryocyte; glycophorin A+, CD45- for erythrocytes; CD33+, CD45+ and CD14+, CD45+ for myeloid differentiation. Flow cytometry will be complemented by observing for morphological differentiation on cytospins stained with May-Grunwald Giemsa. Differentiation will be confirmed by nitroblue tetrazolium staining for myeloid differentiation and with benzidine staining for erythroid differentiation. Based on our knockdown experiments in cell lines and mouse bone marrow (Chapter 2), we would anticipate induction of terminal differentiation followed by a wave of massive cell death. Cell growth rates will be monitored at time of passage by trypan blue exclusion and apoptosis will be assessed bi-weekly by flow cytometry for annexin V-PE and 7AAD and active caspase-3-PE. The ability to differentiate will also be measured by culture in Methocult H4435, methycellulose medium that favours multilineage differentiation of enriched populations.

5.2.1.5 Elucidation of the molecular mechanisms of EAR-2 function?

While we have shown that EAR-2 abrogates differentiation in a DNA binding dependent fashion, other biological functions of EAR-2 have been described as being mediated via protein-protein interactions. Since COUP family transcription factors functions in both a DNA dependent and DNA independent fashion, both the proteins with which EAR-2 interacts with as well as the genomic loci at which EAR-2 binds to are of interest.

5.2.1.5.1 Identification of EAR-2 interacting proteins:

EAR-2 has been shown to bind to and repress the transactivation ability of several transcription factors. Elucidation of the proteins with which EAR-2 interacts with would allow us to determine downstream molecular targets of EAR-2.
Yeast two hybrid may be used to identify EAR-2 interacting proteins. One could use the Matchmaker Gold system by Clontech. Domains spanning the length of the EAR-2 protein, as well as the full length protein, will be fused to the GAL4 DNA-binding domain (DNA-BD) and used as bait in parallel. These will be mated to the “Mate & Plate” pretransformed Human Bone Marrow library of prey proteins that are fused to the GAL4 activation domain (AD). “Mate & Plate” libraries are advantageous because they have been normalized such that abundant cDNAs are reduced and rare sequences are enriched. These libraries have been transformed into the \( \text{MAT} \alpha \) haploid yeast strain, that is compatible for mating with the yeast \( \text{MAT}a \) reporter strain used to express the bait constructs. Upon interaction of the EAR-2 bait with proteins from the prey library, the GAL-4 DNA-BD will cooperate with the AD to activate transcription of four independent reporter genes. The Matchmaker Gold system has two advantages over traditional Y2H. First is the use of the \( \text{AUR1-C} \) gene as a reporter which confers resistance to the antibiotic Aureobasidin A. Use of antibiotic resistance as a reporter is much more definitive, and requires less optimization than nutritional selection. Furthermore the system uses four reporter genes: \( \text{AUR1-C}, \text{ADE2}, \text{HIS3}, \text{and MEL1} \). Hits that will be considered for validation by co-immunoprecipitation will be selected based on known biological roles: Of particular interest are transcription factors and co-activators and co-repressors.

One potential problem may be that as a transcriptional regulator it is possible that EAR-2 will autoactivate or autorepress the reporter. This is why all bait constructs will be tested for autoactivation prior to mating. Constructs that autoactivate the reporters will not be used. Bait domains will be further refined for removal of sequences responsible for autoactivation. Our strategy that employs using multiple domains of EAR-2 as a bait protein will increase the likelihood of finding a reliable bait should autorepression be a problem.

We feel that screening a library derived from bone marrow cells is an appropriate place to begin the screen, giving us more specificity given the hematopoietic phenotype that we are investigating. To increase the depth of screening we could employ the “Mate & Plate” Universal Human Library or the Mate & Plate” HeLa S3 Library.

Identification of EAR interacting proteins may also be achieved using protein mass spectrometry based methods. Mass spectrometry is a powerful tool that is used to determine protein identity. A biological sample is processed using affinity chromatography based
techniques to isolate a protein complex, which is separated into individual proteins using two dimensional gel electrophoresis. Isolated proteins are proteolytically digested and the identity of the peptides determined using mass-spectrometry.

5.2.1.5.2 Epigenetic alterations induced by EAR-2:

I propose that EAR-2 blocks differentiation by altering the epigenetic landscape. Specifically, EAR-2 may shut off genes necessary for lineage commitment through the recruitment of histone deacetylases, biasing the methylation status of bivalent genes towards a state of gene repression. Like other nuclear repressors, we have observed that EAR-2 function is dependent on DNA-binding and the recruitment of HDAC. The inhibition of myeloid cell differentiation by PML-RARα is dependent on global deacetylation of histone 3 (H3K9K14ac) which becomes acetylated upon treatment with retinoic acid (233). Modification of histone marking by the methylation of lysines is necessary for the maintenance of changes in acetylation status. In addition, we observe a much more severe phenotype than that of the PML-RARα transgenic mouse (234), with a profound stem cell phenotype. In hematopoietic stem and progenitor cells, trimethylation of H3 lysine 27 (H3K27me3) and trimethylation of H3 lysine 9 (H3K9me3) is associated with genes that are in a repressed state, while trimethylation of H3 lysine 4 (H3K4me3) is associated with gene activation (235). However, pluripotent cells contain DNA regions where histones are marked by modifications indicative of both activate and repressed genes, these bivalent genes are highly enriched in lineage specific genes, however they are transcriptionally silent. The presence of histone modifications indicative of activation in said genes is thought to poise the undifferentiated cell in an epigenetic state able to commit to a specific lineage. Upon differentiation of human hematopoietic stem into cells of a single lineage (erythroid) there is activation of genes necessary for commitment to the specific lineage (associated with loss of H3K27me3), but also repression of all other possible cell lineage fates (associated with loss of H3K4me3) (235).

The mechanism by which EAR-2 blocks differentiation is not known. Given the role we have observed of EAR-2 in the lineage determination of cells, we hypothesize that EAR-2 binds
to the promoters of bivalent genes and acts as a transcriptional repressor. We wish to address whether this is in fact the case, as well as to determine how it turns off the genes. In the setting of leukemia, does EAR-2 enrich bivalent genes in those histone modifications associated with transcriptional repression, specifically H3K27me3 and H3K9me3 thereby allowing the cell to maintain the H3K27me3 modification hence, allowing them to continue to be poised for possible differentiation? Or does EAR-2 act to silence lineage specific genes in a way similar to the repression of alternate cell fates during terminal differentiation by the removal of H3K27me3? One would envision that the consequences of the latter would be more severe, placing greater constraints on the differentiation ability of the cell. Additionally, we will verify the consequences of the epigenetic changes by looking for DNA occupancy of Polymerase II, which is much more sensitive than looking at changes in RNA. Finally, we know that transcription factors act in complexes, and that EAR-2 inhibits RUNX1. To study the consequences of the epigenetic changes that EAR-2 induces on transcription factor networks we will ask: Does EAR-2 share DNA binding sites with RUNX1, and are these sites subject to epigenetic changes that silence the transcriptional ability of RUNX1.

These studies will help us identify the EAR-2 DNA binding motifs and by coupling these data with data from gene expression profiling using microarrays, we will be able to identify DNA targets of EAR-2.

5.3 EAR-2 expression impairs T cell development:

5.3.1 Significance of data:

We have found a novel gene that regulates the formation of T cells. I have shown that expression of the orphan nuclear receptor EAR-2 severely impairs the development of T cells, suggesting that expression must be decreased in order of T cell lymphopoiesis to proceed. Elucidation of the molecules involved in the formation of T cells is important for understanding of immunology and possible clinical intervention of immunological diseases.
5.3.2 Physiological relevance:

The question of the physiological role of EAR-2 overexpression in blocking lymphopoiesis of T cells is indeed intriguing. Nevertheless, this should not be a surprising observation since the ability of nuclear receptor expression to antagonize T cell development and induce thymic atrophy is well entrenched in the immunological psyche. The ability of EAR-2 to repress the function of other nuclear receptors, including retinoic acid signaling, implies that the phenotype may be partially mediated by inhibiting the anti-apoptotic affects of retinoic acid in activation induced cell death of developing thymocytes. Nevertheless, this only partially explains the phenotype as it does not account for the defect in proliferation observed in early thymic progenitor cells.

Thymic seeding progenitor cells enter the thymus, giving rise to early T cell progenitor cells, multipotent progenitor cells that begin commitment toward the T cell lineage. The fact that these are multipotent cells is very intriguing because essentially what is observed is that in the presence of signals that instruct a cell to progress down a path of T cell development, the cells drastically diminish in number. It is interesting to ponder why the cells not adopt an alternative cell fate, or maintain their current cell lineage of being an undifferentiated progenitor cell. Why do the cells stop proliferating in T cell conditions? What is the nature of this arrest? Is it merely that the microenvironment does not provide the cells with sufficient growth factor stimulation to maintain or adopt a different cellular identity, or is it that there is something toxic to these cells about thymic stimulatory signals? Has the cell’s circuitry been affected such that signals that would otherwise prescribe proliferative now indicate proliferative arrest? And if so, which stimuli might it be? I would predict that Notch signaling would be the critical stimulus, as Notch has been reported to induce proliferative arrest in cancer cells (149, 236-239).

In accordance with the observations made in other hematopoietic cells, it is hypothesized that EAR-2 expression serves as a switch that regulates lineage bifurcation. Many questions arise with respect to the T lymphocyte microcosm: What is the effect of EAR-2 on CD-4 and CD-8 lineage commitment? We observed a further decrease in EAR-2 expression in CD8 single positive cells suggesting that EAR-2 may be involved in CD4 versus CD8 selection. I hypothesize that overexpression of EAR-2 would impair the generation of CD8 SP cells, as observed in the RORγt animal. Another example of EAR-2 expression functioning as a switch
that mediates lineage selection is observed in the generation of Th17 cells, whereas expression of EAR-2 prevents the differentiation of T0 cells to Th17 cells (183). The increase in B lymphocytes that were observed in vivo (Chapter 3) suggests that EAR-2 may have a role in the bifurcation of the common lymphoid progenitor. It would be interesting to assess this directly, either by functional assays in conditions that allow for the differentiation of both lineages or molecularly through gene expression profiling of EAR-2 overexpressing CMP versus controls.

Squelching is a phenomenon in which overexpression of a nuclear receptor binds to co-activators or co-repressors, thereby universally inhibiting nuclear receptor responses. Since we only observe a block in T cell development in animals transplanted with bone marrow that overexpresses EAR-2, and EAR-2 overexpression is similar to RORγt overexpression but no phenotype is reported in the EAR-2 knockout, suggests that these phenomena are associated with overexpression. While squelching is indeed one scenario that might explain the observation, there are several other explanations. The fact that we observe a highly reproducible block of differentiation only in select lineages at specific time points would suggest that this effect is not squelching, as squelching would have a more generalized phenotype, not specific to the DN to DP transition of the T cells. The fact that both overexpression and knockdown of RORγt inhibited T-cell development does not imply that the overexpression phenotype is an artifact, rather it merely suggests that precise regulation of this gene is necessary for T cell development.

5.3.3 Future directions:

5.3.3.1 Mechanism:

Ultimately, the mechanism mediating the severe block in T cell development is worth elucidating. It is likely that EAR-2 functions at many levels of the developing T cell, and hence impinges on various processes. It is likely that the phenotype will not be explained by a single mechanism.

Furthermore, it is not as simple as merely performing expression profiling to elucidate differences in gene expression as one must take into account that the genes that regulate T cell development are differentially regulated in each specific thymocyte subpopulations. For example
BCL11B is expressed exclusively in T cells but does not get turned on until the DN2 subpopulation (27). Therefore, expression profiling needs to compare those specific thymocyte populations in which an effect was observed. Of particular interest is the early T cell progenitor cell, and the DN4/DP cells.

In addition to the proposed Notch induced cell cycle arrest, and phenocopies of RORγt, several other candidate interactors are discussed below.

5.3.3.1.1 GATA3:

Gata3 is another transcription factor that could be involved in the very early decrease in cell numbers observed. Gata3 is expressed in the earliest T cell progenitors, as early as the HSC (240). Decreased expression of Gata3 using either genetic models (241, 242) or in vitro culture (243) showed a reduction in early thymic progenitor cells, similar to the reduction that we observed. The reduction was attributed to the ability of TSP to differentiate into ETP cells, as no differences were observed in cell cycle or apoptosis.

5.3.3.1.2 RORγt:

The similarity between the RORγt transgenic mouse and overexpression of EAR-2 was discussed in Chapter 4. The similarity in the phenotype of EAR-2 overexpression and RORγt overexpression suggests either common downstream pathways or that these molecules belong to the same pathway. Future work would address some of the following questions: Does EAR-2 regulate RORγt expression, and if so under what circumstances? Does EAR-2 bind to its promoter? Does it bind to it directly, and hence recruit co-repressors or coactivators? Does EAR-2 bind to RORγt response elements, directly to DNA or by tethering to RORγt or other molecules in the complex? Are common downstream genes affected including FasL or c-Rel, for example?
5.3.3.1.3 RUNX3:

It is relevant to discuss RUNX because EAR-2 putatively disrupts the transcriptional activation of RUNX (105). Whether, due to sequence homology, EAR-2 also binds to and represses the transactivating ability of RUNX3 is yet to be determined. RUNX proteins have important roles in regulation of T cell development—albeit, they are best known for processes that would not explain the phenotype observed upon EAR-2 overexpression. RUNX3’s effect on Th-POK is critical in the decision of a cell to commit to either the CD4+ or CD8+ T cell lineage (244, 245). Expression of Th-POK seals cells into commitment to the CD4 cell fate (246). RUNX3 silences expression of Th-POK by sitting on the Th-POK silencer thereby repressing CD4+ cell fate choice and committing the cell to a CD8+ cell fate (247). RUNX3 directly binds to the enhancer region of CD8 (248). Interestingly, we observed a decrease in EAR-2 expression in CD8+ SP cells. Presuming that expression of EAR-2 needs to decline in order for CD8+ cell differentiation, in other words, presuming that overexpression of EAR-2 inhibits CD8+ T cell differentiation, one might envision a mechanism by which EAR-2 expression inhibits the transcriptional activity of RUNX3 thereby allowing transcription of Th-POK, that would commit cells to a CD4+ cell fate. RUNX3 is also needed to facilitate differentiation of T helper cells by the regulation of IL4 (249-251) and production of IFNγ (251). By silencing transcription of IL-4 in T-helper cells it acts to facilitate the differentiation of cells into Th1 type T-helper cells, as opposed to Th2 CD4+ cells that secrete IL-4. RUNX3 also transcriptionally activates production of IFNγ. It would be interesting to determine whether EAR-2 expression serves to drive cells toward a Th2 T-helper cell fate, via repression of RUNX3 transcriptional activity.

The ability of EAR-2 to inhibit the differentiation of Th0 cells into Th17 cells due to a decrease in production of IL-17 is well established (183), with RORγt being necessary for the ability of Th0 cells to differentiate into Th17 cells (223). Even though these genes share similar downstream mechanisms they have opposing roles in Th17 cell differentiation and activation. It would be interesting to assess whether this discrepancy can be explained by the ability of EAR-2 to repress the transcriptional activity of RUNX1, as RUNX1 acts co-operatively with RORγt in the production of IL-17 (252).

Furthermore, RUNX1 has important roles in the production of IL-2. It is unclear why the status of CD4+ CD25+ regulatory T cells (T_{reg}) were not assessed in the EAR-2 knockout
animal—I would predict that EAR-2 would inhibit IL-2 production, both via suppression of NFAT (183) and by binding to and interfering with RUNX1’s transcriptional activation of IL-2 (253) would imply that deletion of EAR-2 would increase IL-2 production, and hence decrease the number of T_reg cells. Perhaps the autoimmunity observed in the EAR-2 knockdown animal is not attributed solely to the increase in Th17 cells but also to a decrease in T_reg cells.

5.3.3.1.4 CTIP2/BCL11B:

Coup-TF Interacting Protein 2 (CTIP2), also known as BCL11B is Kruppel-like zinc finger transcription factor that was identified based on binding to the COUP TF and has been shown to interact will all members of the COUP-TF family including EAR-2 (254). BCL11B is a key regulator of T cell differentiation. This gene is fascinating because it functions to downregulate stem cell genes, foreclosing on the undifferentiated state, and committing the cell to the T cell lineage (25-27). Expression of BCL11B is necessary for maintenance of T cell identity, with DN2 cells adopting an NK cell fate even after commitment to the T cell lineage that takes place at the DN2 early-to-late stage in animals with targeting deletions of this gene. While it would be tempting to speculate that BCL11B may be involved in mediating part of the phenotype of EAR-2 overexpression in T cells by repressing BCL11B expression or function, BCL11B is not expressed in early thymic progenitor cells (27). Hence, it is not likely to contribute to the early defect in T cell lymphopoiesis. BCL11B-/- animals also showed a block in progression from DN2 to DN3 (255), which is not what we observed in OP9-DL1 cultures.

5.3.3.2 Clinical implications:

The ability of EAR-2 expression to inhibit T cell development suggests that an EAR-2 agonistic ligand, one that functions to turn on this gene’s transcriptional regulatory abilities, has the potential to be used clinically for purposes of immunomodulation. Granted, there are many outstanding questions: First is the question of the usefulness of a compound that inhibits T cell development, a process that is completed for the most part by puberty, versus T cell activation. While we have shown that EAR-2 inhibits T cell development, we have not shown that EAR-2
expression inhibits T cell activation. Nevertheless, it is very likely that this is the case. Others have shown that EAR-2 potentially inhibits production of IL-2 and IL-17, the former being necessary for T cell activation, which the latter being necessary for activation of Th17 cells, cells which have been implicated in autoimmunity. Furthermore, there may be situations in which it may be desirable to destroy T cells indiscriminately, for example, as a strategy for the prevention of graft-versus host disease following bone marrow transplantation; or as an alternative to thymectomy in patients with Myasthenia gravis. Nevertheless, the most formidable problem to overcome would be the following: If EAR-2 overexpression blocks T cell development as well as causes leukemia, how could an EAR-2 agonistic be developed into a drug that can be safely administered without causing leukemia and wrecking havoc on the hematopoietic system? The solution to this conundrum would be to develop a molecule that serves as a partial agonist. That is, a molecule that turns on those genes that regulate T cell development but that does not turn on the genes necessary for blocking the differentiation of stem cells and leukemogenesis.

5.4 Looking Forward: Identification of an EAR-2 modulator for therapeutic purposes

Nuclear receptors are druggable targets that provide a unique opportunity for the therapeutic manipulation of gene transcription. They function as ligand-responsive transcriptional switches regulated by ligands: Small diffusible lipopholic molecules. The use of natural, or synthetic ligands has been used therapeutically to alter nuclear receptor responses across the spectra of disorders.

The observation that knockdown of EAR-2 induces differentiation in leukemia cell lines is proof of the principle that an EAR-2 antagonist could be used as differentiation therapy in leukemia and MDS. Intriguingly, there might even be a role for EAR-2 in resistance to CML as preliminary experiments show that EAR-2 is downregulated upon treatment with tyrosine kinase inhibitors such as Gleevec (Figure 5.2). This preliminary data begs the question: is EAR-2 involved in resistance to imatinib? And could resistance of CML stem cells to imatinib be mediated by their high EAR-2 expression?
In any case, there are numerous methods of identifying nuclear receptor ligands. One method is to perform high-throughput screening of libraries for identification of lead compounds using a cell based assay where cultured mammalian cells are transfected with a vector encoding for EAR-2 as well as a reporter construct, cells are treated with candidate compounds and assessed for the activity of the reporter construct. More specifically, this could be conducted using the ligand binding domain of EAR-2 fused to the Gal4 DNA binding domain; another method is to conduct virtual screening with the crystal structure of EAR-2 (that has yet to be solved) and high-throughput molecular docking using a compound database; yet another method is to screen for the ability of compounds to induce EAR-2 interaction with co-activator or co-repressor molecules, detected by the generation of a fluorescence signal from energy transfer between fluorescent protein using a fluorescence resonance energy transfer (FRET) based method such as the AlphaScreen assay (Amplified Luminescence Proximity Homogeneous Assay). Yet another approach would be to use a direct binding method where EAR-2 is used as bait to identify compounds it interacts with using mass spectrometry.

The skill comes in identifying compounds with sufficient target specificity and minimal toxicity. EAR-2 is known to be a regulator of nervous system development. The EAR-2 knockout mouse has a neurodevelopmental defect in the locus coeruleus that results in altered pain perception and altered circadian rhythms. Hence, in order to develop an anti-cancer drug that works by altering the activity of EAR-2 in hematopoietic cells but not the nervous system, a selective agonist should be developed.

5.4.1 Development of partial agonists/antagonists:

Development of partial EAR-2 agonists and antagonists would allow for the generation of drugs that are not only effective but that also have sufficient specificity and minimal toxicity. To
Figure 5.2. Tyrosine kinase inhibitors regulate EAR-2 expression. K562 cells were treated with a variety of tyrosine kinase inhibitors, expression of EAR-2 mRNA was assessed 48 hours later.
achieve this end it is necessary to know what the appropriate receptor responses are which need to be modulated. Hence, a systematic approach is necessary as that would serve to generate cartography of sufficient resolution to elucidate the molecular differences that distinguish distinct biological outcomes.

We must understand the differences in protein-protein interactions, hormone response element binding, and cofactor interactions between the different aspects of the phenotypes. Nuclear receptors have cell specific effects that are mediated by expression of cell specific molecular entities. Not only will it be important to determine which co-factors and genomic loci EAR-2 binds to, but it will be useful to determine this in each specific T cell subset, and each hematopoietic subset in which EAR-2 exerts an effect. Ideally, to circumvent off target effects this work would need to be repeated with the two other mammalian paralogues, COUP-TFI and COUP-TFII as well. The degree of homology in the ligand binding domain suggests that a molecule that binds to EAR-2 would also likely bind to the COUP-TFI and COUP-TFII LBD, this is a minor setback that can be overcome by exploiting the differences between the three proteins, and searching for drugs with sufficient specificity to EAR-2.

The work presented in this thesis introduces a novel character to the dramatic story of neoplastic hematopoiesis. EAR-2, an orphan receptor, entrusted with the noble role of deciding the fate of hematopoietic cells. In itself, not sinister at all, and yet overexpression of EAR-2 is sufficient to initiate a rapidly lethal leukemia. If this were a work of literature, EAR-2 would be the classical tragic hero, yet therein resides the beauty, as the fate of this character is not sealed but rather may be changed by the elucidation of partial EAR-2 agonists. The data presented here lies the ground work for the design of drugs that can be used to induce differentiation of leukemia, expansion of bone marrow stem cells, or inhibition of T-cell development. These molecules will not only be effective at regulating EAR-2 function, but can also be designed to be tissue, target and cell type specific. The task is not a trivial one, but one definitely worth embarking upon!
References


42. Davies AR, Schmitt RG. AUER BODIES IN MATURE NEUTROPHILS. *Journal of the American Medical Association* 1968; 203(10): 895-&.


85. Blair A, Hogge DE, Sutherland HJ. Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(-)/HLA-DR. *Blood* 1998 Dec 1; 92(11): 4325-4335.


111. Blair A, Hogge DE, Sutherland HJ. Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(-)/HLA-DR. *Blood* 1998; 92(11): 4325-4335.


135. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations of AML1 are common in therapy-related myelodysplasia following therapy with alkylating agents and are significantly associated with deletion or loss of chromosome arm 7q and with subsequent leukemic transformation. *Blood* 2004 Sep 1; 104(5): 1474-1481.


207. Ichim CV, Atkins HL, Iscove NN, Wells RA. Identification of a role for the nuclear receptor EAR-2 in the maintenance of clonogenic status within the leukemia cell hierarchy. Leukemia Jun 3.


Appendices
Revisiting immunosurveillance and immunostimulation: Implications for cancer immunotherapy

Christine V Ichim*1,2,3

Address: 1Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada, 2Department of Molecular and Cellular Biology, Sunnybrook and Women's College Health Sciences Centre, University of Toronto, 2075 Bayview Avenue, Toronto, Ontario, M4N 3M5 Canada and 3MedVax Pharma Corp, 126 Madison Avenue South, Kitchener, ON N2G 3M6, Canada

Email: Christine V Ichim* - christine.ichim@swri.ca

* Corresponding author

Abstract

Experimental and clinical experience demonstrates that the resolution of a pathogenic challenge depends not only on the presence or absence of an immune reaction, but also on the initiation of the proper type of immune reaction. The initiation of a non-protective type of immune reaction will not only result in a lack of protection, but may also exacerbate the underlying condition. For example, in cancer, constituents of the immune system have been shown to augment tumor proliferation, angiogenesis, and metastases. This review discusses the duality of the role of the immune system in cancer, from the theories of immunosurveillance and immunostimulation to current studies, which illustrate that the immune system has both a protective role and a tumor-promoting role in neoplasia. The potential of using chemotherapy to inhibit a tumor-promoting immune reaction is also discussed.

If only it were all so simple. If only there were evil people somewhere insidiously committing evil deeds, and it were necessary only to separate them from the rest of us and destroy them. But the line dividing good and evil cuts through the heart of every human being, and who is willing to destroy his own heart?

Alexander Solzhenitsyn, The Gulag Archipelago

The notion that the immune system may be manipulated into recognizing and eradicating neoplasia is not new. Heroic efforts to develop a cancer vaccine can be traced as far back as 1777 when the surgeon to the Duke of Kent injected himself with malignant tissue as a prophylaxis against development of cancer. In 1808, another attempt was made to develop a cancer vaccine by the doctor to Louis XVII who inoculated himself with breast cancer in hope of reversing a soft-tissue sarcoma, although no therapeutic effect was observed. However, it was not until 1891 that the first report of successful immunotherapy was published by William Coley, a clinician at the Memorial Sloan Kettering Cancer Institute in New York. Using heat-killed endotoxin-containing bacteria (streptococci and Serratia marcescens), Coley was able to achieve a cure rate of 10% in soft-tissue sarcoma [1,2]. Nevertheless, despite the numerous attempts over the past centuries to use the immune system in the eradication of cancer, the success rate of cellular immunotherapy remains abysmally low.

In light of the successes in the development of vaccines targeting pathogenic agents, this review suggests that lessons learned from the immunology of infectious disease may be applicable to the treatment of neoplasia. The immunology of infectious disease teaches that the clearance of a pathogenic challenge requires the initiation of...
Primary cancer cells exhibit heterogeneity in their proliferative ability. The cancer stem cell (CSC) model accounts for this heterogeneity by proposing that each cancer consists of a small population of CSCs that are capable of unlimited growth and self-renewal and a much larger population of cells, descendants of the CSCs, that have lost self-renewal capacity. The CSC model has important implications for cancer therapy. Eradication of CSCs, the cells responsible for maintenance of the neoplasm, would be necessary and sufficient to achieve cure. By extension, both the frequency of stem cells in a tumor and their propensity to undergo self-renewal ($P_s$) would have a direct impact on the curability of that tumor. The $P_s$ is a critical biological characteristic of CSCs—small differences in $P_s$ have enormous impact on the probability of success in cancer therapy. Differentiation therapy, defined as treatment that reduces the $P_s$ of CSCs, is one approach to targeting CSCs.

Keywords: Cancer stem cells, differentiation, leukemia, cell proliferation, self-renewal, hierarchy

Introduction

Heterogeneity is a hallmark of cancer that is apparent both across the spectra of cancer patients and within the neoplasm of an individual patient. Unlike immortalized cell lines, primary cancer cells are heterogeneous with respect to the number of cell divisions that they are able to execute. Most primary cancer cells, irrespective of their primary site, will not proliferate in vitro. In fact, only a tiny minority of cancer cells can execute sufficient divisions to form a colony in semi-solid medium and a still smaller minority can regenerate a tumor in vivo.

According to the cancer stem cell (CSC) model, cancer is organized in a cellular hierarchy with differences in the differentiation status of the constituent cells of the neoplasm comprising the basis of this cellular heterogeneity (Figure 1). At the apex of the hierarchy is the CSC, a rare cell with indefinite self-renewal and proliferative ability. The CSC may divide to give rise to another CSC (a process known as self-renewal) or it may initiate a program that culminates in differentiated cancer cells that can no longer undergo mitosis. The process of cancer cell differentiation gives rise to an intermediate population of cells, descendants of the CSC that have lost self-renewal capacity and that are able to proliferate for a finite number of cell divisions. Once these cells have executed their allotted number of cell divisions they enter a terminal post-mitotic state. This forms the basis of the cellular hierarchy: rare CSCs with indefinite proliferative ability irreversibly differentiate into non-stem cancer cells with limited proliferative ability; these cells in turn ultimately differentiate to give rise to a much larger population of cells that can no longer proliferate. It is important to specify that while these cells have differentiated, in the sense that they can no longer undergo mitosis, they remain morphologically and functionally immature in comparison to normally differentiated cells of the tissue from which the cancer arose. Furthermore, it is also important to note that even though CSCs are the cells situated at the apex of the cellular hierarchy, recent evidence using retrovirally marked human...
Case report
Progression of myelodysplasia to acute lymphoblastic leukaemia: Implications for disease biology
Patricia Disperati a, b, Christine V. Ichim c, d, Douglas Tkachuk e, f, Kathy Chun e, f, Andre C. Schuh a, c, g, Richard A. Wells c, d, g, *

a Department of Medical Oncology and Hematology, Princess Margaret Hospital, University Health Network, Toronto, Ont., Canada
b Institute of Medical Sciences, University of Toronto, Toronto, Ont., Canada
c Department of Medical Biophysics, University of Toronto, Toronto, Ont., Canada
d Myelodysplastic Syndrome Program, Toronto Sunnybrook Regional Cancer Centre, Medical Oncology and Haematology, 2075 Bayview Avenue, T-Wing, T2-058 Toronto, Ont., Canada M4N 2M5
e Toronto Medical Laboratories, Princess Margaret Hospital, University Health Network, Toronto, Ont., Canada
f Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Ont., Canada
g Department of Medicine, Faculty of Medicine, University of Toronto, Toronto, Ont., Canada

Received 16 June 2005; received in revised form 16 June 2005; accepted 16 June 2005
Available online 25 July 2005

Abstract
Myelodysplastic syndrome (MDS) comprises a group of clonal haematopoietic disorders characterized by peripheral blood cytopenias, bone marrow hypercellularity, and abnormal blood cell differentiation. Approximately 30% of cases of MDS eventually progress to acute myelogenous leukaemia (AML), while progression of MDS into acute lymphoblastic leukaemia (ALL) is rare. In this report, we describe a case of MDS that progressed to ALL, and review the 21 previously reported cases of MDS to ALL transformation. We review the cancer stem cell (CSC) model and its application to these disorders, and discuss the implications of the rarity of transformation of MDS to ALL for the biology of MDS and the pathogenesis of ALL.

Keywords: Myelodysplastic syndrome; Acute lymphoblastic leukaemia; Acute myelogenous leukaemia; Stem cell; Leukaemogenesis; Progression

1. Introduction
Primary cancer cells exhibit heterogeneity in clonogenicity, a measurement of the capacity to proliferate and form colonies in vitro. The cancer stem cell (CSC) model accounts for this cellular heterogeneity by proposing that each malignancy consists of a small population of cells capable of unlimited growth and self-renewal, known as CSCs, and a much larger population of cells, descendants of the CSCs, that have lost self-renewal capacity and are hence undergoing terminal differentiation. Evidence supporting this model has been reported for several malignancies including leukaemia [1], brain cancer [2, 3] and breast cancer [4]. The CSC model has important implications for cancer therapy; eradication of CSCs, the cells responsible for maintenance of the neoplasm, would be necessary and sufficient to achieve cure.

Myelodysplastic syndrome (MDS) is an acquired clonal haematopoietic stem cell disorder. Unlike AML, the abnormal haematopoietic clone in MDS gives rise to mature blood cells, although these cells are functionally and morphologically abnormal. In approximately 30% of cases MDS progresses to acute myelogenous leukaemia (AML), resulting presumably from the acquisition of additional cooperating mutational or epigenetic changes. For this reason MDS has been thought of as a preleukaemic state, and a recognisable intermediate stage in a multi-step pathogenesis of acute leukaemia. Questions concerning the biology of the MDS stem cell, and the nature of the events resulting in progression of MDS to acute leukaemia, are therefore of great interest. In
Identification of a role for the nuclear receptor EAR-2 in the maintenance of clonogenic status within the leukemia cell hierarchy

CV Ichim1,2, HL Atkins3, NN Iscove1,4,5,6,9 and RA Wells1,2,7,8,9

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

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 32Dc13 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. Leukemia (2011) 25, 1687–1696; doi:10.1038/leu.2011.137; published online 3 June 2011

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 clonal 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.
Generation of high-titer viral preparations by concentration using successive rounds of ultracentrifugation

Christine V Ichim¹,² and Richard A Wells¹,²,³,⁴*

Abstract

Background: Viral vectors provide a method of stably introducing exogenous DNA into cells that are not easily transfectable allowing for the ectopic expression or silencing of genes for therapeutic or experimental purposes. However, some cell types, in particular bone marrow cells, dendritic cells and neurons are difficult to transduce with viral vectors. Successful transduction of such cells requires preparation of highly concentrated viral stocks, which permit a high virus concentration and multiplicity of infection (MOI) during transduction. Pseudotyping with the vesicular stomatitis virus G (VSV-G) envelope protein is common practice for both lentiviral and retroviral vectors. The VSV-G glycoprotein adds physical stability to retroviral particles, allowing concentration of virus by high-speed ultracentrifugation. Here we describe a method report for concentration of virus from large volumes of culture supernatant by means of successive rounds of ultracentrifugation into the same ultracentrifuge tube.

Method: Stable retrovirus producer cell lines were generated and large volumes of virus-containing supernatant were produced. We then tested the transduction ability of virus following varying rounds of concentration by ultracentrifugation. In a second series of experiments lentivirus-containing supernatant was produced by transient transfection of 297T/17 cells and again we tested the transduction ability of virus following multiple rounds of ultra-centrifugation.

Results: We report being able to centrifuge VSV-G coated retrovirus for as many as four rounds of ultracentrifugation while observing an additive increase in viral titer. Even after four rounds of ultracentrifugation we did not reach a plateau in viral titer relative to viral supernatant concentrated to indicate that we had reached the maximum tolerated centrifugation time, implying that it may be possible to centrifuge VSV-G coated retrovirus even further should it be necessary to achieve yet higher titers for specific applications. We further report that VSV-G coated lentiviral particles may also be concentrated by successive rounds of ultracentrifugation (in this case four rounds) with minimal loss of transduction efficiency.

Conclusion: This method of concentrating virus has allowed us to generate virus of sufficient titers to transduce bone marrow cells with both retrovirus and lentivirus, including virus carrying shRNA constructs.

Introduction

Viral vectors are commonly used to introduce exogenous genetic material in experimental systems, and have been used successfully in human gene therapy trials to treat patients with primary immunodeficiencies such as X-linked severe combined immunodeficiency (SCID [1-3] and adenosine deaminase deficiency [1-3]. Suitable vectors frequently used in the laboratory and clinical setting include retroviral and lentiviral vectors. However, the ability to transduce difficult-to-infected cells such as primary hematopoietic cells, hematopoietic stem cells, and neuronal cells with these vectors is dependent on the ability to produce stocks of high viral titers [4,5]. Retro- and lentivirus is produced by transfecting producer cell lines with viral plasmids resulting in the production of virions that are released into the supernatant. Target cells may be transduced using the supernatant or alternatively by using supernatant that has been
MODULATION OF NR2F6 AND METHODS AND USES THEREOF

Inventors: Christine Victoria Ichim, Kitchener (CA); Richard Alexander Wells, Toronto (CA)

Correspondence Address:
BERESKIN AND PARR LLP/S.E.N.C.R.L., s.r.l.
40 KING STREET WEST, BOX 401
TORONTO, ON M5H 3Y2 (CA)

Appl. No.: 12/619,290
Filed: Nov. 16, 2009

Related U.S. Application Data
Provisional application No. 61/114,764, filed on Nov. 14, 2008.

Publication Classification
Int. Cl.  
A61K 39/395 (2006.01)  
C12N 5/02 (2006.01)  
A61K 31/7088 (2006.01)  
C12Q 1/02 (2006.01)  
C07H 21/02 (2006.01)

U.S. Cl. ................. 424/130.1; 435/377; 435/375; 514/44 R; 514/44 A; 435/29; 536/23.1

ABSTRACT
The disclosure provides methods of modulating NR2F6 in a cell or animal in need thereof by administering an effective amount of a NR2F6 modulator.
**Kinase Independent Mechanisms of Resistance of Leukemia Stem Cells to Tyrosine Kinase Inhibitors: A Hypothesis**

Christine Victoria Ichim* †

* Department of Medical Biophysics, University of Toronto
† Discipline of Molecular and Cellular Biology, Sunnybrook Research Institute, Toronto, ON, M4N 3M5, Canada

To Whom Correspondence should be addressed:
Christine V. Ichim
Sunnybrook Research Institute
2075 Bayview Avenue, S wing, room S-230
Toronto, ON M4N 3M5
Canada
Ph: 416-480-6100 x3128
Fx: 416-480-5703
Email: ichim@sri.utoronto.ca

**Support**
This work was funded by a CIHR-Canada Graduate Scholarship, an Adel S. Sedra Award of Excellence, a Dr. Joe Connolly Memorial OSOTF Award, a Government of Ontario/Dr. Dina Gordon Malkin Graduate Scholarship in Science and Technology, and a Frank Fletcher Memorial OSOTF Award to CVI.

Word count excluding references: 2326

Running title: Kinase independent effects of BCR-ABL
Abstract

Tyrosine kinase inhibitors such as Gleevec have changed the clinical course of chronic myeloid leukemia (CML). However, the observation that these inhibitors do not target the leukemia stem cell implies that these drugs are not curative and that patients need to maintain life-time therapy. The mechanism of this phenomenon is unclear: the question of whether tyrosine kinase inhibitors are inactive inside leukemia stem cells or whether leukemia stem cells do not require BCR-ABL signalling is currently under debate. We propose an alternate hypothesis: perhaps the leukemia stem cell requires BCR-ABL, but is dependent on its kinase independent functions. While the ability of kinase dead mutants to transform primary cells has previously been evaluated, conditional mutants that lack catalytic activity in stem cells, but then regain their kinase activity in progenitor cells would more accurately recapitulate what this hypothesis proposes occurs in vivo. Mechanisms that might explain a kinase independent role of BCR-ABL include activation of the RAS, PI3kinase/AKT, and/or the src-kinase HCK/STAT5 pathways. It is also worth examining whether conditions beside genotoxic stress allow for nuclear expression of BCR-ABL in leukemia stem cells, as many kinases also have kinase independent roles in regulation of gene expression.

Keywords: leukemia, scaffold, BCR-ABL, tyrosine kinase, stem cell, CML
Introduction

Tyrosine kinase inhibitors (TKI) have dramatically changed the clinical course of chronic myeloid leukemia (CML), improving the five year survival from 35% to greater than 90% with Gleevec (imatinib mesylate) (1, 2). Nevertheless, CML stem cells are refractory to TKIs (3, 4) and so patients are required to continue TKI treatment for life as discontinuation of therapy induces relapse (5, 6). Two contrasting mechanisms have been proposed to explain the reason that leukemia stem cells (LSC) are refractory to TKI: the first suggests that TKI are unable to inhibit kinase activity inside LSC (7, 8). The second mechanism suggests that TKI are able to inhibit kinase activity inside LSCs but that LSCs do not require BCR-ABL signalling for survival (4, 9).

Herein is proposed an alternative model: One in which fundamental differences exist in the signalling cascades orchestrated by BCR-ABL in LSCs versus non-stem leukemia cells. In this model LSCs would indeed require BCR-ABL signalling for survival, but in contrast to non-stem leukemia cells, the signalling that is crucial to the LSC would be independent of BCR-ABL’s catalytic activity. If indeed BCR-ABL is active in LSCs in a kinase-independent fashion then in order to achieve cure we need to devise methods of targeting BCR-ABL that do not rely on inhibition of its catalytic activity.

A kinase independent role for BCR-ABL signalling?

The cancer stem cell model proposes that cancer is composed of stem cells and non-stem cancer cells (10). Oncogenesis hence is akin to a “Darwinian” process where overexpression of oncogenes and silencing of tumour suppressor genes provide a malignant clone with a
competitive advantage. Since natural selection is based on the ability to pass down traits to clonal progeny with proliferative ability, one may hence speculate that oncogenes enhance the fitness of stem cells, given that the cancer stem cell is the only cell that is capable of unlimited proliferative ability.

Herein lies the conundrum: how is it possible that BCR-ABL is necessary and sufficient for the initiation of CML, at least in animal models (11, 12), and yet inhibition of BCR-ABL in stem cells is without consequence? From a Darwinian point of view, it would not make sense that this *sine quo non* oncogene would not enhance the clonal fitness of the CML stem cell. Hence an alternative hypothesis is proposed. It is not that CML stem cells are independent of BCR-ABL, but rather that CML stem cells are not dependent on the catalytic ability of BCR-ABL, relying instead on its kinase independent functions.

We propose that the role of the catalytic ability of BCR-ABL in defined highly purified hematopoietic cell populations be examined. While early studies examining the phenotype of kinase dead mutants of BCR-ABL concluded that this mutant lacks the transforming ability of wt BCR-ABL, these studies do not resemble what we observe in patients: that BCR-ABL kinase activity is not necessary in the LSC compartment but that kinase activity is indeed necessary in the leukemia progenitor cell (LPC) compartment.

It would be interesting, therefore, to assess the effect of a conditional mutant that would lack kinase activity in LSCs but restore regular, kinase dependent signalling in LPCs. Furthermore, earlier studies did not resolve the signalling of these mutants in specific hematopoietic compartments. Given the scarcity of the LSC within the population of hematopoietic cells, previous studies would have been unable to resolve the signalling in the LSCs as this cell population would have been contaminated with non-stem cancer cells that are
far more populous. Nevertheless, in the last decade several advances have been made that beckon a re-examination of earlier data. Specifically the ability to prospectively isolate populations of LSCs, as well as the ability to apply biochemical techniques to samples containing as little as a single cell, will uncover the resistance of LSCs to TKI.

Overview of BCR-ABL Signalling

CML is a clonal myeloproliferative disorder with an incidence of 1-2 per 100,000 per year (13). Molecularly, CML is associated with expression of the specific chromosomal abnormality, the Philadelphia chromosome (Ph) (14) characterized by the reciprocal translocation t(9;22)(q34;q11) (15). The Ph translocation gives rise to the BCR-ABL chimeric protein, in which the first exon of ABL is replaced with the breakpoint cluster region (BCR) gene. This fusion protein is characterized by high levels of tyrosine kinase activity derived from the kinase domain of ABL.

The BCR-ABL protein aberrantly activates a number of pathways that signal for proliferation, genetic instability, suppression of apoptosis and weakened cell adhesion (16-18). BCR-ABL induces activation of the JAK-STAT pathway in the absence of cytokine activation (19-23) by directly stimulating JAK-2(24, 25) and STAT (20, 26). BCR-ABL induces activation of the RAS pathway by binding to the adaptor proteins GRB2, SHC, CRK, and CBL which form a complex and recruit SOS (27-33). SOS then activates the RAS/RAF/MEK/ERK pathway that is upstream of c-MYC (34-36). Furthermore, the complex formed on BCR-ABL with GRB2 recruits GAB2 (17) which in turn activates PI3 kinase/AKT (37). Interestingly, the activation of the RAS pathway and PI3kinase/AKT appears to be independent of the catalytic ability of BCR-ABL, as mutations that disrupt binding of GRB2 prevent activation of these pathways despite
BCR-ABL catalytic ability (17, 31). Nevertheless, the ability of a kinase dead mutant of BCR-ABL to activate Ras was not tested in LSCs. SRC family kinases (SFK) are a family of molecules that are also activated by BCR-ABL (38-40). LYN and HCK are highly activated in TKI resistant patients but not in sensitive patients, implying a kinase-independent but BCR-ABL dependent mechanism (41). Interestingly, HCK is able to be activated by BCR-ABL in a kinase-independent manner (40), and is then able to phosphorylate STAT-5 (42, 43). Activation of STAT-5 is critical for the transforming ability of BCR-ABL (44). BCR-ABL promotes resistance to cell death by increasing the expression of BCL-Xₐ (45, 46) and BCL-2 (47, 48).

Several domains in the BCR-ABL protein are worth highlighting. Constitutive activation of the ABL kinase domain is mediated by dimerization via the coiled-coil domain of BCR, this is required for BCR-ABL’s transformation abilities (49, 50). The last exon domain of ABL contains sites that handle protein-protein interactions with such proteins as Crk, Grb, Nck, ATM, p53 and RB. There are also three nuclear localization sequences (NLS) as well as a DNA binding domain. While ABL is expressed in the both the nucleus and cytoplasm, BCR-ABL is thought to be expressed exclusively in the cytoplasm (51). Hence, the precise function of these domains in BCR-ABL is intriguing. While deletions of regions within the last exon domain of BCR-ABL resulted in increased tyrosine phosphorylation and transformation ability, deletion of the entire last exon prevents transformation. This begs the question of whether BCR-ABL, like ABL, is able to regulate gene transcription. The data to suggest that BCR-ABL is cytoplasmic is not comprehensive: it was conducted in a highly artificial system--cell lines that over-express the BCR-ABL oncogene in cell culture under specific conditions. While it is fair to assume that under some conditions BCR-ABL is cytoplasmic. It has been shown that at least in some other conditions BCR-ABL is able to translocate into the nucleus to affect gene transcription (52).
Gleevec is a TKI that binds to the ABL kinase domain of BCR-ABL and inhibits the protein’s catalytic activity. The development of this drug provided the proof of principle for rational drug design (53): dramatically increased the survival of CML patients while having limited side effects (1, 2). Nevertheless, TKI therapy is not curative: relapse remains a significant problem for patients with CML. Early in the course of therapy, relapse is caused by acquired drug resistance (54, 55). In addition, relapse may also be caused due to the fact that Gleevec and other TKI do not eradicate the LSCs (3, 4).

The phenomenon of acquired resistance is independent of the phenomenon that LSCs are refractory to TKI. Most cases of acquired resistance develop early in the disease from the outgrowth of clones that contain mutations in BCR-ABL that impair its ability to bind the TKI (54, 55). On the other hand, it has been observed that patients that develop a complete cytogenetic response retain BCR-ABL positive clones (2) and that these clones contain leukemia initiating cells (LSCs) that can cause relapse upon discontinuation of imatinib (5, 6, 56). More directly, it was shown that cells with a LSC immunophenotype are able to survive in the presence of TKIs (3, 9).

**Proposed research questions**

**Investigation of the transcriptional modulatory ability of BCR-ABL**

Many kinases are able to translocate to the nucleus to modulate transcription in either a DNA-binding dependent (57) or independent manner (58-60). BCR-ABL is able to translocate to the nucleus at least in conditions of genotoxic stress where it interacts with ATM and ATR, and disrupts ATR signalling(52). Hence it would be interesting to comprehensively investigate whether BCR-ABL has the ability to modulate transcription. It would be interesting to establish...
the conditions needed to induce nuclear localization of BCR-ABL in LSCs. While the localization of BCR-ABL is thought to be cytoplasmic (51), whether this holds true in LSCs is unknown. It is worth examining the localization of BCR-ABL in highly purified LSCs under basal conditions, as well as conditions of stress such as serum starvation, growth factor deprivation and genotoxic stress.

Once conditions that localize BCR-ABL to the nucleus are determined it would be interesting to know the effects of nuclear BCR-ABL on gene transcription, to determine the downstream targets and to validate that BCR-ABL actually regulates transcription. For example, an unbiased approach could be used to determine whether the DBD and NL domains of BCR-ABL are involved in regulation of gene transcription by comparing the gene expression or epigenetic signature of GFP control stem cells and wtBCR-ABL stem cells with ΔdbdBCR-ABL, or ΔnlBCR-ABL deletion mutants. At the very least under conditions of genotoxic stress one would expect that BCR-ABL will be localized in the nucleus of LSCs. It would be exceptionally interesting if the localization of BCR-ABL differed between LSCs and LPCs,

These experiments will prove that BCR-ABL is able to directly impinge on transcription, itself, a novel and exciting finding. Further work creating double mutants will be needed to show that this is a kinase independent effect. If one does not observe an effect, it may be that BCR-ABL is regulating transcription in a DNA-binding independent fashion via binding to a protein-complex. Therefore, it is timely to propose an assay for ΔnlBCR-ABL that will make use of a protocol that pulls down BCR-ABL protein complexes bound to chromatin (61, 62).

Investigation of BCR-ABL signalling in highly purified populations of LSCs and LPCs
The observation that LSCs are refractory to TKI therapy highlights that LSCs differs from non-stem leukemia cells in their fundamental signalling properties. While earlier studies examined the role of BCR-ABL in hematopoietic cells, they do not comprehensively contrast BCR-ABL signalling in highly purified LSC and LPC populations. Corbin et al. commenced characterization of the differences in signalling in LSCs and non-stem leukemia cells, however this work is far from comprehensive as they only examined differences in the phosphorylation of three proteins: Crk, AKT and stat-5 (9). It would be fascinating to comprehensively contrast BCR-ABL signalling in LSC and LPC compartments to identify core differences in BCR-ABL signalling. The JAK/STAT; RAS/RAF/MEK/ERK; and PI3K/AKT would be of particular interest because there is reason to believe that these pathways might be activated in a kinase independent manner by BCR-ABL.

Investigation of Kinase-independent BCR-ABL signalling in highly purified populations of LSCs and LPCs

BCR-ABL acts as a docking site for the protein GRB-2 that is pivotal to the activation of the RAS pathway and PI3K pathway independent of kinase activity; activation of these pathways has never been shown using a kinase dead mutant of BCR-ABL in LSC. Furthermore, while BCR-ABL activates the SRC family member HCK independent of its catalytic ability, the ability of a kinase dead mutant of BCR-ABL to activate HCK and STAT-5 has never been shown in LSCs.

Therefore, it is hypothesized that the kinase inactive mutant of BCR-ABL (K1172R) (31, 63) would demonstrate differences in the signalling of pathways compared to GFP control. However, it is also possible that no effect be observed. In that case, the question of LSC
resistance to TKIs is of such clinical importance that even a negative result would provide useful insight of relevance to human health. Furthermore, a negative result would be interesting as it would suggest that the published kinase independent effects of BCR-ABL are not applicable to LSCs and hence LSCs differ in this regard.

**Significance**

CML is a fatal bone marrow disease that invariably progresses from an indolent disorder to a drug resistant phase that resembles acute leukemia. TKIs have dramatically improved the clinical outcome of CML but are not curative as they do not affect the LSCs. Hence, patients require lifetime TKI therapy. This is expensive, and presents patients with side effects as well as medical complications.

In order to cure the disease it is necessary to understand the fundamental biology underpinning the resistance of LSCs to TKI. Herein, is proposed a novel mechanism to explain why LSCs are refractory to TKI: that BCR-ABL signalling is fundamentally different in LSCs compared to non-stem leukemia cells, and that LSCs rely on kinase-independent BCR-ABL signalling for survival. Kinase independent functions of BCR-ABL have been described in the literature. It is important to determine what if any role these kinase independent functions have in the BCR-ABL signalling of LSCs. This hypothesis will provide important insight in the BCR-ABL signalling pathways in LSCs, and elucidate how these pathways differ in LSCs versus LPCs. At the same time, the proposed work will evaluate the potential of BCR-ABL to directly alter gene transcription. This proposed work will further our understanding of the basic biology of LSCs in CML, that is necessary if we are to cure this disease.
Acknowledgements

This work was funded by a CIHR-Canada Graduate Scholarship, an Adel S. Sedra Award of Excellence, a Dr. Joe Connolly Memorial OSOTF Award, a Government of Ontario/Dr. Dina Gordon Malkin Graduate Scholarship in Science and Technology, and a Frank Fletcher Memorial OSOTF Award to CVI.

Author Contributions:

CVI wrote the manuscript.

Conflict of Interest Disclosures:

The author declares no competing financial interests.
References:


