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Use of RDA analysis of knockout mice to identify myeloid genes regulated *in vivo* by PU.1 and C/EBPα

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ABSTRACT

PU.1 and C/EBPα are transcription factors essential for normal myeloid development. Loss-of-function mutation of PU.1 leads to an absolute block in monocyte/macrophage development and abnormal granulocytic development while that of C/EBPα causes a selective block in neutrophilic differentiation. In order to understand these phenotypes, we studied the role of PU.1 and C/EBPα in the regulation of myeloid target genes *in vivo*. Northern blot analysis revealed that mRNAs encoding receptors for M-CSF, G-CSF and GM-CSF, were expressed at low levels in PU.1$^{-/-}$ fetal liver compared with wild type. To identify additional myeloid genes regulated by PU.1 and C/EBPα, we performed representational difference analysis (RDA), a PCR-based subtractive hybridization using fetal livers from wild type and PU.1 or C/EBPα knockout mice. By introducing a new modification of RDA, that of tissue-specific gene suppression, we could selectively identify a set of differentially expressed genes specific to myeloid cells. Differentially expressed genes included both primary and secondary granule protein genes. In addition, eight novel genes were identified that were upregulated in expression during myeloid differentiation. These methods provide a general strategy for elucidating the genes affected in murine knockout models.

INTRODUCTION

Transcription factors play a major role in cell differentiation, including the development of specific hematopoietic lineages from stem cells (1,2). Mature myeloid cells, consisting of blood monocytes and tissue macrophages, as well as the neutrophilic and eosinophilic granulocytes, develop from a common myeloid precursor. However, the mechanism controlling the development of common myeloid precursors as well as the transition from common precursors into unipotential granulocyte and monocyte precursors has not been fully identified. So far several transcription factors has been implicated in myelopoiesis. These include PU.1, basic leucine zipper (bZIP) CCAAT/enhancer binding protein family (C/EBP), acute myelogenous leukemia 1 (AML1), retinoic acid receptor α (RARα), promyelocytic leukemia zinc finger (PLZF), myeloid zinc finger protein-1 (MZF-1), early response gene-1 (Egr-1), Wilms' tumor suppressor gene (WT-1) and homeobox proteins (2), among which PU.1 and C/EBPα have been shown to be indispensable for myeloid development by gene targeting experiments (3–5).

PU.1 is a member of the Ets transcription family and is predominantly expressed in hematopoietic cells (6). PU.1 mRNA is expressed at low levels in multipotential CD34^{+} cells, and is upregulated with myeloid and B cell differentiation (6–9). Transient transfection studies have shown that PU.1 regulates the promoters of a number of myeloid genes, such as CD11b, primary granule proteins (myeloperoxidase, neutrophilic elastase and proteinase-3), GM-CSF receptor, G-CSF receptor and M-CSF receptor (10–16). Several different loss-of-function experiments revealed that PU.1 is involved in myeloid and lymphoid development (4,5,9,17–19). PU.1 knockout mice completely lack macrophages including osteoclasts, as well as B cells, and show impaired granulopoiesis and T-cell development (4,5,20). However, fetal liver cells from PU.1$^{-/-}$ mice do express mRNA for early myeloid genes (17).

C/EBPα is a member of the C/EBP family, which has a bZIP structure. C/EBPα was originally characterized in liver and adipose tissues, and has been shown to regulate a number of hepatic and adipocyte genes (21–23). Recently, the expression of C/EBPα was shown to initiate with the commitment of multipotential precursors to the myeloid lineage, and be specifically upregulated during granulocytic differentiation (2,24,25). Transient transfection studies have shown that C/EBPα can regulate the promoters of a number of myeloid specific genes, such as G-CSF receptor (15), neutrophil elastase (12) and myeloperoxidase (11). C/EBPα knockout mice die within 8 h of birth because they are unable to properly synthesize and mobilize glycogen and fat (26,27). They also show a selective block in differentiation of neutrophils. Mature neutrophils and eosinophils are not observed in the blood or fetal liver of mutant mice and, instead, myeloid blasts are observed. Like the PU.1 knockout animals, fetal liver cells from C/EBPα$^{-/-}$ clearly express mRNA for some myeloid genes (3). Other hematopoietic lineages are not affected, including monocytes and macrophages (3). These results strongly suggest a critical role for C/EBPα in granulocytic differentiation.

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To understand the impaired myeloid development caused by loss-of-function mutation of PU.1 and C/EBPα, myeloid colony-stimulating factor (CSF) receptors have been suggested as critical targets for PU.1 and C/EBPα (3,17,18). However, the loss-of-function mutant mice of each CSF or CSF receptor do not show defects in myeloid development as severe as those of PU.1 or C/EBPα knockout mice (28–31). It is likely that there exist additional critical targets. In order to identify these target genes for PU.1 and C/EBPα during myeloid development, we have analyzed expression of presumptive myeloid target genes *in vivo* as well as performed representational difference analysis (RDA), a PCR-based subtractive hybridization using wild-type and knockout mice. In contrast with differential display, which amplifies fragments from all represented mRNA species, RDA eliminates those fragments present in both populations, leaving only the difference (32,33). Recently, several groups successfully identified differentially expressed genes using RDA (33–36). In this study, to focus on the differentially expressed genes of myeloid lineage, we tried several new modifications of the RDA procedure.

Here we show the *in vivo* role of PU.1 and C/EBPα in regulating myeloid genes by northern blot analysis, and by using RDA combined with specific gene suppression, we identified a set of myeloid genes, the expression of which is missing or significantly decreased in the mutant fetal liver. These genes included myeloid granule protein genes and eight novel myeloid genes which are new candidate targets for PU.1 and C/EBPα transcription factors.

MATERIALS AND METHODS

Mice, tissues and cells

Targeted disruption of C/EBPα and PU.1 was achieved by homologous recombination in embryonic stem cells and generation of mice from these cell lines as reported previously (5,26). Fetal liver was obtained from embryonic day 19 fetuses. Purification of fetal liver hematopoietic cells was performed by passing fetal liver through 70 μm nylon mesh cell strainers (Becton Dickinson Labware, Franklin Lakes, NJ). Peritoneal exudate cells were harvested by lavage with 10 ml PBS 20, 48 and 72 h after i.p. injection of 1.5 ml 10% thioglycollate broth. Morphological examination revealed that the cells consisted of ~15% monocytes/macrophages and 80% polymorphonuclear granulocytes 20 h after injection, and 50 versus 45%, and 80 versus 15% at 48 and 72 h, respectively.

The murine lymphohematopoietic progenitor cell line EML was maintained in IMDM supplemented with 20% horse serum, glutamine, non-essential amino acids and 10% conditioned medium from BHK cells transfected with rat stem cell factor cDNA (BHK-MKL cells) (37). To induce myeloid differentiation, EML cells were cultured in IMDM supplemented with 10% BHK-MKL conditioned medium, 5% WEHI-3 conditioned medium and 10^{-5} M all-trans retinoic acid (RA) for 72 h. Cells were then washed three times to remove RA and recultured in IMDM supplemented with murine GM-CSF (2.5 ng/ml) for indicated times (37).

RNA preparation and northern blot analysis

Total RNA was isolated by guanidium isothiocyanate extraction followed by CsCl gradient purification (38). Poly(A)$^+$ RNA was purified from total RNA with oligo(dT) cellulose columns (New England Biolabs, Beverly, MA). Fifteen micrograms of each RNA sample were resolved by agarose formaldehyde gel electrophoresis and transferred to Biotrans nylon membranes (ICN, Biomedicals, Inc., Costa Mesa, CA). The blots were hybridized to [α-^{32}P]dCTP-labeled DNA fragments generated by RDA as described previously (8) and exposed for 1–2 days with an intensifying screen. To normalize the loading of RNA samples in each lane, the probe was removed and the blot was rehybridized to an [α-^{32}P]dCTP 3′-end-labeled 18S oligonucleotide (39).

cDNA synthesis

Oligo(dT)-primed double-stranded cDNA was synthesized from 5–10 μg of poly(A)$^+$ RNA using a cDNA synthesis system (GIBCO BRL, Grand Island, NY) according to the manufacturer's instructions. Tester and driver cDNA samples were synthesized in parallel at the same time.

Representational difference analysis

RDA was performed using reagents as described (32–34). The following oligonucleotides were synthesized and used for RDA: R-Bgl-24, 5′-AGCACTCTCCAGCCTCTCACCGCA-3′; R-Bgl-12, 5′-GATCTGCGGTGA-3′; J-Bgl-24, 5′-ACCGACGTCGACTATCCATGAACA-3′; J-Bgl-12, 5′-GATCTGTTCATG-3′; N-Bgl-24, 5′-AGGCAACTGTGCTATCCGAGGGAA-3′; and N-Bgl-12, 5′-GATCTTCCCTCG-3′. cDNA was digested with *Dpn*II and ligated to the R-Bgl-12/24 adaptors. Amplicons were made by PCR amplification of the ligated *Dpn*II cDNA fragments for 20 cycles using the R-Bgl-24 as a primer. Driver DNA was prepared by digesting amplicons with *Dpn*II. Tester DNA was prepared by gel-purification of digested amplicons between 150 and 2000 bp followed by ligation to J-Bgl-12/24 adaptors. First subtractive hybridization was performed using 400 ng tester and 40 μg driver (tester:driver = 1:100). An aliquot of the hybridization mixture was amplified by PCR for 10 cycles using the J-Bgl-24 as a primer. The PCR products were then digested with mung bean nuclease (New England Biolabs) at 30°C for 35 min and further amplified for 18 cycles. These PCR products are the first difference products (DP1). The difference products were digested with *Dpn*II and ligated to a new adaptor, N-Bgl-12/24 (after the first hybridization) or J-Bgl-12/24 (after the second hybridization), and the procedure was repeated twice using tester:driver ratios of 1:800 and 1:4000–400 000 for the second and third round of hybridization, respectively.

In some experiments (see Results), previously generated cDNA fragments were suppressed by adding 150–300 ng of each DNA fragment without adaptors to each round of hybridization. The suppression of liver genes was performed by adding 40 μg driver prepared from adult liver to each round of hybridization. The suppression of mature myeloid genes was performed by adding 30 μg driver prepared from peritoneal exudate cells collected 20 or 72 h after i.p. injection of thioglycollate.

DNA sequences of novel cDNA clones identified by RDA have been submitted to the GenBank database (accession nos AA720492–AA720501).

RESULTS

Expression of transcription factors in mutant mice

To check the involvement of PU.1 or C/EBPα in the regulation of transcription factors likely to play critical roles in myeloid

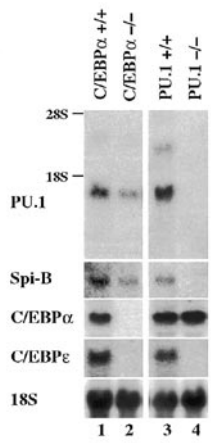

Figure 1. Northern blot analysis of mRNA of transcription factor genes. Total RNA (15 µg) from wild-type (lanes 1 and 3), C/EBPα$^{-/-}$ (lane 2) and PU.1$^{-/-}$ (lane 4) day 19 fetal liver was analyzed using species- and gene-specific 5′ cDNA probes of PU.1 and Spi-B (8), a 3′ cDNA probe of C/EBPα (26) and exon 3 of murine C/EBPε (41).

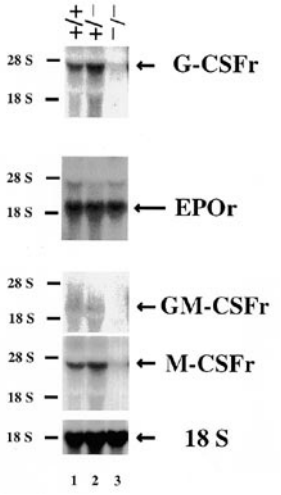

Figure 2. Northern blot analysis of mRNA of growth factor receptors in PU.1 knockout mice. Total RNA (20 µg) from wild-type (lane 1), PU.1 +/– (lane 2), or PU.1$^{-/-}$ (lane 3) day 19 fetal liver was electrophoresed in 1% agarose/formaldehyde gels, transferred to a nylon membrane and probed with murine cDNAs corresponding to M-CSF receptor (M-CSFr) (59); G-CSF receptor (G-CSFr) (60); GM-CSF receptor α (GM-CSFr) (61); erythropoietin receptor (EPOr) (62); and 18S oligonucleotide (39).

development, we analyzed the expression of PU.1, Spi-B, C/EBPα and C/EBPε (40,41), all of which have been shown or postulated to play a role in myeloid development (2), in the mutant fetal livers (Fig. 1). PU.1 knockout mice were made by disrupting the DNA binding domain, inserting the neo gene within exon 5 (5), but no PU.1-related transcripts were detected in PU.1$^{-/-}$ fetal liver by a probe specific to the 5′ end of the PU.1 cDNA (8), confirming that this is indeed a null phenotype. PU.1 expression was decreased ~50% in C/EBPα$^{-/-}$ fetal liver, consistent with the presence of a significant number of immature myeloid cells in these animals (3). Spi-B is an Ets transcription factor closely related to PU.1 but now known to be expressed primarily in B cells (8,42). Spi-B expression was slightly decreased in C/EBPα$^{-/-}$ fetal liver, but was undetectable in PU.1$^{-/-}$ fetal liver. C/EBPα expression was not affected in PU.1$^{-/-}$ fetal liver, but it is very hard to detect a difference in hematopoietic cells because of the high C/EBPα expression by hepatocytes and adipocytes. Another myeloid specific C/EBP transcription factor, C/EBPε, which is critical for terminal myeloid maturation (41), was not expressed in both mutant fetal livers. These findings are consistent with the lineage-specific expression of Spi-B and C/EBPε in B cell and granulocyte lineages, respectively, and suggest that they might be regulated by PU.1 and/or C/EBPα. Alternatively, the knockout cells might be blocked in their differentiation and do not become mature enough to express Spi-B or C/EBPε.

mRNA expression of myeloid CSF receptors in mutant mice

We have previously shown that G-CSF receptor mRNA is selectively downregulated in C/EBPα knockout mice by northern blot analysis, whereas M-CSF receptor and GM-CSF receptor mRNA levels are not impaired (3). This suggests that impaired G-CSF signaling might be in part responsible for the selective block of neutrophilic differentiation. To determine the role of myeloid CSF receptors in the defect found in PU.1$^{-/-}$ mice, we analyzed their expression in PU.1$^{-/-}$ fetal liver by northern blot analysis (Fig. 2). The expression of M-CSF receptor and G-CSF receptor mRNA was markedly decreased but detectable, consistent with previous reports indicating the presence of early myeloid gene expression in PU.1$^{-/-}$ fetal liver cells (17). GM-CSF receptor mRNA was not detectable in PU.1$^{-/-}$ fetal liver by northern blot analysis, although it has been detected by RT–PCR (17). These findings are consistent with promoter studies demonstrating a functional PU.1 site in transient transfection analysis (14–16). The levels of erythropoietin receptor mRNA were not affected in PU.1$^{-/-}$ fetal livers compared with wild type, in keeping with the lack of a consistent effect of PU.1 disruption on erythropoiesis (4,5). Although PU.1 and C/EBPα knockout mice have no detectable, or very low, levels of CSF receptor expression, their defects in myeloid development are more severe than those of loss-of-function mutant mice of CSFs or CSF receptors (28–31). This suggests that there are additional genes regulated by PU.1 and C/EBPα whose altered expression lead to the observed phenotype. Therefore, we performed RDA to identify these additional genes regulated by these transcription factors.

Identification of differentially expressed genes between C/EBPα +/+ and –/– fetal livers by RDA

As noted above, C/EBPα-deficient mice show a selective block in differentiation of neutrophils. Other hematopoietic lineages, including monocytes, are not affected (3). To identify C/EBP-α-regulated genes during neutrophilic differentiation, we performed RDA using C/EBPα-deficient mice. The cDNA in which the differentially expressed cDNAs are to be found is called ‘tester’ cDNA, and the reference cDNA is called ‘driver’ cDNA. The cDNA from each population is digested with a restriction endonuclease, ligated to adaptors, and then amplified by PCR. The products of amplification are called amplicons. To isolate amplicons unique to the tester cDNA, tester amplicon was ligated to new adaptors and hybridized to an excess of driver amplicon. PCR with primers for the new adaptors preferentially amplifies tester–tester homoduplexes. This process is repeated several

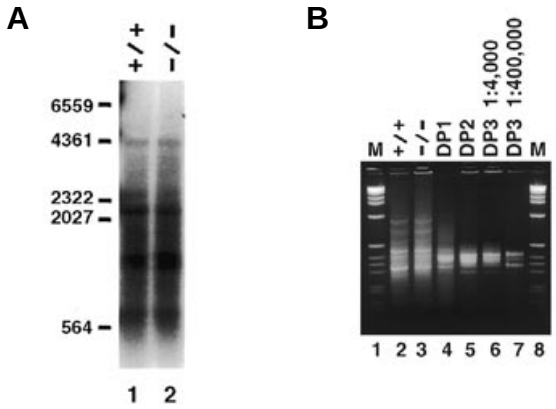

Figure 3. (**A**) Alkaline agarose gel analysis of second strand cDNA synthesis. α-^{32}P-labeled second strand cDNAs made from wild-type (lane 1) or C/EBPα$^{-/-}$ (lane 2) day 19 fetal liver mRNA were electrophoresed on a 1.4% alkaline agarose gel, and the dried gel was exposed to X-ray film. (**B**) Agarose electrophoresis of difference products generated by RDA. Amplicon from wild-type day 19 fetal liver cDNA (lane 2), amplicon from C/EBPα$^{-/-}$ day 19 fetal liver cDNA (lane 3), first difference product (DP1) with tester to driver ratio of 1:100 (lane 4), DP2 with tester to driver ratio of 1:800 (lane 5), DP3 with tester to driver ratio of 1:4000 (lane 6) and 1:400 000 (lane 7) and DNA molecular markers (lanes 1 and 8) were electrophoresed on a 2.0% agarose gel.

times with increasing driver-to-tester ratios until only fragments unique to the tester remain (32,33).

Since C/EBPα mutant mice die soon after birth, we used day 19 fetal liver as material for RDA. Because C/EBPα also regulates transcription of hepatocyte- and adipocyte-specific genes, these genes as well as myeloid-specific genes were candidates for identification from fetal liver by RDA. Poly(A)$^+$ RNA was purified from fetal livers of day 19 embryos and double-stranded cDNA was synthesized using an oligo(dT) primer. After ligating adaptors to *Dpn*II-digested cDNAs, the tester and driver amplicons were generated by PCR amplification. It is important that preparations to be subtracted are as similar as possible as shown in Figure 3. If the quality of cDNA and amplicon varies between subtracted populations, this can result in amplification of false positives. The tester was subtracted with the driver, and the difference was selectively amplified by PCR. This process was repeated three times until the difference products (DPs) showed clear bands with little background visible by ethidium bromide staining (Fig. 3B). The third subtraction was performed with different hybridization ratios. The third difference product (DP3) with a higher stringency (tester:driver = 1:400 000) showed fewer bands than that with lower stringency (1:4000), indicating that more genes were suppressed in the third subtraction by increasing ratios of tester to driver (Fig. 3B). After digesting with *Dpn*II, DP2 (1:800) and DP3 (1:400 000) were separated on an agarose gel and each band was excised out and subcloned. The subcloned inserts were used in northern blot analysis as probes to check mRNA expression of identified genes.

Table 1 shows the profile of genes identified by RDA in this screening. DP3 with a higher stringency (1:400 000) contained C/EBPα, demonstrating that the RDA procedure was selecting differentially expressed genes. The northern blot analysis revealed that all genes were truly differentially expressed, i.e. expressed in the C/EBPα$^{+/+}$ fetal liver, but not or at lower levels in C/EBPα$^{-/-}$ fetal liver (Table 1). As expected, DP3 contained both myeloid-specific and liver-specific genes, including neutrophilic elastase, which has been previously characterized in transient transfection studies as one of the targets for C/EBPα (12). DP2 (1:800) contained more genes than DP3, and most of them were distinct from DP3 except for contrapsin and protein C. Interestingly, DP2 contained three additional differentially expressed genes, but the expression of the remaining seven genes showed no difference between C/EBPα +/+ and –/– fetal liver or was unexpectedly upregulated in C/EBPα$^{-/-}$ fetal liver (Table 1). These results indicate that although RDA could selectively amplify differentially expressed genes, to keep high specificity, it requires a high stringency, which causes a limit on the number of genes amplified.

Table 1. DNA fragments generated by RDA, C/EBPα +/+ minus –/– with different stringencies

Gene (accession no.)	Expression E19 fetal liver C/EBPα +/+	–/–	BM	Adult liver
DP3 (tester:driver = 1:400 000				
[a]C/EBPα (M62362)	+	–	+	+
[a]Neutrophilic elastase (U04962)	2+	–	3+	–
[a]Contrapsin (X55147)	2+	–	–	3+
[a]Protein C (D10445)	5+	+	–	6+
[a]Unknown 1	+	–	–	+
DP2 (tester:driver = 1:800)				
[a]Contrapsin (X55147)				
[a]Protein C (D10445)				
[a]Haptoglobin (S67972)	2+	–	+	2+
[a]Apolipoprotein A-I (L04151)	4+	+	–	3+
[a]Eosinophil chemotactic factor (X15313)	+	–	3+	–
Pref-1 (L12721)	+	5+	–	–
Unknown 2	+	+	–	+
Unknown 3	+	+	–	+
Unknown 4	+	+	–	2+
Unknown 5	+	+	–	+
Unknown 6	2+	2+	–	3+

Relative expression was evaluated by northern blot analysis. Evaluation of mRNA levels are consistent for each gene but cannot be compared among genes.
[a]Genes differentially expressed.

Suppression of liver genes during RDA subtractive hybridization

As shown in Table 1, RDA using C/EBPα$^{-/-}$ fetal liver amplified liver genes more than myeloid genes, possibly because day 19 fetal liver contains more liver RNA than myeloid RNA. To preferentially amplify myeloid genes, we modified the RDA technique. First of all, we enriched hematopoietic cells by using cell strainers, nylon mesh devices with 70 μm pore size which select for cell size. We could enrich hematopoietic cells ~3–4-fold by passing fetal livers through cell strainers (data not shown). We then prepared liver-specific DNA fragments generated in the previous RDA (Table 2) and driver from adult liver, and added either of them in the hybridization mixture to suppress the amplification of liver-specific genes. We used a high stringency of 1:400 000 for the third round of subtractive hybridization. The DP3 showed several clear bands on an agarose gel, and the profile of bands was

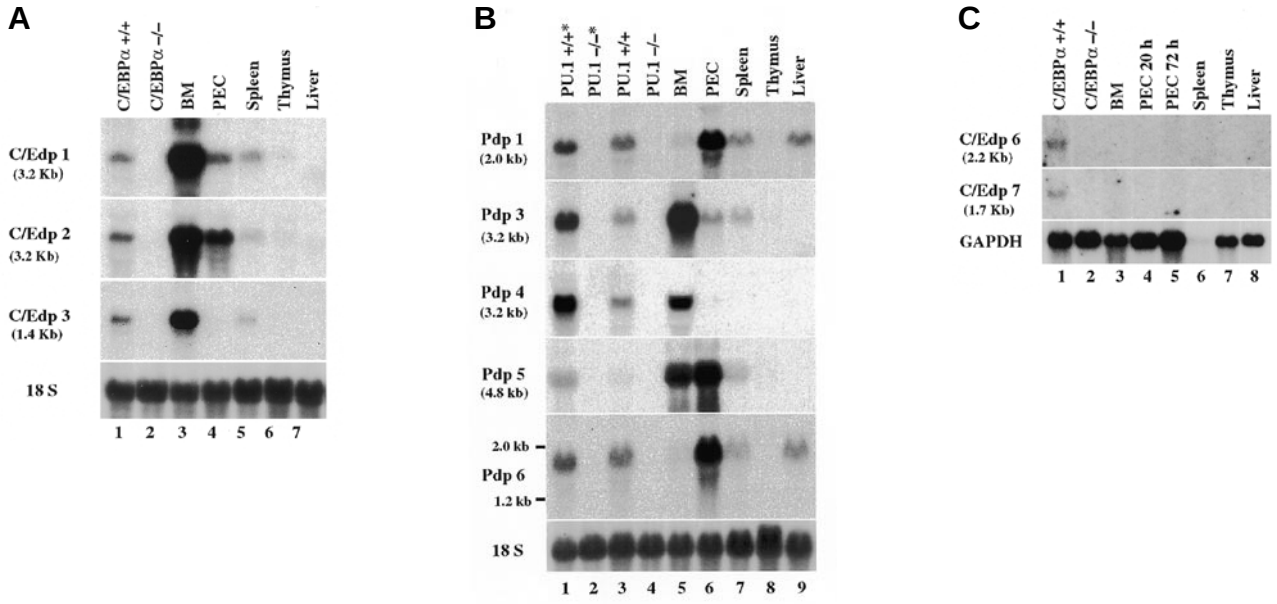

Figure 4. Northern blot analysis of mRNA of novel genes generated by RDA. (**A**) Total RNA (15 µg) from wild-type (lane 1) and C/EBPα$^{-/-}$ (lane 2) day 19 fetal liver, bone marrow (lane 3), peritoneal exudate cells 48 h after thioglycollate stimulation (lane 4), spleen (lane 5), thymus (lane 6) and adult liver (lane 7). (**B**) Total RNA (15 µg) from wild-type (lanes 1 and 3) and PU.1$^{-/-}$ (lanes 2 and 4) day 19 fetal liver treated (lanes 1 and 2) and untreated (lanes 3 and 4) with cell strainers, bone marrow (lane 5), peritoneal exudate cells 48 h after thioglycollate stimulation (lane 6), spleen (lane 7), thymus (lane 8) and adult liver (lane 9). (**C**) Poly(A)$^+$ RNA (3 µg) from wild-type (lane 1) and C/EBPα$^{-/-}$ (lane 2) day 19 fetal liver, bone marrow (lane 3), peritoneal exudate cells 20 h (lane 4) and 72 h (lane 5) after thioglycollate stimulation, spleen (lane 6), thymus (lane 7) and adult liver (lane 8). DNA fragments generated by RDA were used as probes.

very similar with each type of suppression. Nucleotide sequence analysis of DP3 revealed that the suppression of liver-specific genes worked very well (Table 2); liver genes were dramatically suppressed during the subtractive hybridization. DP3 with specific suppression by liver-specific DNA fragments contained only two liver-specific genes, and DP3 with suppression by the driver prepared from adult liver contained no liver-specific genes. The successful suppression of liver genes led to the amplification of more myeloid genes. Many genes for primary and secondary granule proteins of neutrophils were identified, including myeloperoxidase and neutrophilic elastase, targets for C/EBPα identified by transient transfection assays (11,12) (Table 2). In addition to known myeloid genes, five novel genes were identified (C/Edp 1–5, Table 2). C/Edp 2 and 3 showed high nucleotide sequence similarity to human neutrophil collagenase (79%) and human ficolin (72%), respectively, suggesting that C/Edp 2 and 3 are putative murine homologues of these genes. The other genes showed no significant similarity to any known genes. Northern blots revealed that all of these unknown genes were differentially expressed and were preferentially expressed in the BM and/or peritoneal exudate cells consisting of mature neutrophils and monocytes (Fig. 4A). These results demonstrate that liver genes could be successfully suppressed by using specific gene fragments or adult liver driver, and this suppression facilitates the amplification of myeloid-specific genes.

Identification of differentially expressed genes between PU.1 +/+ and –/– fetal livers by RDA

PU.1-deficient mice show impaired myeloid and lymphoid development (4,5). The mutant mice lack mature neutrophils, monocytes/macrophages and lymphoid cells in the blood or fetal liver, and die from septicemia within 2 days of birth. However, antibiotic-treated mice could survive for 2 weeks and show the development of normal appearing T cells and a few cells with the characteristics of neutrophils (5). To identify PU.1-regulated genes during myeloid differentiation, we performed RDA using day 19 fetal liver of PU.1-deficient mice. We prepared cDNAs from whole fetal liver and enriched hematopoietic cells by a cell size selection using cell strainers, and compared the profile of amplified genes. Because PU.1 is not expressed in hepatocytes, only myeloid and lymphoid genes were expected to be identified from the fetal liver by RDA.

Table 3 shows the profile of genes identified by RDA. We used a high stringency of 1:400 000 for the third round of subtractive hybridization. As expected, most of the genes contained in DP3 were myeloid-specific genes, including those specific to the neutrophil and/or monocyte/macrophage lineage, and others were lymphoid genes. There was no significant difference in the profile between DP3 from whole fetal liver and purified hematopoietic cells, suggesting that this procedure is very sensitive, but we could amplify different genes by using different materials. We identified five unknown genes (Pdp 1 and Pdp 3–6). They showed no significant similarity to any known genes. Interestingly, two of them were the same genes as those identified by RDA using C/EBPα knockout mice. Northern blots revealed that all of these unknown genes were differentially expressed and also preferentially expressed in the BM and/or peritoneal exudate cells (Fig. 4B). Therefore, RDA is sensitive and specific enough to identify the difference in a small subpopulation from materials comprised of heterogenous cell populations.

Table 2. DNA fragments generated by RDA, C/EBPα +/+ minus –/– with the suppression of liver-specific genes

Gene (accession no.)
Suppression with liver gene fragments ([a])
Lactoferrin (D88510)
Myeloid bectenecin (U95002)
Lipocortin I (M24554)
Neutrophil gelatinase associated lipocalin (X81627)
Eosinophil chemotactic factor (X15313)
Neutrophilic elastase (U04962)
C/Edp 4 (Pdp 3)
C/Edp 5 (Pdp 4)
[b]Haptoglobin (M96827)
C4 complement protein (M11789)
Suppression with adult liver cDNA
Lactoferrin (D88510)
Myeloid bectenecin (U95002)
Lipocortin I (M24554)
Neutrophil gelatinase associated lipocalin (X81627)
Eosinophil chemotactic factor (X15313)
Myeloperoxidase (X15313)
Gelatinase B (D12712)
Stefin 1 (M92417)
C/Edp 1 (AA720492)
C/Edp 2 (AA720493)
C/Edp 3 (AA720494)
C/Edp 4 (Pdp 3; AA720498)
C/Edp 5 (Pdp 4; AA720499)

[a]DNA fragments previously generated by RDA (contrapsin, protein C, haptoglobin, apolipoprotein A-I) were used for suppression.
[b]This haptoglobin fragment is a different one from that used for suppression.
C/Edp, Pdp: Unknown differentially expressed gene isolated by RDA, C/EBP +/+ minus –/– (C/Edp) and PU.1 +/+ minus –/– (Pdp).

Suppression of mature myeloid genes leads to the amplification of immature myeloid-specific genes

The absence of C/EBPα and PU.1 causes a block at an early stage of myeloid differentiation. The critical targets responsible for this differentiation block are expected to be also expressed at early stage. To focus on the early targets during myeloid differentiation, we used fetal liver hematopoietic cells enriched by cell size selection, and performed suppression of mature myeloid genes. We prepared driver amplicons from peritoneal exudate cells collected 20 and 72 h after i.p. injection of thioglycollate. The former cells consisted of ~80% of neutrophils, while the latter consisted of 80% of monocytes/macrophages. Driver from peritoneal exudate cells 20 h after stimulation was added to the hybridization mixture of C/EBPα +/+ and –/– to suppress neutrophilic genes, and both drivers were added to that of PU.1 +/+ and –/– to suppress both neutrophilic and monocyte/macrophage genes. We also performed specific gene suppression using DNA fragments from our novel RDA clones and myeloid granule proteins (Table 4), because most of them are expressed only in immature myeloid cells, i.e. from myeloblasts to band cells, but not in mature myeloid cells including peritoneal exudate cells (reviewed in 43). Liver genes were suppressed by adult liver driver as described above. We used lower stringencies, 1:40 000 for the third round of subtractive hybridization, because the expression of differentially expressed genes specific to immature myeloid cells was expected to be weaker than before. As shown in Table 4, we obtained quite a different profile of genes. Although we failed to suppress gelatinase B and Pdp 4 with specific DNA fragments, suppression with mature myeloid cDNA and other specific DNA fragments worked well. We isolated a new *Dpn*II fragment of MPO, eosinophil peroxidase (EPO), proteinase-3 and gelatinase B. They are myeloid granule proteins and are expressed in immature myeloid cells (43,44). Others were a Kupffer cell-specific gene, a B-cell gene and three unknown genes. The unknown genes showed no significant similarity to any known genes. Northern blots revealed that C/Edp 6 and 7 were differentially expressed (Fig. 4C), but unknown 7 was not (data not shown). Because the expression of C/Edp 6 and 7 was relatively weak, poly(A)$^+$ RNA northerns were required for identification. C/Edp 6, 7 and Pdp 4 were not expressed in peritoneal exudate cells at all (Fig. 4B and C). These results demonstrate that suppression of mature myeloid genes facilitates the amplification of genes preferentially expressed in immature myeloid cells.

Table 3. DNA fragments generated by RDA, PU.1 +/+ minus –/–

Gene (accession no.)
Whole fetal liver
Lysozyme M (M21050)
Bacteria binding macrophage receptor (U18424)
Complement subcomponent C1q α-chain (X58861)
Lactoferrin (D88510)
Eosinophil chemotactic factor (X15313)
Ig λ-chain (M30387)
Pdp 1 (AA720497)
Pdp 3 (C/Edp 4; AA720498)
Pdp 4 (C/Edp 5; AA720499)
Pdp 5 (AA720500)
Pdp 6 (AA720501)
Enriched fetal liver hematopoietic cells
Lysozyme M (M21050)
Bacteria binding macrophage receptor (U18424)
Complement subcomponent C1q α-chain (X58861)
Lactoferrin (D88510)
Myeloperoxidase (X15313)
Myeloid bectenecin (U95002)
gp91phox (U43384)
LAPTm 5 (U51239)
MHC class II H2–1A-α (M11357)
Ig λ-chain (M30387)
Pdp 1 (AA720497)
Pdp 6 (AA720501)

C/Edp, Pdp: unknown differentially expressed gene isolated by RDA, C/EBPα +/+ minus –/– (C/Edp) and PU.1 +/+ minus –/– (Pdp).

Table 4. DNA fragments generated by RDA with the suppression of mature myeloid cDNAs

C/EBPα +/+ minus –/–	PU.1 +/+ minus –/–
Myeloperoxidase (X15313)[a]	myeloperoxidase (X15313)[a]
Eosinophil peroxidase (D78353)	gelatinase B (D12712)
Proteinase-3 (U43525)	Kupffer cell receptor (D88577)
	procathepsin E (X97399)
C/Edp 6 (AA720495)	Pdp 4 (AA720499)
C/Edp 7 (AA720496)	
Unknown 7	

Drivers prepared from peritoneal exudate cells and previously generated DNA fragments (C/Edp 1–3, Pdp 1–6, myeloperoxidase, neutrophilic elastase, lactoferrin, gelatinase B, myeloid bectenecin, neutrophil gelatinase associated lipocalin, lipocortin I and eosinophil chemotactic factor) were used for suppression.
[a]This MPO fragment is different from that in Tables 2 and 3.

Expression of myeloid granule protein genes in mutant mice

In this study, we identified many myeloid granule protein genes, including primary granule protein genes (myeloperoxidase, neutrophilic elastase and proteinase-3); secondary granule protein genes [lactoferrin, neutrophil gelatinase associated lipocalin (NGAL), putative murine homologue of neutrophilic collagenase C/Edp 2, gelatinase B and myeloid bectenecin]; and lysozyme M, which is localized in both primary and secondary granules. Among them, myeloperoxidase and neutrophilic elastase have been characterized as common targets for PU.1 and C/EBPα by transient transfection assays (11,12). To determine the *in vivo* role of PU.1 and C/EBPα in the regulation of myeloid granule protein genes, we analyzed their expression by northern blotting. The expression of many of them was very low or undetectable in both mutant fetal livers *in vivo* (Fig. 5). Specifically, myeloperoxidase and proteinase-3 were expressed at very low levels in both mutant fetal livers, NGAL and C/Edp2 at low levels in PU.1$^{-/-}$ fetal liver, and lysozyme M at low levels in C/EBPα$^{-/-}$ fetal liver (Fig. 5). Although mRNAs encoding myeloid granule proteins are confined to myeloid cells, lysozyme M is abundantly expressed also in non-hematopoietic tissues, particularly in the lung (45), in which C/EBPα is highly expressed (46). Interestingly, the expression of lysozyme was markedly downregulated in C/EBPα$^{-/-}$ newborn lung but not in PU.1$^{-/-}$ newborn lung (Fig. 5). These findings suggest the critical role of PU.1 and C/EBPα in the regulation of myeloid granule protein genes *in vivo*.

Myeloid-specific expression of novel genes

To clarify the lineage-specific expression of novel genes, we analyzed their expression in lymphohematopoietic tissues. Most of them were preferentially expressed in the bone marrow or peritoneal exudate cells, but not in spleen, thymus or adult liver (Fig. 4A and B), suggestive of their preferential expression in myeloid cells.

We further analyzed their expression during the myeloid differentiation of EML cells. EML is a stem cell factor-dependent lymphohematopoietic progenitor cell line immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor (37). Myeloid differentiation is suppressed in EML cells, but common progenitors for neutrophils and macrophages are

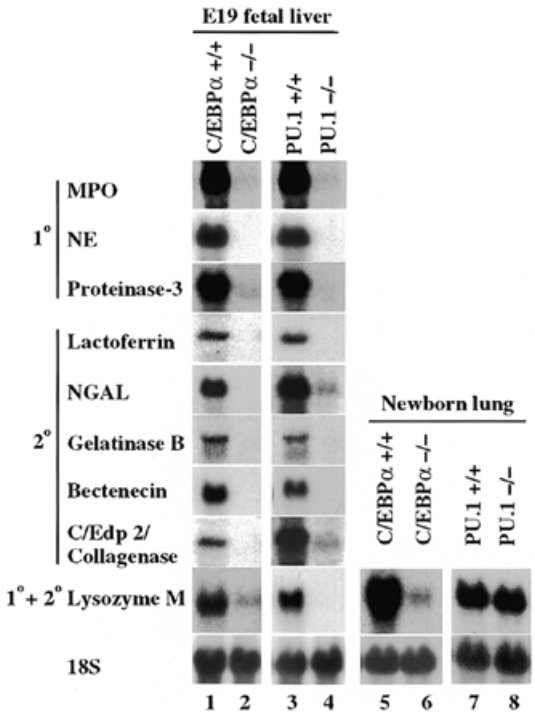

Figure 5. Northern blot analysis of mRNA of myeloid granule protein genes. Total RNA (15 µg) from wild-type (lanes 1 and 3), C/EBPα$^{-/-}$ (lane 2), PU.1$^{-/-}$ (lane 4) day 19 fetal liver, and wild-type (lanes 5 and 7), C/EBPα$^{-/-}$ (lane 6) and PU.1$^{-/-}$ (lane 8) newborn lung. DNA fragments generated by RDA were used as probes.

generated by treatment with high concentrations of retinoic acid. These myeloid progenitors differentiate into neutrophils and macrophages in response to GM-CSF, but still neutrophilic differentiation is blocked around the promyelocyte to myelocyte stages and only few mature neutrophils could be observed. Differentiated neutrophilic cells appeared on day 3 after treatment with GM-CSF (blasts 14.3%, promyelocytes 53.0%, myelocytes/metamyelocytes 24.5% and monocytes/macrophages 8.0%), reached a peak on day 6 (blasts 6.0%, promyelocytes 28.0%, myelocytes/metamyelocytes 47.5%, band/segmented cells 8.5% and monocytes/macrophages 9.7%), and then decreased, while macrophages gradually increased and dominated on day 10 (blasts 3.5%, promyelocytes 19.7%, myelocytes/metamyelocytes 8.1% and monocytes/macrophages 69.8%). As shown in Figure 6, the expression of C/Edp 1–3 and Pdp 3 and 4 were strongly induced during myeloid differentiation, and downregulated on day 10. The 2.0 kb transcript of Pdp 6 was weakly induced, while the 1.0 kb transcript, which is a minor transcript in peritoneal exudate cells (Fig. 4B), was strongly upregulated. The expression of Pdp 5 was upregulated 3 days after GM-CSF stimulation and maintained during differentiation. The analysis of the expression of these novel myeloid genes in other hematopoietic cell lines revealed that they were expressed in myeloid cells but not in T cells, B cells or erythroid cells (data not shown). Therefore, RDA selectively amplified differentially expressed genes which are preferentially expressed during myeloid differentiation. The expression of C/Edp 6 and 7 was not detected in EML cells or other hematopoietic cell lines (data not shown); therefore, they may be preferentially expressed in fetal liver.

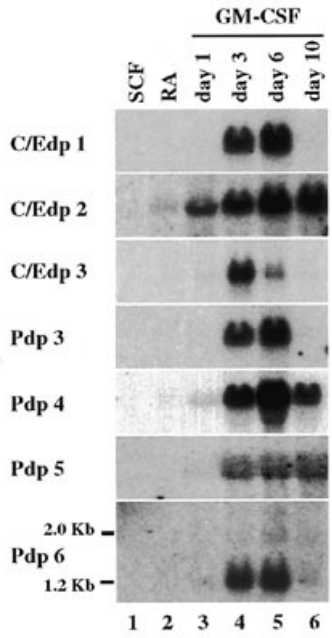

Figure 6. Northern blot analysis of mRNA of novel genes identified by RDA during myeloid differentiation of EML cells. Total RNA (15 µg) from before stimulation (lane 1), after treatment with RA (10^{-5} M) and IL-3 for 3 days (lane 2), after 1 day (lane 3), 3 days (lane 4), 6 days (lane 5) and 10 days (lane 6) of culture in the presence of GM-CSF. The same probes as in Figure 4 were used.

DISCUSSION

The receptors for the myeloid colony-stimulating factors, M-CSF, GM-CSF and G-CSF have been proposed to be critical targets for the impaired myeloid development in PU.1 and C/EBPα mutant mice (3,17,18). We have previously shown by northern blot analysis that G-CSF receptor mRNA is remarkably downregulated in C/EBPα knockout mice, whereas mRNAs for M-CSF receptor and GM-CSF receptor are not impaired (3), suggesting that impaired G-CSF signaling might be responsible for selective block of neutrophilic differentiation. On the other hand, M-CSF receptor mRNA was undetectable by RT–PCR analysis of differentiated PU.1$^{-/-}$ ES cells (17,18). In this study, we analyzed the expression of myeloid CSF receptors in PU.1$^{-/-}$ fetal liver by northern blot analysis, and noted that the expression of all three is markedly decreased (Fig. 2). However, at least M-CSF receptor and G-CSF receptor are still expressed at low levels, suggesting that PU.1$^{-/-}$ fetal liver cells could express at least low levels of myeloid CSF receptors. No complementation assays to rescue the defects by using myeloid CSF receptor transgenes have been reported. Therefore, the role of myeloid CSF receptors in both mutant mice still remains to be defined. In addition, the myeloid defects of PU.1 or C/EBPα knockout mice do not completely match those of loss-of-function mutant mice of each CSF or CSF receptor (28–31). These findings suggest the presence of additional genes regulated by PU.1 and C/EBPα.

In this study, we extended the studies of CSF receptor expression and identified additional genes regulated by PU.1 and C/EBPα. We employed RDA, a PCR-based subtractive hybridization. RDA eliminates those fragments present in both populations and amplifies only the difference. RDA is sensitive enough to isolate genes expressed in only a very small percentage of cells (32,33). Therefore, this technique was suitable for our cloning approach using fetal livers as materials in which myeloid cells compose only a small percentage of the total cell population.

Our data demonstrated that this procedure truly amplified differentially expressed genes, and was able to amplify genes expressed at low levels as well. Most of the genes identified were expressed in the fetal liver at much lower levels than in the bone marrow or peritoneal exudate cells (Fig. 4). However, because so many liver genes are differentially expressed in C/EBPα$^{-/-}$ fetal liver, we amplified more liver genes than myeloid genes (Table 1). To suppress the amplification of liver genes, we first tried suppression by liver-specific DNA fragments. Suppression of expected difference products by specific DNA fragments has been reported to facilitate the amplification of new gene fragments (32,33). We prepared liver-specific DNA fragments generated in the previous RDA, and added these to the hybridization mixture. This suppression worked well, but other liver genes were still amplified (Table 2). To get complete suppression of liver genes and preferentially amplify myeloid genes, we prepared driver amplicon from adult liver and added it into each round of subtractive hybridization. As shown in Tables 2 and 4, liver genes were completely suppressed and this facilitated the amplification of myeloid genes. This modification was also successfully applied to suppression of mature myeloid genes to amplify immature myeloid genes (Table 4). Our data demonstrate that genes expressed in a certain cell population or at a specific stage of differentiation could be completely suppressed by the appropriate driver, and this suppression facilitates the amplification of differentially expressed genes in other cell populations or differentiation stages. RDA combined with this kind of gene suppression would be helpful to focus on genes specific to a certain cell population in materials consisting of heterogenous cells.

Although RDA is an effective technique, it still has some limitations. First, our data clearly showed that some of the differentially expressed genes are lost during the repeated subtractive hybridization by increasing the stringencies (Table 1 and Fig. 3B). Difference products with a low stringency could contain more differentially expressed genes but many more false positives as well. On the other hand, difference products with a high stringency limit the number of fragments generated. This problem could partially be resolved by suppression of expected difference products by specific DNA fragments or additional drivers as we performed in this study. Secondly, RDA preferentially amplifies genes with significant differences in expression. Most of the differentially expressed genes identified were not expressed in the mutant fetal liver. Only several genes were still expressed in mutant fetal liver at low levels. Decreasing the stringencies was not as effective (Table 1). New modifications will be needed to amplify genes with small differences. A minor limitation is that the technique tends to isolate small portions of the full length cDNA.

By RDA using PU.1 and C/EBPα knockout mice, we identified many differentially expressed genes, including myeloid- and liver-specific genes. The expression of several liver genes have already been shown to be downregulated in the fetal and newborn liver of C/EBPα knockout mice (26,27). We identified six additional liver genes which are differentially expressed, and they are presumably new targets for C/EBPα in hepatocytes. Interestingly, we happened to find that the expression of pref-1, a pre-adipocyte transmembrane protein, is upregulated in C/EBP-

α$^{-/-}$ fetal liver. During adipocyte differentiation, pref-1 is reported to be downregulated, while C/EBPα is upregulated (23,47). Our finding suggests that C/EBPα negatively regulates the expression of pref-1, and pref-1 might be a new direct target for C/EBPα in adipocytes. This finding also suggests the existence of other negatively regulated genes by C/EBPα or PU.1. To selectively identify these genes, reverse RDA, i.e. mutant fetal liver minus wild-type fetal liver, would be an approach to be taken in the next step.

With some modifications of RDA, we could preferentially identify myeloid-specific genes. Because PU.1 knockout mice show impaired development of neutrophils as well as monocytes in the fetal liver, many neutrophil-specific genes were identified from both PU.1 and C/EBPα knockout mice (Tables 1–4). Among known genes, primary granule protein genes myeloperoxidase and neutrophilic elastase are known as common targets for both PU.1 and C/EBPα (11,12), and another primary granule protein gene, proteinase-3, is a target for PU.1 (13). Myeloperoxidase mRNA is expressed in CD34^{+} multipotential cells (48,49) and at high levels in myeloid progenitors at the promyelocytic and promonocytic stages of myeloid differentiation (50,51), while neutrophilic elastase and proteinase-3 mRNAs are expressed in the promyelocytic stage (52). Although myeloid progenitors are present in both knockout mice (3,17), and monocytic cells are intact in C/EBPα$^{-/-}$ fetal liver (3), the expression of myeloperoxidase mRNA was significantly low in both mutant fetal livers (Fig. 5), indicating that both PU.1 and C/EBPα are critical for the transcription of myeloperoxidase *in vivo*. Neutrophilic elastase mRNA was also missing and proteinase-3 mRNA was markedly downregulated in both mutant mice. They are also likely *in vivo* targets of these two transcription factors. These findings suggest cooperative regulation of myeloid primary granule genes by PU.1 and C/EBPα *in vivo*.

The expression of secondary granule protein genes were also undetectable in both mutant mice (Fig. 5), possibly because of the lack of expressing cells, such as myelocytes, metamyelocytes and band cells. Low levels of mRNA of NGAL and neutrophilic collagenase (C/Edp2) were detected only in PU.1$^{-/-}$ fetal liver. This might well represent the development of a few neutrophilic cells in PU.1$^{-/-}$ fetal liver, consistent with the reported development of cells characteristic of neutrophils in PU.1$^{-/-}$ bone marrow (5). Recently, CCAAT displacement protein has been reported to repress the expression of secondary granule protein genes (53,54), but transcription factors that directly activate their transcription have not been well characterized. Because the expression of PU.1 and C/EBPα is maintained during granulocytic differentiation, they are candidate regulators of secondary granule expression, as is C/EBPε (40,41).

Lysozyme M is a myeloid granule protein localized in both primary and secondary granules. Its expression is already detectable in myeloblasts and upregulated during myeloid differentiation, including both granulocytic and monocytic lineages (43). PU.1 is reported to activate the myeloid-specific enhancer of the chicken lysozyme gene (55), and C/EBPβ interacts with another enhancer and mediates lipopolysaccharide-induced expression of the chicken lysozyme gene (56). Northern blot analysis showed that lysozyme M expression was absent in PU.1$^{-/-}$ fetal liver and markedly impaired in C/EBPα$^{-/-}$ fetal liver (Fig. 5). In addition, lysozyme M mRNA was immediately upregulated after induction of C/EBPα expression in an immature hematopoietic cell line (A.Iwama and D.G.Tenen, unpublished data). These data suggest an important role of these transcription factors in the regulation of lysozyme M expression. Moreover, we found that lysozyme M expression was impaired in the C/EBPα$^{-/-}$ newborn lung. Lysozyme is expressed in type II alveolar pneumocytes and alveolar macrophages in rodent lung (57), while C/EBPα mRNA is localized to type II pneumocytes (58) and C/EBPα$^{-/-}$ mice show hyperproliferation of type II pneumocytes (27). C/EBPα is also expressed in activated macrophages (A.Iwama and D.G.Tenen, unpublished data). Taken together with the fact that PU.1 is not expressed in type II pneumocytes, C/EBPα could be a major regulator for lysozyme M expression in the lung, particularly in type II pneumocytes. These findings indicate that C/EBPα plays a major role in the regulation of lysozyme M in non-hematopoietic cells, and suggest the possibility that C/EBPα is a key transcription factor in the regulation of genes specific to type II pneumocytes, such as surfactant protein genes.

The expression analysis of transcription factors *in vivo* indicated that C/EBPα does not affect the expression of PU.1, because the reduction of PU.1 mRNA in C/EBPα$^{-/-}$ fetal liver is likely to parallel the decrease in mature granulocytic cells. On the contrary, PU.1 might be important in the regulation of C/EBPα, and it is possible that impaired granulopoiesis in PU.1 knockout mice is caused by defective C/EBPα expression. The expression analysis of C/EBPα in the neutrophilic cells of PU.1$^{-/-}$ bone marrow would help to address this question. The specific absence of Spi-B mRNA only in PU.1$^{-/-}$ fetal liver is consistent with our previous data of its B-cell-specific expression (8), and the absence of C/EBPε mRNA in C/EBPα$^{-/-}$ fetal liver as well as PU.1$^{-/-}$ fetal liver confirms its granulocyte-specific expression (40). Spi-B and C/EBPε might be regulated by PU.1 or both PU.1 and C/EBPα in each cell lineage.

We identified eight novel myeloid genes differentially expressed between wild-type and mutant fetal livers. Among them, C/Edp 2 and 3 are likely to be the murine homologues of neutrophil collagenase and ficolin, respectively. C/Edp 1 and Pdp 3, and Pdp 1 and 6 seem to be different *Dpn*II fragments from the same gene, respectively, because they showed the same mRNA expression profile (Figs 4A and B, and 6). Northern blot analysis showed that most of the novel genes were preferentially expressed in the bone marrow or peritoneal exudate cells, but not in spleen, thymus or adult liver (Fig. 4A and B). They were undetectable or only weakly expressed in other tissues, such as brain, heart, lung, kidney, skeletal muscle and testis (data not shown), indicating that they are hematopoietic-specific genes. Only Pdp 1 and 6 were expressed in adult liver (Fig. 4B), but they were not differentially expressed between wild type and C/EBPα$^{-/-}$ fetal liver (data not shown), suggesting that they are expressed in macrophages, including Kupffer cells, in the liver. Most of them were upregulated during myeloid differentiation of the multipotential hematopoietic cell line, EML (Fig. 6), suggesting these genes are good candidate targets for PU.1 and C/EBPα. Although C/Edp 6 and 7 were differentially expressed between wild-type and mutant fetal liver, we could not detect any apparent expression in the bone marrow, peritoneal exudate cells, adult liver or other adult organs. It is possible that they are specifically expressed in the embryonic stage.

Our data confirmed the critical role of PU.1 and C/EBPα *in vivo* in the regulation of myeloid genes, including myeloid CSF receptors and myeloid granule proteins. Using RDA combined with specific gene suppression, we further identified novel myeloid genes, the expression of which are missing in the mutant

fetal livers. These novel genes are new candidate targets for PU.1 and C/EBPα. Characterization of their roles in myeloid development as well as their transcriptional regulation in relation to PU.1 and C/EBPα will be helpful in elucidating the mechanism of impaired myeloid development caused by loss-of-function mutation of PU.1 and C/EBPα. Additional studies, including isolation and characterization of the promoter elements, will be required to establish whether these genes are directly or indirectly regulated by PU.1 and C/EBPα.

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