Sino Biological
Biological Solution Specialist
Tag Antibody As Low As
$59
Only $38/ each for
Hot Cytokines & Growth Factors
50% off
for All ELISA Kits and Pair Sets

This information is current as of October 30, 2017.

E2F4 Modulates Differentiation and Gene Expression in Hematopoietic Progenitor Cells during Commitment to the Lymphoid Lineage

Megan E. Enos, Simona A. Bancos, Timothy Bushnell and Ian N. Crispe

J Immunol 2008; 180:3699-3707; ;
doi: 10.4049/jimmunol.180.6.3699
http://www.jimmunol.org/content/180/6/3699

Why *The JI*?

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

**average*

References This article **cites 32 articles**, 13 of which you can access for free at: http://www.jimmunol.org/content/180/6/3699.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2008 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017

E2F4 Modulates Differentiation and Gene Expression in Hemopoietic Progenitor Cells during Commitment to the Lymphoid Lineage[1]

Megan E. Enos,* Simona A. Bancos,† Timothy Bushnell,‡ and Ian N. Crispe[2]*

The E2F4 protein is involved in gene repression and cell cycle exit, and also has poorly understood effects in differentiation. We analyzed the impact of E2F4 deficiency on early steps in mouse hemopoietic development, and found defects in early hemopoietic progenitor cells that were propagated through common lymphoid precursors to the B and T lineages. In contrast, the defects in erythromyeloid precursor cells were self-correcting over time. This suggests that E2F4 is important in early stages of commitment to the lymphoid lineage. The E2F4-deficient progenitor cells showed reduced expression of several key lymphoid-lineage genes, and overexpression of two erythromyeloid lineage genes. However, we did not detect effects on cell proliferation. These findings emphasize the significance of E2F4 in controlling gene expression and cell fate. ***The Journal of Immunology,*** **2008, 180: 3699–3707.**

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017

Hemopoietic stem cells (HSC)[3] must choose between self-renewal and differentiation; if they differentiate they can become common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). It is still unclear how environmental signals (1) and lineage-specific transcription factors work together to control the frequency with which dividing HSC either undergo self-renewal or commit to one or the other lineage. Transcription factors expressed in HSC can drive commitment to either the lymphoid or the myeloid lineage (2). For example, factors of the Ikaros family specifically favor differentiation down the lymphoid pathway (3), whereas other factors, such as GATA-1 and C/EBPα, favor differentiation down the myeloid pathway (4, 5).

We are particularly interested in mechanisms that influence the choice between self-renewal and differentiation. Thus we study the E2F family of transcription factors, which promotes cell cycle progression and exit; the latter is associated with terminal differentiation in many cell types (6, 7). The E2F family includes eight genes, and several of their products are susceptible to differential splicing, generating distinct isoforms (8). The activating E2Fs, namely E2F1, E2F2, and E2F3a, promote the G_1-to-S phase transition during cell cycle progression (9), interacting with the basal transcriptional machinery to enhance expression of cyclin E, DNA polymerase α, thymidine kinase, and other genes that advance the cell cycle (10). In contrast, the repressing E2Fs, namely E2F3b, E2F4 and E2F5, have the ability to bind similar promoter regions to those bound by the activating E2Fs (11), but are simultaneously bound by pocket proteins (pRb, p107, or p130) that physically prevent interaction with the transcriptional machinery (12). However, phosphorylation of the pocket proteins bound to E2F3b-5 can de-repress the transcription of the promoter regions to which they bind. The final subset of E2Fs, E2F6, E2F7, and E2F8 lack a transactivation domain and do not interact with pocket proteins, and therefore have the potential to silence promoter regions (13, 14).

The repressing E2Fs could favor differentiation simply by promoting cell cycle exit (7). Among these, E2F4 is found at high concentrations in hemopoietic cells (15). Overexpression of E2F4 promotes terminal differentiation of cells in vitro (16), whereas E2F4-deficient animals show reduced neonatal viability, with very few animals surviving to adulthood (15, 17). These animals died due to opportunistic infections and anemia, but they were also smaller than either heterozygous or wild-type (WT) littermates (15, 17). Recently, E2F4 has been shown to play a role during fetal erythropoiesis, explaining the anemia that is seen in the fetuses and neonates (18). However, the impact of E2F4 deficiency on thymocyte development has been controversial (15, 17). Both the erythroid and the lymphoid defects in E2F4-deficient mice could be explained if the defect occurred at the level of the HSC, which gives rise to both lineages.

In these studies we focused extensively on two populations of cells. The first is a subset that is enriched for HSC activity, which we identified using the phenotype first proposed by Spangrude et al. (19); they are cells lacking multiple lineage-specific (Lin^-) markers, but expressing stem cell Ag-1 (sca-1) and the stem cell factor receptor c-kit. This cell population overlaps functionally with HSC, but because in these experiments we are identifying them by phenotype, we use the term LSK cells to denote the Lin^-, sca-1^+, c-kit^+ phenotype. This term is previously used to denote this population. The second population of cells is one that has been proposed to correspond to bone marrow B cell precursors but that also has shown pro-T potential, rendering them CLP

*Department of Microbiology and Immunology, and the David H Smith Center for Vaccine Biology and Immunology, †Department of Environmental Medicine, Lung Biology and Disease Program, and ‡Department of Pediatrics, Center for Pediatric Biomedical Research, The James P. Wilmot Cancer Center, University of Rochester, Rochester, NY 14620

Received for publication January 8, 2008. Accepted for publication January 8, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant R01GM056689 from the National Institutes of Health (to I.N.C.).

[2] Address correspondence and reprint requests to Dr. Ian N. Crispe, David H Smith Center for Vaccine Biology and Immunology, University of Rochester, 601 Elmwood Avenue, Rochester, NY 14620. E-mail address: Nick_Crispe@urmc.rochester.edu

[3] Abbreviations used in this paper: HSC, hemopoietic stem cell; LSK, Lin^-sca-1^+c-kit^+; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythroid progenitor; DN, double negative; WT, wild type.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

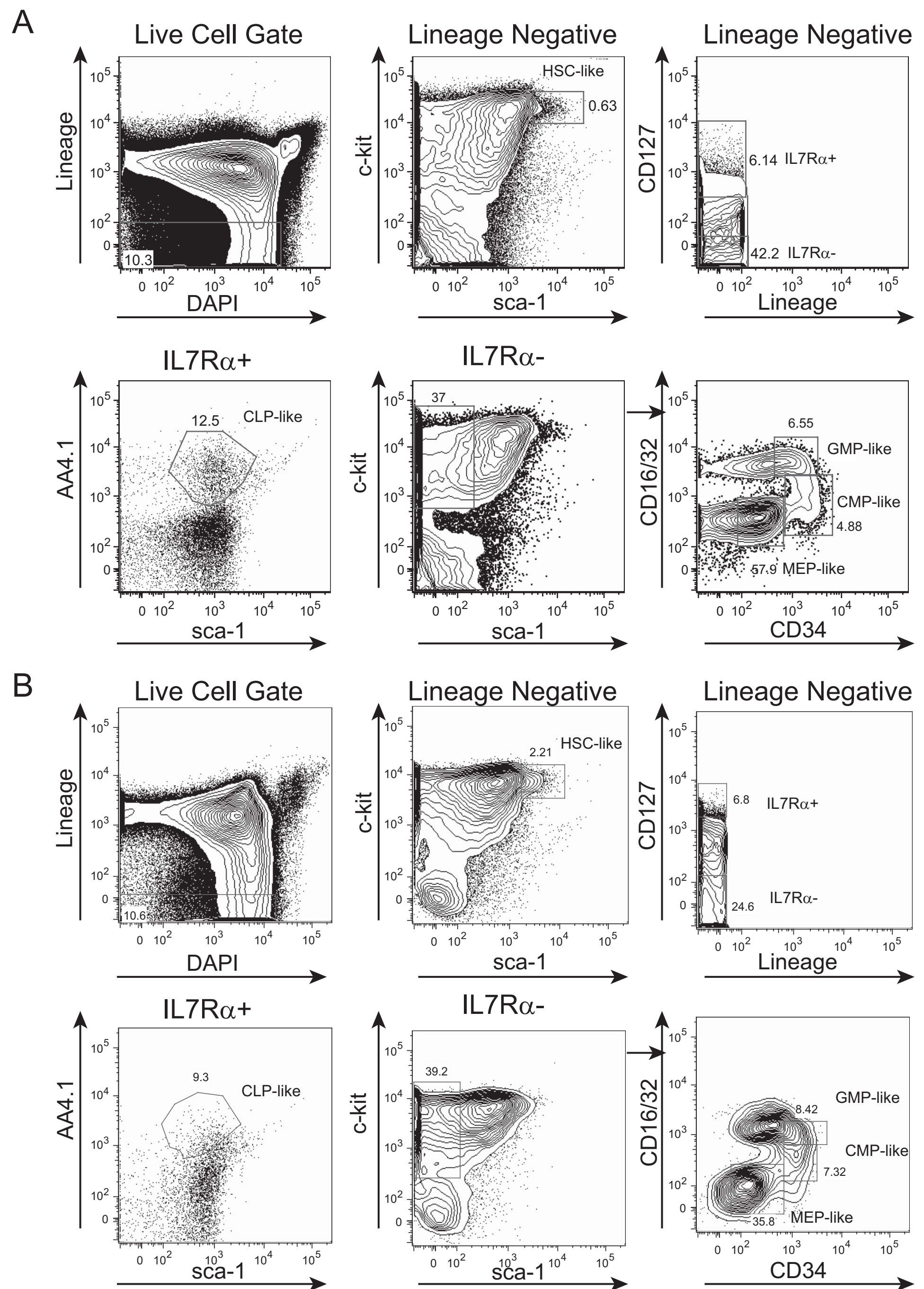


FIGURE 1. Identification of progenitors in fetal liver. *A*, Identification of HSC-like, CLP-like, and CMP-like cells in WT fetal liver. Multicolor flow cytometry and a sequential gating strategy identify first Lin$^-$ viable cells, then cells coexpressing c-kit and sca-1. *B*, The same subsets of cells appear in E2F4-deficient fetal liver.

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017

(20). These cells are also Lin $^-$ but express the IL-7R α-chain and the marker AA4.1, together with an intermediate level of sca-1. Because we are not assaying the differentiation population of these cells, and because the exact in vivo potential of cells with this phenotype is still under discussion, we will avoid naming these cells CLP. However, there is no accepted operational acronym for this population, so we will use the operational term "CLP-like" cells.

In this paper, we report that E2F4-deficient fetal liver cells showed a selective defect in lymphoid differentiation. This defect was not due to obvious differences in the expression of the *e2f4* gene in lymphoid vs myeloid cells. However, the *e2f4*$^{-/-}$ LSK cells were deficient in *il7ra* and *pax5* gene expression, whereas the *e2f4*$^{-/-}$ CLP-like cells were deficient in the expression of the key lymphoid lineage transcription factor, *ikaros*. Conversely, *e2f4*$^{-/-}$ CMP-like cells overexpressed *gata1* and *gata2*. This argues that E2F4 acts during hemopoiesis by controlling the expression of lineage-specific factors.

Materials and Methods

Mice

C57BL/6 (CD45.2$^+$) mice and B6.SJL-Ptprca Pep3b/BoyJ (CD45.1$^+$) mice were purchased from the The Jackson Laboratories. *Rag2*$^{-/-}$ (B6.SJL(129S6)-Ptprca/BoCrTac-Rag2^{tm1Fwa}N10) mice were purchased from Taconic Farms. *e2f4*$^{+/-}$ mice were a gift from Dr. J. Nevins (Duke University, Durham, NC). These mice were bred as heterozygotes. Mice were kept under specific pathogen-free conditions in the University of Rochester Animal Housing Facility, and all of these experiments were approved by the Animal Care and Use Committee for the University of Rochester.

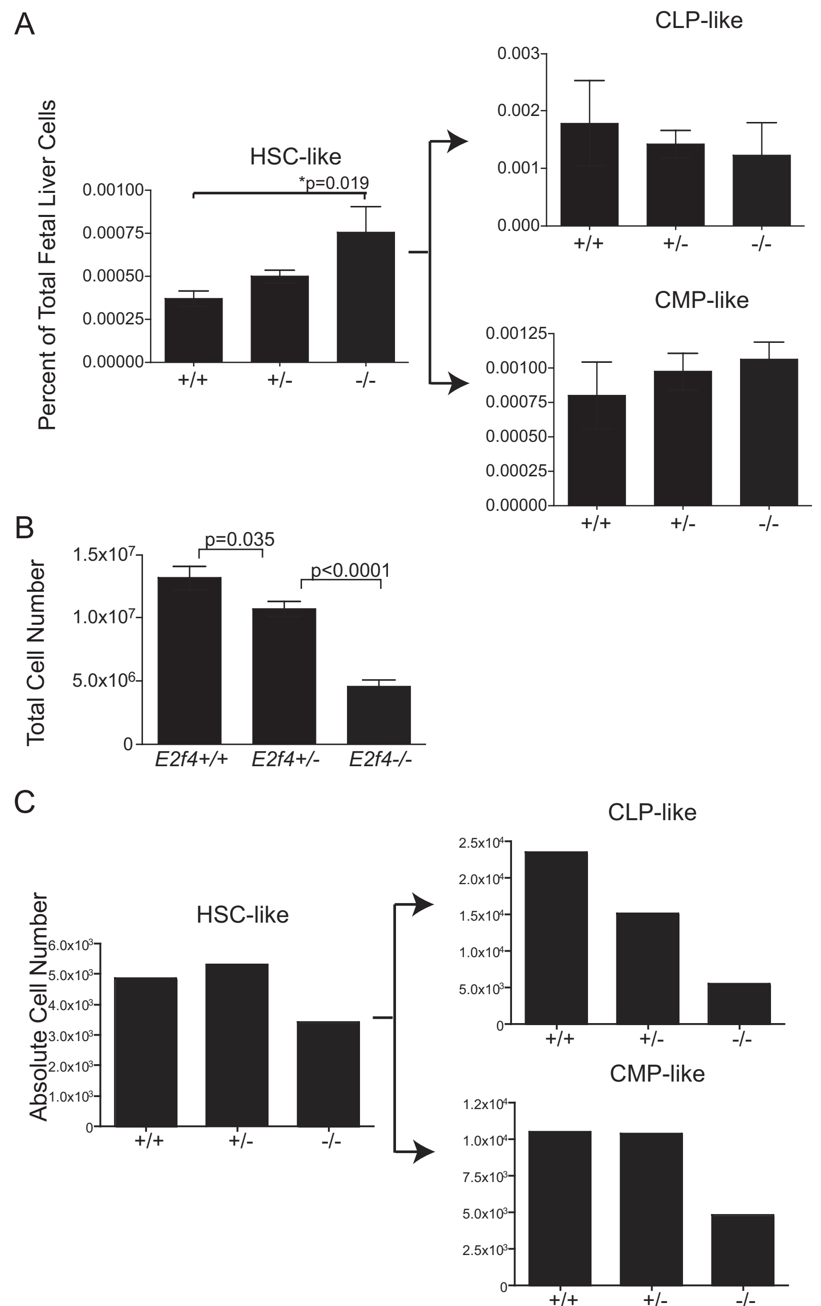


FIGURE 2. Frequency and abundance of progenitor cell subsets. *A*, Flow cytometric analysis of fetal livers yielded an increase in the frequency of cells with the HSC-like phenotype, with no effect on the frequency of fetal CLP-like and CMP-like cells. Data shown are mean percentage ± SEM from the analysis of three litters of fetuses, $e2f4^{+/+}$ (n = 4 mice), $e2f4^{+/-}$ (n = 12 mice), and $e2f4^{-/-}$ (n = 6 mice). Statistical significance was determined by the Mann-Whitney *U* test. *B*, Fetal liver cells were counted based on trypan blue exclusion. $e2f4^{+/-}$ (n = 26), $e2f4^{+/-}$ (n = 52), and $e2f4^{-/-}$ (n = 19). Data shown represent mean cell number ± SEM. A normal Mendelian distribution of genotypes arose from $e2f4^{+/-}$ matings. *C*, Estimated absolute number of cells in each progenitor subset, based on multiplying mean fetal liver cell number by mean percentage.

Repopulation

For competitive repopulation experiments, $e2f4^{+/-}$ (CD45.2$^+$) and $e2f4^{+/+}$ (CD45.1$^+$) females were mated with males of the same genotype to generate fetuses distinct in *e2f4* expression and CD45 allotype. On day 14.5 of gestation, fetal livers were mechanically disrupted to make single cell suspensions, and a small amount of fetal tissue was taken for PCR genotyping. DNA was isolated using the Qiagen DNeasy kit, following the manufacturer's instructions, and PCR was conducted using previously described primers (15). Fetal liver cells were resuspended at a concentration of 2.5 × 10^6 cells/ml in sterile PBS. They were pooled based on *e2f4* genotype and then mixed at a 1:1 ratio with competitor CD45.1$^+$ fetal liver cells. Recipient $Rag2^{-/-}$ (CD45.1$^+$) mice were irradiated with 6.0 Gy and injected i.v. with 5 × 10^5 fetal liver cells. There were three groups of experimental mice, which received $e2f4^{+/+}$ (CD45.2$^+$) cells mixed with $e2f4^{+/+}$ (CD45.1$^+$) cells, $e2f4^{+/-}$ (CD45.2$^+$) cells mixed with $e2f4^{+/+}$ (CD45.1$^+$) cells, or $e2f4^{-/-}$ (CD45.2$^+$) cells mixed with $e2f4^{+/+}$ (CD45.1$^+$) cells. Recipients reconstituted for 4, 8, and 16 wk before tissue harvest, and received 0.16 mg/ml sulfamethoxazole and 0.32 mg/ml trimethoprim in the drinking water for 4 wk.

For noncompetitive repopulation experiments, recipient $Rag2^{-/-}$ (CD45.1$^+$) mice were irradiated with 6.0 Gy and injected i.v. with 1 × 10^6 CD45.2$^+$ fetal liver cells that were either $e2f4^{+/-}$ or $e2f4^{-/-}$. Recipients reconstituted for 4, 8, or 16 wk before harvest. These recipients also received antibiotics in the drinking water for 4 wk.

Staining

Single cell suspensions were obtained by mechanical dissociation of the thymus. To obtain bone marrow, the ends of femurs and tibias were cut off and the bones were flushed with RPMI 1640 medium using a 26-gauge needle. Cells were added to 96-well plates at a concentration of 5–10 × 10^6 cells/well. Ab mixes were added at 1/100 dilutions in staining buffer (PBS with 1% BSA), and cells were stained for 30 min at 4°C in 50 μl/well. Abs used for this staining include TCRβ-FITC, TCRδ-PE, CD44-PE Cy5, CD25-allophycocyanin, CD127-FITC, CD45.2-biotin, CD117-allophycocyanin, CD2-FITC, CD16/32-FITC, CD34-PE (all from BD Biosciences). Abs from eBioscience include CD127-biotin, CD45.2-PE Cy5.5, CD45.1-PE Cy7, CD4-allophycocyanin Cy7, AA4.1-PE, sca-1-PE Cy5.5, CD45.1-PE Cy7, CD19-allophycocyanin Cy5.5, CD3-allophycocyanin

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017

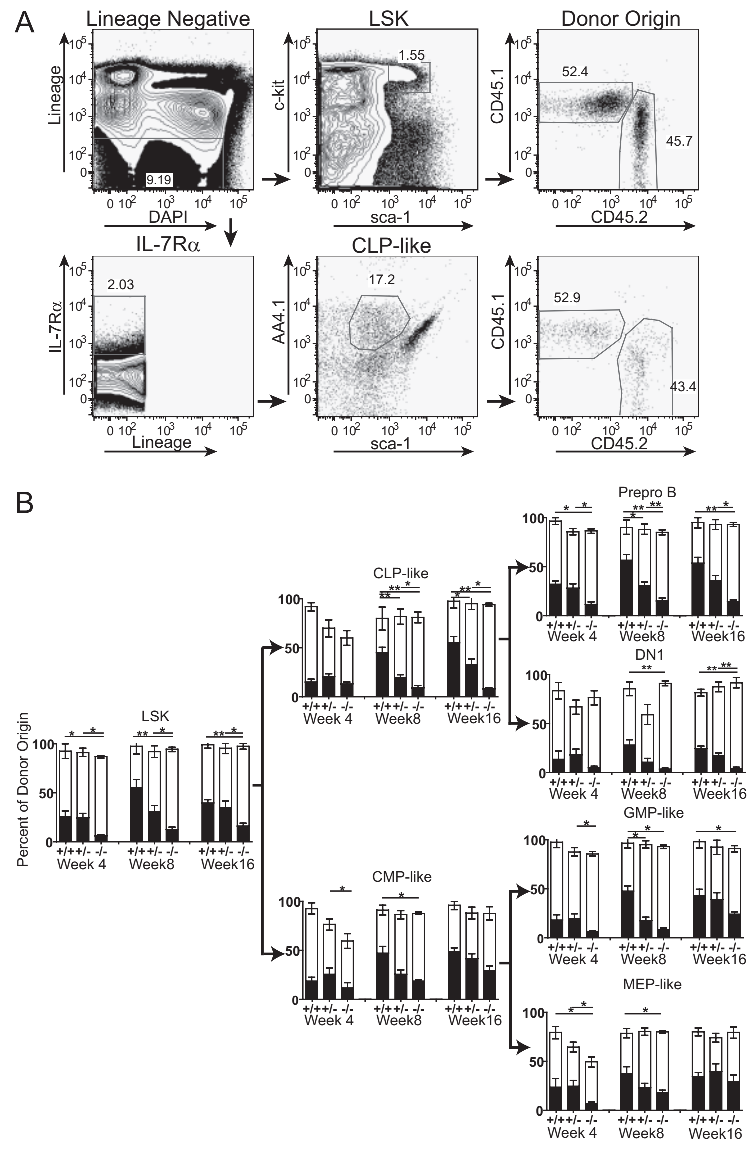


FIGURE 3. Differentiation of E2F4-deficient fetal liver cells. Competitive repopulation experiments revealed a defect in the *e2f4*$^{-/-}$ progenitor cells to reconstitute irradiated hosts. *A*, Multicolor flow cytometry and a sequential gating strategy to identify LSK cells and CLP-like cells, as well as donor identification, based on expression of the CD45 allotypic marker. *B*, The gating strategy is the basis for determining the percentage of any cell type that is derived from one of the fetal liver donor cell populations. CD45.1^{+} (□) competitors were *e2f4*$^{+/+}$ and CD45.2^{+} (■) cells were derived from fetuses from *e2f4*$^{+/-}$ matings. Hosts were CD45.1^{+} *rag2*$^{-/-}$ mice that were given 6.0 Gy before i.v. injection with a 1:1 mixture of fetal liver cells; a total of 5×10^5 cells were injected. Hosts were allowed to reconstitute for 4, 8, or 16 wk, at which time thymuses, femurs, and tibias were harvested. Reconstitution analysis for LSK cells, CLP-like cells, CMP-like cells, DN1 thymocytes, pre-B and pro-B cells, GMP-like cells, and MEP-like cells is shown. Data shown represent mean percentage ± SEM for each genotype. *, $p < 0.05$ and **, $p < 0.005$ as determined by the Mann-Whitney *U* test. Data were pooled from two to three independent experiments for *e2f4*$^{+/+}$ ($n = 4$), *e2f4*$^{+/-}$ ($n > 9$), and *e2f4*$^{-/-}$ ($n > 10$) mice.

Cy7, Ter119-allophycocyanin Cy7, GR1-allophycocyanin Cy7, B220-allophycocyanin Cy7, CD19-allophycocyanin Cy7, IgD-PE, IgM-biotin, CD117-PE Cy5, B220-allophycocyanin, CD45.2-allophycocyanin Cy5.5, CD127-allophycocyanin. Cells were washed and resuspended in a 1/100 dilution of streptavidin-PE Texas Red for 20 min. Cells were then washed in staining buffer and resuspended in a 1/5000 dilution of 5 μM DAPI (4′,6′-diamidino-2-phenylindole) for 20 min. Cells were then washed twice and resuspended in fixative. Flow cytometric data were acquired on an LSR-II using FACSDiva software (both from BD Biosciences), and analyzed using FlowJo (Tree Star).

RNA extraction and PCR

LSK cells and CLP-like cells from reconstituted mice were sorted using a FACSAria cell sorter (BD Biosciences). Cells were lysed and RNA was isolated using TRIzol, following the manufacturer's instructions. Total RNA was used for real-time quantitative RT-PCR. All quantitative RT-PCR was performed at the University of Rochester Functional Genomics Core Facility. Real-time quantitative RT-PCR to measure *e2f4* RNA expression levels was performed using cells sorted from C57BL/6 mice (The Jackson Laboratory).

Results

Mice lacking the E2F4 transcription factor fail to thrive; few of these mice survive to adulthood. Therefore, to study the influence of E2F4 on hemopoiesis, we were constrained to study fetal liver progenitor cells rather than adult bone marrow-derived cells.

Fig. 1 shows the flow cytometric identification of different hemopoietic progenitor cell populations in the liver of WT and E2F4-deficient fetuses. We first identified these cells in WT fetal liver using multicolor flow cytometry and a sequential gating strategy to identify first Lin^{-} viable cells, then cells coexpressing c-kit and sca-1 (Fig. 1*A*). This subset we term LSK cells. The cell population with this phenotype contains self-renewing HSC, thus we also call these cells "HSC-like". To identify the precursors of the lymphoid and the myeloid lineages, we identified Lin^{-} cells with expression of CD127 (the α-chain of the IL-7R) and the AA4.1 marker. These cells correspond to the CLP identified in adult bone marrow. In this experiment, we use the term "CLP-like" to define these cells phenotypically. We also identified a subset of early myeloid cells that were Lin^{-}, sca-1^{-}, and c-kit^{+}; these cells were further subdivided based on CD16 and CD34 expression into subsets corresponding to CMP, granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP) (21). Because our experiments use the surface phenotype to define these subsets of cells, we use the operation

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017

terms GMP-like, CMP-like, and MEP-like. Fig. 1*B* shows that all of these populations were present, and readily identified, in fetal liver cells from $e2f4^{-/-}$ fetuses.

Within the E2F4-deficient fetal liver, LSK cells were significantly more abundant ($p = 0.019$) than in WT fetal liver (Fig. 2*A*). There was a trend toward a decreased frequency of CLP-like cells and an increased frequency of CMP-like cells, but neither of these was statistically significant. At first sight, these data are consistent with a partial block in HSC differentiation in the E2F4-deficient cells. The total cellularity of E2F4-deficient fetal livers was substantially reduced (Fig. 2*B*). Furthermore, the E2F4 heterozygous mice were significantly hypocellular, compared with cells from WT fetal livers ($p = 0.035$). These measurements of the frequency of each cell subset, together with the total fetal liver cell count, allowed us to estimate the absolute number of HSC-like, CLP-like and CMP-like cells in the fetal livers of each genotype (Fig. 2*C*). This calculation shows no clear effect of E2F4 deficiency on the number of HSC-like cells, a small effect in attenuating the number of CMP-like cells, and the greatest defect in the number of the CLP-like cells.

To test the hypothesis that the lack of E2F4 was causing impairment of the first differentiation steps undertaken by HSC, we performed competitive repopulation studies in sublethally irradiated Rag2-deficient mice. Thus, we crossed E2F4 heterozygous mice to generate fetuses of all three genotypes: $e2f4^{+/+}$, $e2f4^{+/-}$ and $e2f4^{-/-}$. Fetuses were harvested at 14.5 days of gestation, and typed by PCR; fetal liver cells were mixed with an equal number of normal fetal liver cells differing in CD45 allotype, and the cell mixture was adoptively transferred into the recipient mice. Subsets of hemopoietic precursor cells were identified by flow cytometry at 4, 8, and 16 wk after reconstitution.

Fig. 3*A* illustrates the gating scheme used to generate these data. Thus, Lin^- cells were further analyzed to identify the sca-1^+, c-kit^+ subset (i.e., LSK cells), and then this population was further classified based on their expression of either CD45.1 or CD45.2. Similarly, the IL-7Rα^+ subset of Lin^- bone marrow cells was further dissected to identify the AA4.1^+ sca-1 intermediate cells, which we term CLP-like. These cells were also analyzed for CD45 allotype expression to establish the ratio of E2F4-genotyped fetal liver cells to normal competitor.

It would be logical to expect that, when the typed fetal liver cells were $e2f4^{+/+}$, the ratio of test to competitor cells would be 1:1. Fig. 3*B* shows the ratio of test to competitor cells for E2F4-intact, E2F4-heterozygous, and E2F4-deficient cells. At the 4 wk time point, the normal competitor cells (Fig. 3*B*, open histogram) were generally in excess; this finding was probably due to a contribution from radio-resistant precursor cells in the irradiated host. However, at 8 and 16 wk, the ratio of $e2f4^{+/+}$ to normal competitor cells approximated to 50:50 in the earlier hemopoietic precursor populations, namely LSK, CLP-like, and CMP-like cells. Against this background, we were able to see effects of the E2F4 genotype of the donor fetal liver cells.

In Fig. 3*B*, each grouping of data show the ratio of typed to competitor cells at the 4, 8, and 16 wk time point. Differences that are statistically significant at the level of $p < 0.05$ and differences that are significant at the level of $p < 0.005$ are indicated. Examining the LSK cells, it was clear that E2F4 influenced the abundance of these cells in the repopulation model. Thus at 4, 8, and 16 wk there was a significant difference in the effectiveness of competitive repopulation between E2F4-intact and E2F4-deficient cells.

The immediate differentiation products of LSK cells are CLP and CMP. When we examined the abundance of the phenotypic counterparts of these precursors in competitive repopulation, a

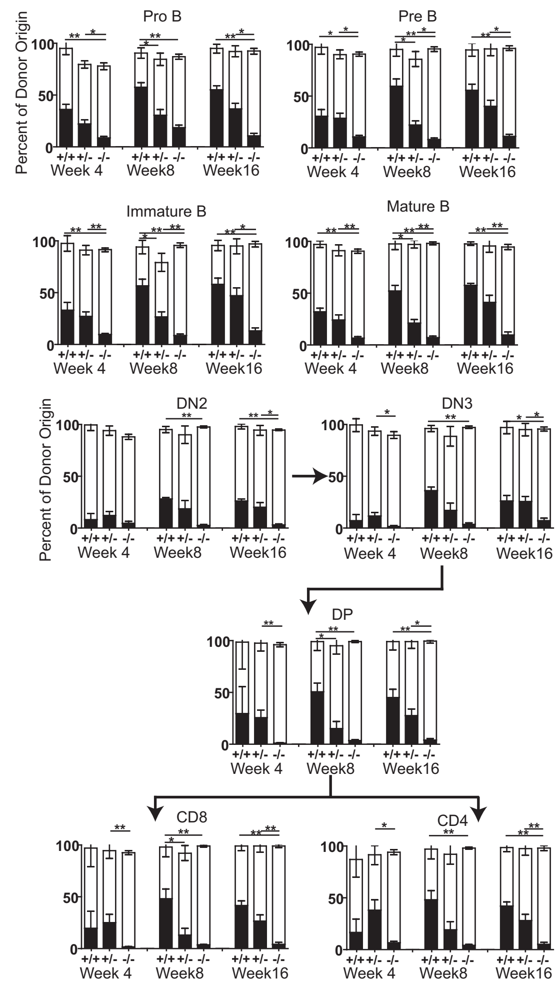


FIGURE 4. Repopulation of bone marrow B cells and thymocytes. Using the competitive repopulation strategy outlined in *Results* for Fig. 2, we determined the contribution of $e2f4^{+/+}$, $e2f4^{+/-}$, and $e2f4^{-/-}$ fetal liver cells, vs normal competitor cells, to the repopulation of bone marrow B cells and thymocytes. Data show that the defective repopulation characteristic of pre-B and pro-B cells and DN1 thymocytes (shown in Fig. 2) was propagated down both the B and T lineages.

clear difference emerged. In the case of CLP-like cells, there was no clear difference at 4 wk but at 8 and 16 wk, the E2F4-deficient cells showed a highly significant ($p < 0.005$) disadvantage compared with normal competitor cells. In contrast among the CMP-like cells, a significant ($p < 0.05$) effect of E2F4 at weeks 4 and 8 was lost by week 16.

To further evaluate the significance of this apparent difference, we evaluated the effect of *e2f4* genotype on the effectiveness of repopulation of the next compartments in the differentiation cascade: bone marrow pre-B and pro-B cells and double negative (DN)1 thymocytes as the differentiation products of CLP, and GMP-like and MEP-like cells as the products of CMP differentiation. In the case of both pre-B and pro-B cells and DN1 cells, the genotype of the typed fetal liver cells had a highly significant ($p < 0.005$) effect on the efficiency with which these cells competed with normal cells, and this effect was sustained at 8 and 16 wk of repopulation. We followed the effects of *e2f4* genotype on more differentiated B and T cells in the bone marrow and thymus; the effect of *e2f4* was sustained in all of these populations out to 16 wk (Fig. 3*B*). In contrast to the sustained effect on lymphocytes, the effect of fetal liver cell genotype on competition for the GMP-like cell compartment was significant ($p < 0.05$) at 8 and 16 wk, but

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017

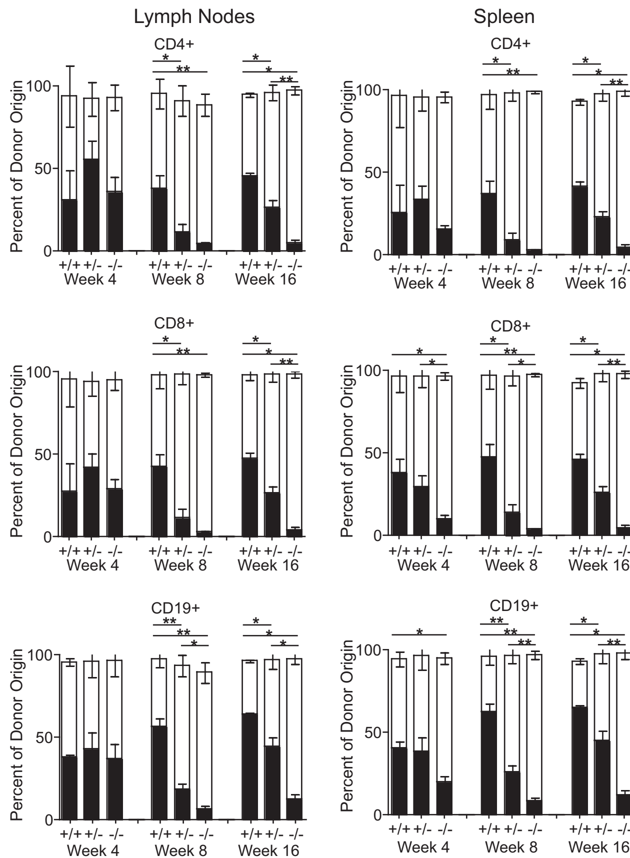


FIGURE 5. Repopulation of spleen and lymph node lymphocytes. The competitive repopulation of peripheral CD4$^+$ T cells, CD8$^+$ T cells, and CD19$^+$ B cells by *e2f4*$^{+/+}$, *e2f4*$^{+/-}$, and *e2f4*$^{-/-}$ fetal liver cells. This experiment extends data shown in Fig. 4 by revealing that no peripheral mechanisms compensate for the deficiency of T and B lineage cells in the primary lymphoid organs.

the significance of the fetal liver cell *e2f4* genotype in the MEP-like cell compartment was lost at 16 wk.

This experiment revealed a sustained effect of *e2f4* genotype on the capacity of HSC to repopulate the lymphoid lineages. In contrast, the effects on myeloid development were most evident at early time points, and tended to disappear as the reconstituted hemopoietic systems approached equilibrium. To test whether the effects on early progenitors of the B and T lineage could explain the immune deficiency of *e2f4*$^{-/-}$ mice, we analyzed the repopulation of more differentiated subsets of bone marrow B cells (Fig. 4) and thymocytes (Fig. 4). In both of these lineages, the capacity of *e2f4*$^{-/-}$ cells to compete with WT cells was severely impaired.

These defects were also observed in the secondary lymphoid organs. Thus, we analyzed the competitive efficiency of *e2f4*$^{+/+}$, *e2f4*$^{+/-}$, and *e2f4*$^{-/-}$ fetal liver cells to repopulate the CD4$^+$, CD8$^+$, and CD19$^+$ cells in the lymph nodes and spleen (Fig. 5). There was a statistically significant deficit in the repopulation of all three lymphocyte subsets by E2f4-deficient cells at 8 and 16 wk. We conclude that the early differentiation defect in *e2f4*$^{-/-}$ cells cannot be corrected at any later stage of T or B cell development.

These data stand in contrast to some published work in E2F4-deficient mice. Thus, the abundance of thymocytes and T cells has been reported to be normal in surviving adult *e2f4*$^{-/-}$ mice (17), but deficient in neonatal mice (15). We would now interpret the lack of thymocytes, described in *e2f4*$^{-/-}$ neonatal mice, to be an effect of a cell-intrinsic differentiation defect. The apparent recovery of the T cell lineage in older mice could be explained by the remarkable capacity of the T cell system to "fill space" through homeostatic expansion (22). Our data are not immediately consistent with the well-authenticated observation that *e2f4*$^{-/-}$ precursor cells are defective in erythroid differentiation, leading to anemia in surviving E2F4-deficient mice (15, 17). To reconcile these observations with our own, we need to consider that possibility that E2F4 acts in HSC self-renewal, in the HSC-to-CLP differentiation process, but also at a later stage in the erythroid lineage.

Because E2F4 appeared to be promoting the HSC-CLP-to-lymphoid lineage to a greater extent than the CMP-to-erythromyeloid lineage, we considered the possibility that *e2f4* gene expression was lineage-restricted. To test this consideration, we FACS-sorted LSK cells, CLP-like cells, and multiple other cell populations down the erythromyeloid and lymphoid lineages. The expression of *e2f4* message was compared with *gapdh* as an internal control, using quantitative real-time RT-PCR (Fig. 6). Rather to our surprise, the expression of *e2f4* gene was strongest in the erythromyeloid cells. Among the lymphoid cells, there was no difference between LSK cells and CLP-like cells, but a relative reduction in *e2f4* gene expression in pre-B and pro-B cells, followed by an increase of ~10-fold in later B cell subsets. Among the early T lineage cells, the expression *e2f4* message was similar in CLP-like cells and DN1, but the expression then increased 10-fold among later thymocyte stages. We conclude that the greater importance of E2F4 in lymphoid lineage cells does not simply reflect its selective expression in these cells.

During cell cycle control, E2F4 acts as a transcriptional repressor, binding to E2F-responsive promoter sequences in association with the pocket proteins p130 and p107. These pocket proteins are subject to phosphorylation by cyclin-dependent kinases, resulting in dissociation of the E2F4 from the promoter, and its export from the cell nucleus. Such de-repressed promoters are then available for transcription, under the control of the structurally related transcription factors, E2F1, E2F2, and E2F3a. It was important to determine whether the effects we observed on hemopoietic differentiation were linked to effects on the cell cycle. Therefore, we stained different subsets of bone marrow cells in fetal liver-reconstituted mice to determine their DNA content, and thus to calculate the percentage of cells in cycle (Fig. 7). At this

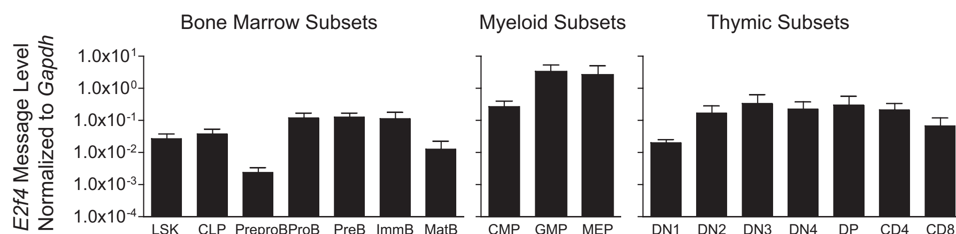
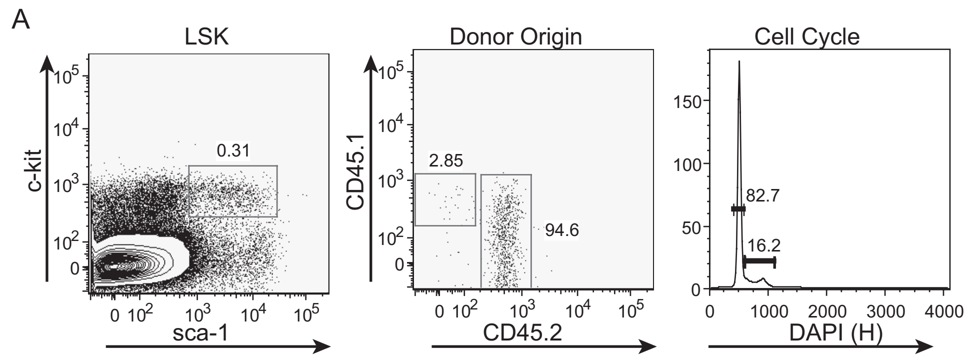


FIGURE 6. *e2f4* gene expression in hemopoietic cells. The *e2f4* message was present throughout lymphoid and myeloid development. Cell subsets were sorted and lysed to isolate mRNA. Quantitative real-time RT-PCR was used to determine the message level of *e2f4* transcripts. The *e2f4* message level was normalized to *gapdh* levels. Results are pooled from three independent experiments using $n > 5$ mice. Data shown represent mean ± SEM.

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017



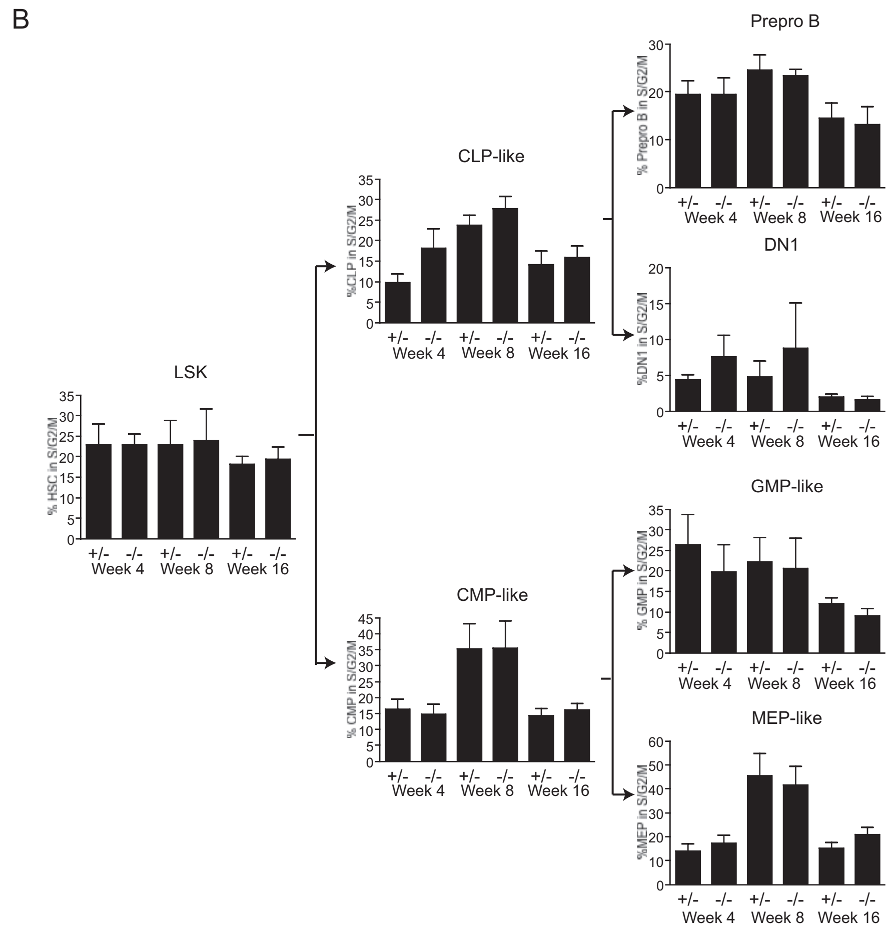


FIGURE 7. Cell cycle analysis. *A*, In noncompetitive repopulation experiments, subsets of early progenitor cells were identified by gating within the Lin^- population of bone marrow cells, as shown for LSK cells (*left*). Within these cells, donor-derived cells were identified based on CD45.2 expression (*center*). In these cells, DAPI staining was used to determine DNA content (*right*). Cells with a greater than 2n DNA content were defined to be in S, G_2, or M phase. *B*, The frequency of cells in S-G_2-M among seven subsets of early hemopoietic cells of either *e2f4*$^{+/-}$ or *e2f4*$^{-/-}$ genotype are shown. At weeks 4, 8, and 16 of repopulation, there was no effect of *e2f4* genotype on the frequency of cycling cells.

level of analysis, there was no obvious effect of E2F4 on hemopoietic precursor cell proliferation.

The influence of E2F4 over early hemopoietic lineage commitment could have been mediated through gene repression, similar to the effects on the cell cycle in other cell types. To test this observation, we isolated LSK, CLP-like, and CMP-like cells by flow cytometric cell sorting from fetal liver-reconstituted mice that had received *e2f4*$^{+/+}$ or *e2f4*$^{-/-}$ cells. In each of these six cell populations, the expression of a panel of key genes associated with either lymphoid or erythromyeloid differentiation was measured by quantitative real-time RT-PCR, using the expression of *gapdh* in each sample as an internal standard. The results of this analysis are shown in Fig. 8.

Among genes that are strongly associated with the lymphoid differentiation pathway, we measured *aiolos*, *ikaros*, *il7ra*, *pax5*, and *rag2* (23–25). In the case of *aiolos*, there was a trend toward lower expression in *e2f4*$^{-/-}$ cells, but this difference was only significant ($p < 0.05$) in CMP-like cells. The expression of *ikaros* was significantly lower in both CLP-like cells and CMP-like cells of *e2f4*$^{-/-}$ genotype, whereas in the case of *il7ra* the significant difference was in the LSK cells. The early B cell differentiation factor *pax5* was undetectable in *e2f4*$^{-/-}$ LSK cells, but not influenced by *e2f4* genotype in more differentiated cells. Finally, the expression of the *rag2* gene was not significantly altered in the absence of E2F4. In the case of these lymphoid-associated genes, the timing and magnitude of the *e2f4* effect was different in each case, but the shared feature was the reduced expression of the gene of interest, relative to *gapdh*.

We also examined genes with effects predominantly on erythromyeloid cells, or with effects on both pathways. Among these effects, the expression of *pu.1* was significantly reduced in LSK and CMP-like cells of *e2f4*$^{-/-}$ genotype. The role of the PU.1 factor is complex because it promotes either the myeloid or the lymphoid lineage based partly on its expression levels, and partly

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017

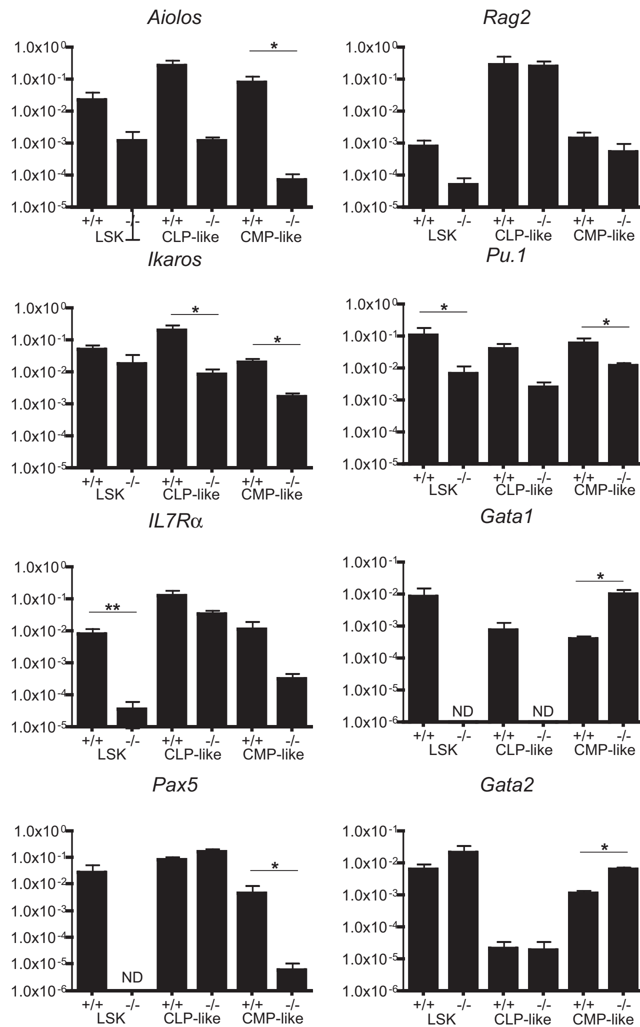


FIGURE 8. E2F4 influences the expression of lineage factors. Expression levels of several lymphoid or erythromyeloid differentiation-promoting genes were modified in $e2f4^{-/-}$ progenitor cells. Host mice were reconstituted for 16 wk with either $e2f4^{+/+}$ or $e2f4^{-/-}$ fetal liver cells. LSK cells, CLP-like cells, and CMP-like cells were sorted and lysed for mRNA analysis. Transcript levels for specific differentiation factors were determined by quantitative real-time RT-PCR and mRNA levels were normalized to *gapdh* expression levels. For the results, $e2f4^{+/+}$ samples ($n = 6$) for LSK, ($n = 8$) for CLP, and ($n = 4$) for CMP are used. Among $e2f4^{-/-}$ samples, $n = 6$ for LSK, $n = 6$ for CLP, and $n = 7$ for CMP are used. Data are mean ± SEM. statistical significance was determined by the Mann-Whitney *U* test.

on the concentrations of the other transcription factors with which PU.1 interacts (26–28). The GATA-1 factor is strongly associated with erythroid differentiation (29). Message for *gata1* was undetectable in LSK and CLP-like cells of $e2f4^{-/-}$ genotype, but significantly increased in the $e2f4^{-/-}$ CMP-like cells. The GATA-2 factor is important in stem cell self-renewal, and also has effects later in the megakaryocyte pathway (30–32). The expression of *gata2* was not modified by *e2f4* genotype in the LSK cells and the CLP-like cells, but similar to *gata1*, the expression was significantly increased in CMP-like cells. Not all of the lineage-associated genes we analyzed showed such straightforward effects. Thus, expression of c-*myc* was not significantly modified by the *e2f4* genotype in LSK, CLP-like, or CMP-like cells, and *c/ebpa* gene expression was reduced in $e2f4^{-/-}$ cells (data not shown), consistent its association with erythromyeloid differentiation but out of step with the effects on *gata1* and *gata2*.

Discussion

The findings suggest that the cell cycle control protein, E2F4, is involved in HSC differentiation through an action on the level of expression of genes associated with either the lymphoid or the erythromyeloid fate. Thus, competitive repopulation experiments revealed a selective defect in the capacity of $e2f4^{-/-}$ fetal liver cells to differentiation from cells corresponding to multilineage stem cells (the LSK cells) to cells corresponding to lymphoid precursors (CLP-like cells). In parallel, we observed effects on transcription factor expression in early hemopoietic progenitor cells that could explain the effects on differentiation. However, this effect could not be due to direct transcriptional repression because the lack of E2F4 was associated not with gene overexpression, but with gene underexpression. Therefore, we must conclude that the mechanism of action was indirect.

Among these effects, the impact of *e2f4* genotype on *ikaros*, *il7ra*, and *pax5* gene expression was consistent with its effect on the impaired repopulation of the lymphoid compartment by $e2f4^{-/-}$ cells. However, these effects are not simply explained by the E2F4 protein acting as a repressor of gene expression because in many cases the expression of the gene was decreased in $e2f4^{-/-}$ cells, and not increased as would be expected if a repressive factor were lost. The expression and modulation of *pax5* calls for special mention. This gene is regarded as B cell-specific, but we have detected its expression by adequately controlled, *gapdh*-standardized real-time quantitative RT-PCR in multiple FACS-sorted isolates of CMP-like cells. We are not aware of a prior study of *pax5* expression in nonlymphoid cells that has been conducted with this level of precision. Therefore, we believe the expression of *pax5* in CMP-like cells is a valid, novel observation.

Among transcription factors associated with erythromyeloid differentiation, the increased expression of *gata1* and *gata2* genes in $e2f4^{-/-}$ CMP-like cells is consistent with E2F4 protein acting as a repressor in these cells, and with respect to these genes. Thus, during the process of myeloid lineage commitment, E2F4 could promote the lymphoid cell fate by repressing these genes. However, there is no reason to believe that either the GATA1 or the GATA2 proteins act as repressors of lymphoid genes such as *ikaros*, *il7ra*, or *pax5*. Our observations are best accommodated by a model in which the process of lineage specification within the LSK compartment involves the direct repression by E2F4 of key genes associated with the erythromyeloid lineage, most strikingly *gata1*, and also the indirect de-repression of lymphoid genes. The E2F4 protein could then act as a repressor of a repressor, rendering accessible such genes as *ikaros*, *il7ra*, and *pax5*.

Cycling mammalian cells enter proliferative quiescence though the cessation of the action of cyclin-dependent kinases, often through their inhibition by specific inhibitors of the cip/kip or the INK families. In the absence of cyclin-dependent kinase action, pocket proteins (pRb, p107, and p130) become dephosphorylated. This leads to the binding of E2F factors, including E2F4. The active E2F transcription factors E2F1, E2F2, and E2F3a are immobilized in the cytoplasm, whereas E2F4 is translocated to the nucleus, where it binds to promoters and causes gene repression. As a result of this study, we found that a deficiency of E2F4 strongly influences the expression of both lymphoid-associated and erythromyeloid lineage-associated genes in early hemopoietic progenitor cell subsets, with corresponding effects on the differentiation fate of the cells in vivo. Thus, a molecular mechanism that is elsewhere linked to cell cycle exit does not have this function, but instead is associated with differentiation, in early hemopoietic cells.

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017

Acknowledgments

We thank Dr. Joseph Nevins for the gift of the E2F4 mutant mice. We also thank Nathan Laniewski for sorting cell populations for several experiments, and Drs. Jim Miller, Tim Mosmann, and Craig Jordan for critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Adams, G. B., and D. T. Scadden. 2006. The hematopoietic stem cell in its place. *Nat. Immunol.* 7: 333–337.
2. Warren, L. A., and E. V. Rothenberg. 2003. Regulatory coding of lymphoid lineage choice by hematopoietic transcription factors. *Curr. Opin. Immunol.* 15: 166–175.
3. Yoshida, T., S. Y. Ng, J. C. Zuniga-Pflucker, and K. Georgopoulos. 2006. Early hematopoietic lineage restrictions directed by Ikaros. *Nat. Immunol.* 7: 382–391.
4. Zhang, D. E., S. Hohaus, M. T. Voso, H. M. Chen, L. T. Smith, C. J. Hetherington, and D. G. Tenen. 1996. Function of PU. 1 (Spi-1), C/EBP, and AML1 in early myelopoiesis: regulation of multiple myeloid CSF receptor promoters. *Curr. Top. Microbiol. Immunol.* 211: 137–147.
5. Du, J., M. J. Stankiewicz, Y. Liu, Q. Xi, J. E. Schmitz, J. A. Lekstrom-Himes, and S. J. Ackerman. 2002. Novel combinatorial interactions of GATA-1, PU.1, and c/EBPε isoforms regulate transcription of the gene encoding eosinophil granule major basic protein. *J. Biol. Chem.* 277: 43481–43494.
6. Trimarchi, J. M., and J. A. Lees. 2002. Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell Biol.* 3: 11–20.
7. Attwooll, C., E. Lazzerini Denchi, and K. Helin. 2004. The E2F family: specific functions and overlapping interests. *EMBO J.* 23: 4709–4716.
8. Dimova, D. K., and N. J. Dyson. 2005. The E2F transcriptional network: old acquaintances with new faces. *Oncogene* 24: 2810–2826.
9. Wu, L., C. Timmers, B. Maiti, H. I. Saavedra, L. Sang, G. T. Chong, F. Nuckolls, P. Giangrande, F. A. Wright, S. J. Field, et al. 2001. The E2F1–3 transcription factors are essential for cellular proliferation. *Nature* 414: 457–462.
10. La Thangue, N. B. 2003. The yin and yang of E2F-1: balancing life and death. *Nat. Cell Biol.* 5: 587–589.
11. Araki, K., Y. Nakajima, K. Eto, and M. A. Ikeda. 2003. Distinct recruitment of E2F family members to specific E2F-binding sites mediates activation and repression of the E2F1 promoter. *Oncogene* 22: 7632–7641.
12. Dyson, N. 1998. The regulation of E2F by pRB-family proteins. *Genes Dev.* 12: 2245–2262.
13. Cartwright, P., H. Müller, C. Wagener, K. Holm, and K. Helin. 1998. E2F-6: a novel member of the E2F family is an inhibitor of E2F-dependent transcription. *Oncogene* 17: 611–623.
14. Maiti, B., J. Li, A. de Bruin, F. Gordon, C. Timmers, R. Opavsky, K. Patil, J. Tuttle, W. Cleghorn, and G. Leone. 2005. Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation. *J. Biol. Chem.* 280: 18211–18220.
15. Rempel, R. E., M. T. Saenz-Robles, R. Storms, S. Morham, S. Ishida, A. Engel, L. Jakoi, M. F. Melhem, J. M. Pipas, C. Smith, and J. R. Nevins. 2000. Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Mol. Cell* 6: 293–306.
16. Persengiev, S. P., I. I. Kondova, and D. L. Kilpatrick. 1999. E2F4 actively promotes the initiation and maintenance of nerve growth factor-induced cell differentiation. *Mol. Cell Biol.* 19: 6048–6056.
17. Humbert, P. O., C. Rogers, S. Ganiatsas, R. L. Landsberg, J. M. Trimarchi, S. Dandapani, C. Brugnara, S. Erdman, M. Schrenzel, R. T. Bronson, and J. A. Lees. 2000. E2F4 is essential for normal erythrocyte maturation and neonatal viability. *Mol. Cell* 6: 281–291.
18. Kinross, K. M., A. J. Clark, R. M. Iazzolino, and P. O. Humbert. 2006. E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. *Blood* 108: 886–895.
19. Spangrude, J. G., I. Aihara, I. L. Weissman, and J. Klein. 1988. The stem cell antigens Sca-1 and Sca-2 subdivide thymic and peripheral T lymphocytes into unique subsets. *J. Immunol.* 141: 3697–3707.
20. Kouro, T., K. L. Medina, K. Oritani, and P. W. Kincade. 2001. Characteristics of early murine B-lymphocyte precursors and their direct sensitivity to negative regulators. *Blood* 97: 2708–2715.
21. Traver, D., T. Miyamoto, J. Christensen, J. Iwasaki-Arai, K. Akashi, and I. L. Weissman. 2001. Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. *Blood* 98: 627–635.
22. Rocha, B., N. Dautigny, and P. Pereira. 1989. Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. *Eur. J. Immunol.* 19: 905–911.
23. Rothenberg, E. V., and R. Pant. 2004. Origins of lymphocyte developmental programs: transcription factor evidence. *Semin. Immunol.* 16: 227–238.
24. Nutt, S. L., B. Heavey, A. G. Rolink, and M. Busslinger. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* 401: 556–562.
25. Morgan, B., L. Sun, N. Avitahl, K. Andrikopoulos, T. Ikeda, E. Gonzales, P. Wu, S. Neben, and K. Georgopoulos. 1997. Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation. *EMBO J.* 16: 2004–2013.
26. Nerlov, C., and T. Graf. 1998. PU. 1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev.* 12: 2403–2412.
27. Dahl, R., and M. C. Simon. 2003. The importance of PU.1 concentration in hematopoietic lineage commitment and maturation. *Blood Cells Mol. Dis.* 31: 229–233.
28. Dahl, R., J. C. Walsh, D. Lancki, P. Laslo, S. R. Iyer, H. Singh, and M. C. Simon. 2003. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPα ratio and granulocyte colony-stimulating factor. *Nat. Immunol.* 4: 1029–1036.
29. Takahashi, S., T. Komeno, N. Suwabe, K. Yoh, O. Nakajima, S. Nishimura, T. Kuroha, T. Nagasawa, and M. Yamamoto. 1998. Role of GATA-1 in proliferation and differentiation of definitive erythroid and megakaryocytic cells in vivo. *Blood* 92: 434–442.
30. Ikonomi, P., C. E. Rivera, M. Riordan, G. Washington, A. N. Schechter, and C. T. Noguchi. 2000. Overexpression of GATA-2 inhibits erythroid and promotes megakaryocyte differentiation. *Exp. Hematol.* 28: 1423–1431.
31. Labbaye, C., M. Valtieri, T. Barberi, E. Meccia, B. Masella, E. Pelosi, G. L. Condorelli, U. Testa, and C. Peschle. 1995. Differential expression and functional role of GATA-2, NF-E2, and GATA-1 in normal adult hematopoiesis. *J. Clin. Invest.* 95: 2346–2358.
32. Ling, K. W., K. Ottersbach, J. P. van Hamburg, A. Oziemlak, F. Y. Tsai, S. H. Orkin, R. Ploemacher, R. W. Hendriks, and E. Dzierzak. 2004. GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. *J. Exp. Med.* 200: 871–882.

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017