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Estrogen-regulated Conditional Oncoproteins: Tools to Address Open Questions in Normal Myeloid Cell Function, Normal Myeloid Differentiation, and the Genetic Basis of Differentiation Arrest in Myeloid Leukemia

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Neutrophils, monocytes and dendritic cells are effectors of innate immunity and essential coactivators in the acquired immune response. Understanding the biochemical basis of their mature cell functions, their differentiation from hematopoietic progenitors, and the mechanisms by which myeloid leukemia oncogenes block their differentiation programs, continue to be areas of active research. Four major problems limit progress in these fields. First, the biochemical analysis of mature cells is limited by the time and cost of purifying neutrophils, monocytes, or dendritic cells from wild-type and genetically modified mouse strains. Second, while immortal myeloid cell lines are used to understand the transcriptional basis of normal terminal differentiation following their treatment with differentiation-promoting agents (e.g. G-CSF, IL-6, RA, TPA), these cells contain stable defects responsible for their immortalization, and the degree to which they model normal differentiation is often incomplete. Third, these same inducible cell lines are used as model systems to determine how myeloid oncoproteins prevent differentiation; however, oncoproteins that block differentiation of marrow progenitors cultured in GM-CSF or IL-3 but permit their differentiation in response to G-CSF or RA, do not score effectively in these assays (e.g. Hoxa9, Mll-Enl). Fourth, there is no reproducible method to derive myeloid progenitor lines that execute predictable terminal differentiation to neutrophils, monocytes, or dendritic cells. Developing this type of system is needed to evaluate how myeloid gene inactivation by knockout technologies alters lineage-specific differentiation and mature cell function.

Conditional myeloid oncoproteins provide a tool to solve these research problems by providing a predictable and inexpensive means of expanding, in culture, GM-CSF– or IL-3-dependent myeloid progenitors from any genotype, and by permitting their synchronous differentiation to neutrophils, monocytes, or dendritic cells under defined culture conditions following inactivation of the conditional oncoprotein. This system of conditionally immortalizing normal bone marrow precursors provides the large numbers of normal cells required for analysis of cell biology and protein biochemistry, and further provides a model system in which to study the genetic mechanisms controlling terminal differentiation and how specific oncoproteins expressed in the cell lines prevent this differentiation program. The ability to derive conditionally-immortalized progenitor lines from knock-out mice provides cell lines for the reconstitution of knockout gene function and subsequent dissection of knockout protein function by mutational analysis. Finally, conditional myeloid cell lines can be established from both ES cells and from d10 fetal liver cells, allowing for the analysis of embryonic lethal mutants on both the maturation and terminal differentiation of mature myeloid cells. In this review, we summarize the importance and limitations of current approaches in myeloid cell research, and how estrogen-regulated conditional oncoproteins help to solve these problems.

Keywords: Estrogen-related oncoproteins; Myeloid function; Myeloid differentiation; Myeloid leukemia differentiation

Abbreviations: DC, dendritic cells; DMSO, dimethylsulfoxide; ES, embryonic stem cells; FLT3-ITD, FLT3 internal tandom duplication; RA, retinoic acid; TPA, tetradecanoyl phorbol myristal acetate

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Gene abbreviations: MRP8, migration inhibitor related protein 4; MRP14, migration inhibitor related protein 8; Ly6G (GR1), leukocyte antigen 6G; gp91phox, respiratory burst oxidase subunit 91; Cnlp, cathelin-like protein; NE, neutrophil elastase; LF, lactoferrin; MPO, myeloperoxidase; NOP52, nucleoporin 52; SR-A1, macrophage scavenger receptor A1; MCSF-R, macrophage colony-stimulating factor 1; NG, neutrophil gelatinase; NC, neutrophil collagenase; NOP56, nucleolar protein 56; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-3, interleukin-3; SCF, stem cell factor; STAT, signal transducer and activator of transcription; mAKRa, aldoketo reductase a; IRF2, interferon response factor 2; IRF8, interferon response factor 8, aka IFCSBP; MHC, major histocompatability complex; ICAM, integrin cell adhesion molecule; SIGLEC, sialic acid-recognizing animal lectins of the immunoglobulin superfamily; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; PPAR, peroxisome proliferator-activated receptor; Gfi-1, Growth factor independent 1

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THE IMPORTANCE AND LIMITATIONS OF NORMAL PROGENITORS FOR STUDYING NORMAL MYELOID CELL FUNCTION

The response of granulocytes, monocytes and myeloid dendritic cells (DCs) to foreign pathogens include their ability to (1) bind chemoattractants (e.g. complement C5a) and bacterial peptides, to bind endothelium, extravasate and migrate to inflammatory sites [1], (2) to bind, engulf and kill pathogens using pore-forming peptides [2] (beta defensins and cathelicidins) and reactive oxygen species [3] as part of the innate immune response, (3) to process both foreign and self-antigens and present them to T-lymphoid cells as part of the acquired immune response [4] and (4) to modulate both the innate and acquired immune responses by secreting pro-inflammatory and anti-inflammatory cytokines [4,5]. Three factors limit the pace of research focused on understanding mature myeloid cell function. First, it is costly and time-consuming to isolate neutrophils, monocytes, or DCs from mice, compounding the expense of transgenic and gene knockout approaches to evaluate mature myeloid cell function. Second, the low abundance of mature cells limits the scope of experimentation. Third, because transgenes can not be introduced and expressed quantitatively in post-mitotic myeloid cells, transfection can not be used as a tool to interrogate cellular functions, signal transduction cascades, or the regulation of gene transcription in mature myeloid cells. Consequently, one can not restore knockout protein function in mature cells and delineate their essential domains and biochemical functions by mutation analysis. The use of conditional oncoproteins can solve these problems.

THE IMPORTANCE AND LIMITATIONS OF MYELOID LEUKEMIA CELL LINES IN MODELING NORMAL MYELOID COMMITMENT, TERMINAL DIFFERENTIATION AND CELL CYCLE ARREST

Studies that defined the origin and maturation of myeloid cells also used total marrow. Specific (G-CSF, M-CSF, GM-CSF) and broadly synergistic (IL-3, SCF, FLT-3) cytokines were identified that support the proliferation and differentiation of clonal myeloid progenitors committed to neutrophil (CFU-G), monocyte (CFU-M), or biphenotypic (CFU-GM) fates [6]. Leukemia cell lines proved useful to define Pu.1, c-Myb, c/EBPα and AML1 as transcription factors that cooperate in activation of early myeloid-specific genes [7–9] (MPO [10], NE [11], and the receptors for G-CSF [12], GM-CSF [13] or M-CSF [7]). Genetic analysis proved the importance of these factors in myeloid commitment (AML1 [14]), expansion (c-Myb [15]) and terminal differentiation (Pu.1 [16], c/EBP alpha [17]). The limitation of using leukemic cell lines to model transcriptional regulation in myeloid progenitors is the uncertainty that intrinsic differentiation defects responsible for their immortal proliferation may yield an incomplete picture of transcriptional regulation.

Mechanisms that regulate gene transcription accompanying terminal differentiation and cell cycle exit are studied in myeloid leukemia lines (e.g. 32Dcl3, HL60, NB4, U937, M1AML, FDCP1) that execute different subsets of morphologic and genetic differentiation to neutrophils or monocytes in response to physiologic (e.g. G-CSF, IL-6), or non-physiologic stimuli (e.g. supra-physiologic levels of RA or vitamin D3, DMSO, TPA). For example, granulocyte-committed 32Dcl3 progenitors fail to differentiate in IL-3, but upregulate LF, NG, and NC when cultured in G-CSF, and can be used to study terminal differentiation mechanisms such as transcriptional activation by c/EBPϵ or transcriptional derepression by CDP/cut [18]. By contrast, biphenotypic HL60 progenitors are defective in upregulation of LF, NG, or NC [19–21], but can upregulate TLR [22] and C5aR [23], and offer the advantage of evaluating the molecular basis of commitment because they can be induced to differentiate into either granulocytes or monocytes. The major limitation of these approaches is also the uncertainty that intrinsic differentiation defects responsible for their immortal proliferation may yield an incomplete picture of terminal differentiation mechanisms.

A final disadvantage of using inducible cell lines for studying terminal differentiation mechanisms is that differentiation inducers, such as TPA, or RA, may activate transcription of genes unrelated to those that regulate differentiation, complicating a differential gene transcription approach to understanding the early stages of differentiation. Conditional oncoproteins can solve these

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problems by permitting terminal differentiation to proceed in the absence of other genetic defects or differentiation inducers.

THE IMPORTANCE AND LIMITATIONS OF MYELOID LEUKEMIA CELL LINES FOR MODELING THE MECHANISM BY WHICH MYELOID LEUKEMIA ONCOGENES ARREST DIFFERENTIATION OF NORMAL MYELOID PROGENITORS

Myeloid leukemia arises from cooperation between oncogenes that block differentiation (discussed below) and oncogenes that activate signal transduction pathways and circumvent cytokine-dependence (e.g. Bcr–Abl [24] or FLT3-ITD [25]). The major, single-hit activity reported for myeloid oncoproteins is preventing neutrophil and monocyte differentiation of GM-CSF or IL-3-dependent marrow progenitors, producing immortal, GM-CSF- or IL-3-dependent cell lines (e.g. Nup98/HoxA9 [26], Hoxa9 [27], Hoxa7, MLL/ENL [28], MLL/ELL [29], E2a/Pbxl [30], v-Myb, and PML/RAR [31]). In no case has the function of an oncoprotein in primary marrow progenitors been described as blocking G-CSF or IL-6-induced differentiation of primary marrow progenitors, creating a G-CSF or IL-6-dependent cell lines. Despite this fact, the question of how myeloid oncoproteins prevent differentiation has focused on how they prevent G-CSF-induced granulocytic differentiation of 32Dclone3 [32] cells arrested by Aml1-Eto [33], Ear-2 [34], IRF-2 [35], c-Myc [36], dominant-negative STAT3 [36], v-Myb [37], tal-1 [38], Notchl [39], Evi 1 [40]) or IL-6-induced differentiation of M1AML cells (differentiation arrested by Hoxb8 or v-Myb, dominant-negative STAT3 [41]). There are problems with relying on these immortalized cell lines to reveal oncoproteins mechanisms of differentiation arrest. First, they do not reveal how an oncoprotein blocks differentiation in response to GM-CSF or IL-3, which remains an unsolved question in leukemogenesis. Second, they cannot be used to define mechanisms of differentiation arrest by oncoproteins such as Hoxa9, Mll-Enl, or Evi-1, which block differentiation in response to GM-CSF but permit neutrophil differentiation in response to G-CSF [27,28] or monocyte differentiation in response to M-CSF or IL-6 [40]. Conditional oncoproteins provide a tool to address each of these problems by establishing a regulated differentiation system in GM-CSF– and IL-3– responsive progenitors.

CONDITIONAL MYELOID ONCOPROTEINS PROVIDE AN OPTIMAL TOOL FOR STUDYING MYELOID DIFFERENTIATION AND ONCOGENESIS

What components define the ideal cell system in which to study myeloid cell function, myeloid differentiation, and oncoprotein-mediated differentiation arrest? The ideal cell system must model normal and terminal myeloid differentiation in response to GM-CSF or IL-3. It should permit researcher-controlled differentiation from the transformed (undifferentiated) progenitor to the fully normal and terminally differentiated granulocyte or monocyte and should permit an evaluation of the ability of multiple oncoproteins to block this differentiation program in the absence of coexisting oncoproteins and in the absence of inducers that activate broad patterns of gene expression. In this manner, the mechanism of gene activation accompanying differentiation can be identified and the means through which different oncoproteins prevent this activation mechanism can be determined and interrelated. NIH3T3 fibroblasts, for example, have permitted a methodical assessment of mechanisms used by mitogenic oncoproteins because their interconversion between untransformed (contact-inhibited) and transformed (focal) morphologies allow for both biochemical and genetic approaches to understanding how multiple mitogenic oncoproteins induce proliferation, ultimately, establishing the tyrosine kinase, Ras, Raf, Fos/Jun signal transduction cascade of gene activation. The conundrum that arises in developing such a model within the myeloid lineage is that myeloid cell lines can be generated only by blocking their differentiation, and such cell lines are subsequently incapable of normal differentiation. This conundrum can be resolved by converting an oncoprotein capable of immortalizing GM-CSF or IL-3-dependent myeloid progenitors into a conditional form dependent on a small molecule activator, such as estrogen. By removing the activator, functional inactivation of the oncoprotein permits analysis of normal terminal differentiation mechanisms, and heterologous oncoproteins can be expressed in trans to establish new differentiation blocks.

THE MYELOID ONCOPROTEIN E2A-PBX1, FUSED TO THE ESTROGEN-BINDING DOMAIN (EBD) OF THE ESTROGEN RECEPTOR, ESTABLISHES AN IDEAL CELL SYSTEM FOR UNDERSTANDING THE GENETIC BASIS OF MYELOID CELL FUNCTION, OF MYELOID DIFFERENTIATION IN GM-CSF OR IL-3, AND OF THE MECHANISM OF DIFFERENTIATION ARREST BY MYELOID ONCOPROTEINS

Myb [42], Hoxa9 [27] and E2a-Pbx1 [30,43] are oncoproteins that immortalize primary GM-CSF or IL-3-dependent myeloblasts and have been rendered estrogen-dependent by fusion to the EBD. Among these oncoproteins, the conditional behavior of progenitors immortalized by E2a-Pbx1-EBD have been characterized most extensively [43]. E2a-Pbx1 is a chimeric oncoprotein formed by the t(1;19) translocation of human pre-B cell leukemia [44]. E2a is a bHLH transactivation protein, while Pbx1 is a homeodomain protein that can heterodimerize with Hox and Meis partners [45,46]. E2a-Pbx1

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contains the transactivation domain of E2a fused to Pbx1 sequences, and retains its ability to heterodimerize with Hox but not Meis cofactors [46]. Because Hox proteins regulate transcription of genes controlling both embryonic development and hematopoiesis, the ability of E2a-Pbxl to deregulate myeloid differentiation and cause myeloid leukemia [47] may lie in its ability to maintain persistent activation of genes that specify the immature cell type.

E2a-Pbx1-EBD Establishes a System for Analysis of the Genetic Basis of Myeloid Differentiation and of Differentiated Cell Function

Fusion of E2a-Pbx1 to the EBD subjects its transcriptional functions to positive regulation by estrogen [43]. Following infection of murine marrow progenitors with E2a-Pbx1-EBD retrovirus, uninfected progenitors differentiate to adherent monocytes or to short-lived granulocytes, while myeloblasts expressing E2a-Pbx1-EBD are blocked in differentiation and emerge as immortalized progenitor populations within 2 weeks. Upon removal of estrogen, individual clones exhibit neutrophil (Fig. 1, panels A and B), monocyte (panels C and D), or biophenotypic differentiation.

Transcription factor genes required for myeloid terminal differentiation (Pu.1, AML1, c/EBPa, c/EBPe, c-Myb) are expressed persistently in these cell lines (see accompanying manuscript); therefore, selective deficiencies in these factors is not an explanation for differentiation arrest by E2a-ER-Pbx1. To delineate a transcriptional mechanism of differentiation arrest by E2a-Pbx1, suppression-selection PCR (RDA-PCR) and Affymetrix arrays were used to identify genes upregulated within 48 h following removal of estrogen from progenitors immortalized by E2a-Pbx1-EBD (accompanying paper). A 10- to 80-fold up regulation of myeloid genes encoding Ca2 + -binding proteins (MRP8 and MRP14), proteinase inhibitors (Stefin 1), anti-microbial peptides (Bactinecin and Cnlp), chemotactic factors (YM1), and that regulate myeloid cell extravasation (Lipocortin 1) and other myeloid functions (PDP-4, LF, Ly6G) were identified (see accompanying manuscript).

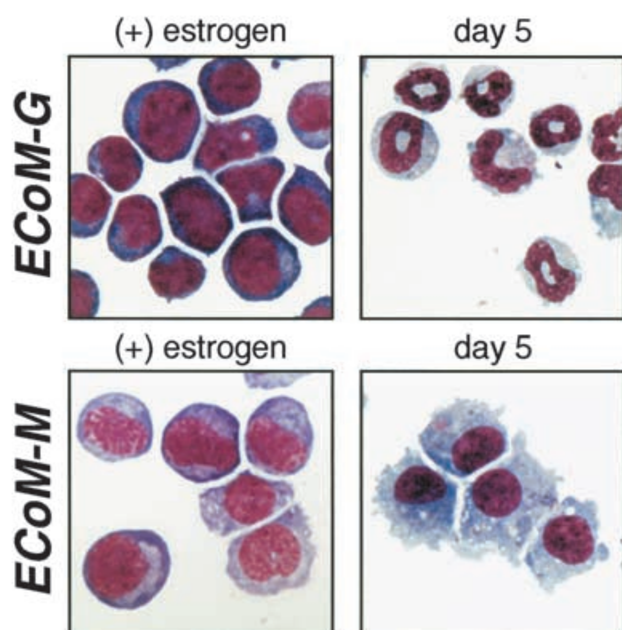

FIGURE 1 ECoM cell clones differentiate morphologically to granulocytes, monocytes, and dendritic cells. Wright-Giemsa stains of granulocyte-committed (panels A and B), and monocyte-committed (panels C and D) before (panes A and C), or after (panels B and D) removal of estrogen for 9 or 12 days, respectively.

Myeloid gene up regulation was accompanied by cell cycle arrest, down regulation of c-myc, c-myb and Gfi-1, and down regulation of their target genes NE, NOP52 and nucleophosmin 1, and up regulation of the p15ink gene, which is repressed by c-Myc. Gfi-1 is a transcriptional repressor expressed in T lymphoid progenitors that prevents PMA-induced cell cycle downregulation in T cells, and induces T cell lymphoma when persistently expressed. Of 10,400 genes evaluated by analysis of Affymetrix arrays, more than 97% evidenced less than a three-fold change 48 h following removal of estrogen (see accompanying paper). This limited genetic response indicates that these cells comprise an excellent model for pursuing how normal terminal differentiation proceeds when instructed through the receptors for GM-CSF or IL-3 and how E2a-Pbx1 prevents this differentiation mechanism.

The use of E2a-Pbx1-EBD-immortalized progenitors solves many of the technical difficulties encountered in understanding normal myeloid cell function and differentiation. They solve the problems of cost and time in purification of the non-abundant differentiated cells required to analyze mature myeloid cell function. They solve the problem of limited cell numbers that preclude certain types of analysis in mature cells, because these cell lines can generate an unlimited number of neutrophils or monocytes, allowing one to address all questions concerning mature cell function or the regulation of gene transcription accompanying terminal differentiation. They solve the problem of studying differentiation arrest in the context of instruction through the GM-CSF or IL-3 receptors, because terminal differentiation now proceeds normally in the context of GM-CSF or IL-3 signaling. They solve the problem of studying myeloid differentiation in cell lines that contain intrinsic differentiation defects, because by inactivating the single conditional oncoprotein, differentiation proceeds through normal pathways in the absence of other intrinsic oncoproteins.

E2a-Pbx1-EBD Progenitors Provide a means to Express Heterologous Proteins in Mature Myeloid Cells in Order to Interrogate Their Impact on Cellular Functions, Signal Transduction Cascades, and Gene Transcription

By using E2a-Pbx1-EBD to generate conditionally immortalized progenitors from mice containing knockout

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alleles for genes controlling myeloid differentiation or mature myeloid cell function (e.g. Siglec or TLR gene products), one can derive a model system in which to study the function of the knockout protein. Functional defects can be characterized in conditionally-immortalized myeloid (−/−) progenitors derived from the knockout mouse, or in mature cells generated from these progenitors following removal of estrogen. Exogenous expression of the knockout gene in conditionally-immortalized (−/−) progenitors can be evaluated for its ability to correct the defect in progenitors or in their mature cell progeny. Using this system, functional domains of the knockout protein can be mapped by a mutational approach. If the protein is a transcription factor, the ability of an essential domain to reconstitute gene expression can be verified, and cofactors that bind the domain can be identified and cloned using a yeast two-hybrid approach and differentiation-specific libraries.

E2a-Pbx1-EBD Progenitors Provide a Tool to Analyze the Genetic Basis of Granulocyte-specific vs. Monocyte-specific Gene Expression

Genes specific for neutrophil or monocyte differentiation were activated specifically in E2a-Pbx1-EBD cell lines committed to granulocyte (LF in ECoM-G cells) or monocytic (Lysozyme M, SR-A1, macrosialin, MCSF-R, and F4/80 in ECoM-M cells [43]) differentiation (see accompanying paper). Genes possibly involved in neutrophil vs. monocyte commitment (mAKRa) or known to be associated with monocyte commitment (IRF8) were expressed selectively in ECoM-G and ECoM-M progenitors, respectively. These matched cell lines should be particularly useful for further refining the transcriptional basis of neutrophil vs. monocyte commitment by a differential gene expression approach, and because both lines are immortalized by the same oncogene, irrelevant differences identified during differential gene expression analysis should be minimized.

E2a-Pbx1-EBD Progenitors Provide a Means of Generating and Studying Dendritic Cells

Dendritic cells (DCs) are one type of antigen-presenting cell (APCs) that efficiently initiates the primary immune response by processing and presenting antigens to T-cells [48]. Bone marrow DCs (BM-DCs) can be derived by culturing primary marrow [49,50] or CD34-positive human umbilical cord blood cells [51] in GM-CSF and IL-4. Other sources of APCs include crude splenocytes, and fibroblast cell lines engineered to express the proper MHC and co-stimulatory molecules [52–54]. The ability of ECoM cell lines to differentiate into dendritic cells was tested because they would represent a convenient source of cells to investigate DC biology and would provide a low cost alternative to sacrificing adult mice as a source of splenic APC. Following differentiation in GM-CSF plus IL-4, ECoM clones exhibited a dendritic cell morphology and expressed dendritic cell surface markers [48] at levels at least as abundant as those on thioglycollate macrophages, splenocytes, or BM-DCs (MHCI, MHCII, CD11c, co-stimulatory molecules CD80 (B7-1), CD86 (B7-2), and ICAM (CD54); Fig. 2, panels A–H and manuscript in preparation). Stimulation of T cell proliferation by ECoM-derived dendritic cells was comparable to that of BM-DCs, the best source of APCs (not shown). The cells are also convenient as they can be derived from any mouse (and thus any MHC background) to permit antigen presentation to responding T cells and T cell lines that have been similarly isolated from mice expressing a variety of MHC genotypes.

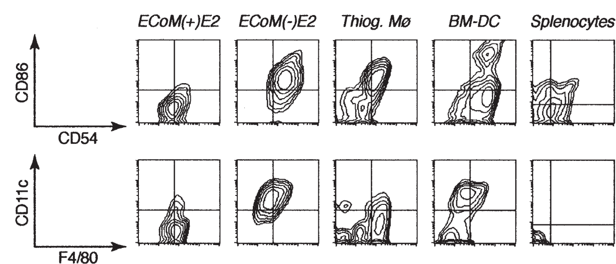

FIGURE 2 Differentiated ECoM cells are a useful source of antigen presentation cells. Expression of dendritic cell surface antigens in ECoM progenitors cultured in the presence of GM-CSF plus IL-3 estrogen (+Es; panels A and F) or following withdrawal of estrogen for 8 days (−Es; panels B and G), or on thioglycollate macrophages (panels C and H), BM-DCs (derived by 12-day culture in GM-CSF; panels D and I), or total splenocytes (Panels E and J).

E2a-Pbx1-EBD Immortalize Myeloid Progenitors from ES Cells and from Day 11 Fetal Liver, Permitting the Analysis of Myeloid Gene Function from Embryonic Lethal Genotypes

Knockouts of certain genes involved in myeloid biology produce an embryonic lethal phenotype that disallows analysis of the −/− genotype on myeloid cell function or differentiation. In these cases, if development proceeds to d10–d12, myeloid progenitors isolated from day E10–E12 fetal liver (FL) can be immortalized by E2a-Pbx1-EBD retrovirus and the impact of the −/− phenotype evaluated in progenitors and during terminal differentiation, as described above. Using this approach, we derived cell lines from day E11 SMRT knockout mice, which normally die at day E12.

ES cells provide a second means to derive conditionally-immortalized myeloid progenitor lines from embryonic lethal genotypes. ES cells can be differentiated to embryoid bodies, and myeloid-committed precursors can be isolated by culturing disrupted embryoid bodies in SCF plus IL-3 [55]. Using ES cells containing homozygous PPARγ −/− and PPARδ −/− genomes, immortalized myeloid progenitors were derived that exhibit monocyte differentiation following

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inactivation of E2a-Pbx1-EBD in medium containing SCF and M-CSF (Ajay Chawla, personal communication). This approach, coupled with the reconstitution of expression described above, permits analysis of PPARγ and PPARδ functions in mature monocytes and during monocytic maturation.

E2a-Pbx1-EBD Progenitors Provide a Method to Determine How E2a-Pbx1 Blocks Differentiation

Progenitors conditionally immortalized by E2a-Pbx1-EBD allow one to investigate how E2a-Pbx1 blocks differentiation in a model system that contains no other intrinsic differentiation defects. By identifying how E2a-Pbx1 prevents activation of differentiation genes, one can backtrack to direct E2a-Pbx1 target genes responsible for these normal genetic responses. Reconstitution of wild-type E2a-Pbx1 expression, followed by mutational analysis of E2a-Pbx1, allows one to map essential E2a-Pbxl domains/biochemical functions required for differentiation arrest. The benefit of this approach, verses that of using primary marrow, is that the expression of mutant E2a-Pbx1 proteins can be verified by Western blotting following expression in conditionally-immortalized cell lines, and the rationale behind why certain mutants fail to reestablish differentiation arrest can be determined (such as failure to localize to the nucleus). The use of primary marrow progenitors does not provide sufficient cells to routinely evaluate the level of expression of non-transforming mutants, and the fact that these progenitors terminally differentiate prevents one from determining why non-transforming E2a-Pbx1 proteins fail to block differentiation.

E2a-Pbx1-EBD Progenitors Provide a Method to Determine How other Oncoproteins Prevent Differentiation of GM-CSF or IL-3-dependent Progenitors

Conditional differentiation of certain E2a-Pbx1-EBD cell lines is re-blocked by myeloid oncoproteins Hoxa9 or Hoxa7, creating the first efficient cell line-based system in which to study the biochemical and genetic mechanisms by which Hoxa9 and Hoxa7 suppress differentiation and maintain proliferation during GM-CSF or IL-3 signaling. Hoxa9 domains required to prevent differentiation can be identified, and interacting factors defined by the technique of yeast two-hybrid selection. The cooperative function of such factors in Hoxa9 differentiation arrest can be proven by demonstrating that their elimination, by antisense mRNA approaches or in single cells with neutralizing antisera, prevent Hoxa9 function. Myeloid differentiation genes whose expression is prevented in the parental cell lines by E2a-ER-Pbx1, as well as in the Hoxa9 derivatives following estrogen removal, contain promoters that integrate common mechanisms of differentiation arrest. This example illustrates how ECoM cells can be used as tools to identify and interrelate mechanisms of differentiation arrest by multiple oncoproteins.

EBD FUSIONS OF OTHER MYELOID ONCOGENES PRODUCE SIMILAR IMMORTAL CELL LINES THAT EXECUTE NEUTROPHIL-SPECIFIC OR MONOCYTE-SPECIFIC DIFFERENTIATION UNDER DIRECTION BY GM-CSF OR IL-3

Fusion of Hoxa9 to the EBD also subjects both its transcriptional properties and myeloid immortalization potential to estrogen-dependence. When estrogen is removed, clones immortalized by EBD-Hoxa9 exhibit granulocyte, monocyte, or biphenotypic differentiation (Fig. 3). Genes whose transcription is prevented by E2a-ER-Pbx1 will be evaluated in these Hoxa9-ER conditional systems to identify common targets. Likewise, genes identified in the Hoxa9-ER system as being Hoxa9-response will be evaluated in the E2a-ER-Pbx1 cell lines. Continued application of this approach to other myeloid oncoproteins, such as MLL fusion proteins, should ultimately reveal both the specific and interrelated mechanisms by which myeloid oncoproteins block normal myeloid differentiation.

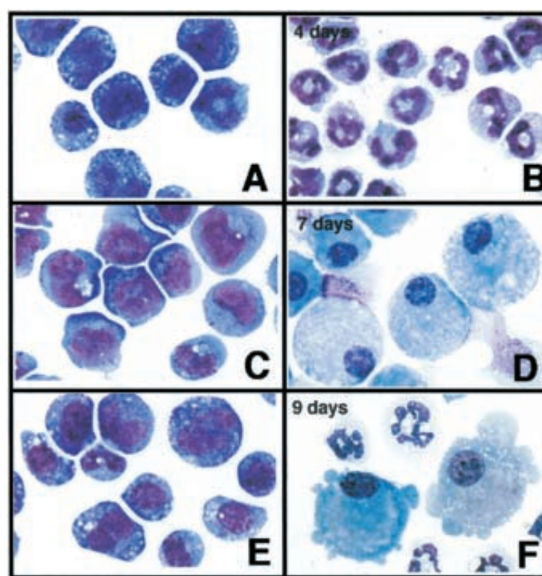

FIGURE 3 Hoxa9-EBD progenitors differentiate morphologically to granulocytes and monocytes. Wright-Giemsa stains of granulocyte-committed (panels A, B), monocyte-committed (panels C, D), and biphenotypic (panels E, F) progenitors cultured before (panes A, C, E), or after (panels B, D, F) removal of estrogen for 4, 7, or 9 days, respectively.

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COMPARISON OF EBD-ONCOPROTEIN CELL LINES WITH DOMINANT NEGATIVE RETINOIC ACID RECEPTOR (RAR) CELL LINES AS TOOLS FOR MODELING MYELOID CELL FUNCTION, NORMAL MYELOID DIFFERENTIATION, AND THE GENETIC BASIS OF DIFFERENTIATION ARREST IN MYELOID LEUKEMIA

Dominant negative RARα403 immortalizes multipotent progenitors (EML cells) and IL-3-dependent promyelocytes (MPRO cells), which differentiate into granulocytes in response to RA and recapitulate terminal myeloid differentiation [56–58]. The differentiation properties of MPRO cells appear comparable to granulocyte-committed myeloid progenitor cell lines immortalized E2a-Pbx1-EBD or Hoxa9-EBD. The advantage of using RARα403 is that EML cells, or EML-like derivatives from other genetic backgrounds, can also recapitulate multilineage erythroid, myeloid, and lymphoid differentiation. The disadvantage of studying myeloid differentiation in RARα403-immortalized M-PRO cells is that high concentrations of RA are required to inactivate dominant negative RARα403 and might activate the expression of other cellular genes controlled by endogenous RA receptors, complicating a differential gene transcription approach to understanding the genetics of early differentiation. In addition, MPRO cells have not been reported to detect the ability of heterologous oncoproteins to block differentiation in trans, and could be problematic for this application if activation of endogenous RA receptors circumvented the heterologous differentiation block.

PERSPECTIVE

Oncoprotein-EBD proteins provide a rapid means of conditionally immortalizing progenitors from bone marrow, fetal liver, embryonic stem cells, and various mouse strains for the purpose of studying the differentiation and the mature functions of neutrophils, monocytes, and dendritic cells, as well as how their differentiation programs are arrested by human leukemia oncoproteins. A comprehensive understanding of this process will provide the best foundation for identifying new drug targets for the treatment of myeloid leukemia.

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