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Gene Expression Profiling Related to the Enhanced Erythropoiesis in Mouse Bone Marrow Cells

Hee-Young Yang,[1,2] Dong Kee Jeong,[3] Seok-Ho Kim,[1,2] Kyoung-Jin Chung,[4] Eun-Jin Cho,[1,2] Cheng Hao Jin,[2] Ung Yang,[2] Sang Ryeul Lee,[2] Dong-Seok Lee,[5] and Tae-Hoon Lee[2]*

[1]Department of Molecular Medicine, Chonnam National University, Gwangju, South Korea
[2]Department of Oral Biochemistry, Dental Science Research Institute, The 2nd Stage of Brain Korea 21 for Dental School, Chonnam National University, Gwangju, South Korea
[3]Department of Animal Science and Biotechnology, College of Applied Life Sciences, Cheju National University, Jeju, South Korea
[4]Graduate School of Biological Sciences and Technology, Chonnam National University, Gwangju, South Korea
[5]Department of Animal Biotechnology, Animal Resource Science, Kangwon National University, Chuncheon, South Korea

Abstract Peroxiredoxin II knockout (Prdx II$^{-/-}$) mice had a spontaneous phenotype of hemolytic anemia. In this study, we found that Ter-119^{+}CD71^{+} cells increased in Prdx II$^{-/-}$ mice bone marrow (BM) at 8 weeks of age. We examined the differential expression profiles to bone marrow cells (BMCs) between Prdx II$^{+/+}$ and Prdx II$^{-/-}$ mice using a cDNA microarray. We identified the 136 candidates were differentially expressed a greater twofold increase or decrease than EPO receptor. In this study, we focused on the up-regulated NBPs during erythropoietic differentiation. According to cDNA microarray results, six NBPs except *zfp-127* were up-regulated during erythropoiesis in Prdx II$^{-/-}$ mice. Among the six candidates, *eIF3-p44*, *hnRNPH1*, *G3bp*, and *Zfpm-1* were dramatically increased at day 7 of the in vitro erythropoietic differentiation of human CD34^{+} cells. However, *DJ-1* and *Rbm3* were slightly increased only at day 12. Our results suggest that up-regulated NBPs might be involved during erythropoietic differentiation. J. Cell. Biochem. 104: 295–303, 2008.
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Key words: Prdx II knockout mice; cDNA microarray; hematopoietic stem cell; erythropoiesis; nucleotide binding protein

Erythropoiesis is a dynamic process in which mature red blood cells (RBCs) are derived from haematopoietic stem cells (HSC) [Shivdasani and Orkin, 1996; Ho, 2005]. Although there are a wide variety of factors that participate in erythropoiesis, their mechanism and function is unclear [Lacombe and Mayeux, 1998; Cantor and Orkin, 2002; Koury et al., 2002]. Of these factors, erythropoietin (EPO) is the well-known factor for the survival, proliferation, and differentiation of erythroid progenitor cells [Welch et al., 2004]. Moreover, the EPO level in peroxiredoxin II knockout (Prdx II$^{-/-}$ mice) was twice more than in Prdx II$^{+/+}$ mice at 8 weeks after postnatal. The number of normoblast was also higher in the bone marrow cells (BMCs) of Prdx II$^{-/-}$ mice at same time [Lee et al., 2003]. Even though Prdx II-deficiency has no the direct evidence related to hemolytic anemia, our previous studies presented that Prdx II$^{-/-}$ mice have phenotypes of hemolytic anemia with Heinz body formation of RBC, the increase of reticulocytes, and splenomegaly

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*Correspondence to: Tae-Hoon Lee, PhD, Associate Professor, Department of Oral Biochemistry, School of Dentistry, Chonnam National University, 300 Yongbong-Dong, Buk-Ku, Gwangju 500-757, South Korea.
E-mail: thlee83@chonnam.ac.kr

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[Lee et al., 2003]. According to our previous results, peripheral blood cell contents are not changed except reticulocytes and RBC in Prdx $II^{-/-}$ mice [Lee et al., 2003]. In an erythropoietic point of view, we think Prdx $II^{-/-}$ mice are the acceptable model for identification of differentially expressed genes during erythropoiesis. In the present study, we identified the differentially expressed genes between Prdx $II^{-/-}$ and Prdx $II^{+/+}$ mice BMCs. In addition, we elucidated the gene expression of six nucleotide binding proteins (NBPs) in the BMCs of Prdx $II^{-/-}$ mice and on in vitro differentiation system from human $CD34^+$ cells to proerythrocytes.

MATERIALS AND METHODS

Prdx $II^{-/-}$ Mice and Human $CD34^+$ Cell Culture

Prdx $II^{-/-}$ mice and Prdx $II^{+/+}$ mice with C57BL6/J background were used in this study [Lee et al., 2003]. All the mice were bred under specific-pathogen free (SPF) conditions and were cared for according to animal care regulations (ACR) of Chonnam National University. The isolation and differentiation of human $CD34^+$ cells was previously described [Yang et al., 2007].

Fluorescence-Activated Cell Sorting (FACS) Analysis

The mice BMCs (1×10^6 cells) at 8 weeks or 6 months of age were stained with a biotin-conjugated Lin antibody cocktail (CD11b, Ly6G, B220, NK1.1, CD2, and Ter-119; Pharmingen, San Diego, CA) and streptavidin-peridinin-chlorophyll-protein complex (PerCP; Pharmingen). And then, the mice BMCs were analyzed with phycoerythrin (PE)-conjugated anti-Sca-1 and fluorescein isothiocyanate (FITC)-conjugate anti-c-Kit (Pharmingen).

cDNA Microarray

The BMCs of the Prdx $II^{-/-}$ ($n = 14$) and Prdx $II^{+/+}$ ($n = 10$) mice at 8 weeks of age were prepared by flushing the femurs with diethylpyrocarbonate (DEPC) treated phosphate buffered saline (PBS) (DEPC/PBS). The collected BMCs were centrifuged at 1,500 rpm for 5 min at 4°C and washed twice with DEPC/PBS. The total RNA from the BMCs of Prdx $II^{-/-}$ and Prdx $II^{+/+}$ mice was isolated using RNeasy Midi Kit (Qiagen, Valencia, CA). The yield and purity of the RNA were measured by spectrophotometry analysis. Twenty micrograms of the total RNA was used for cDNA synthesis. And then hybridization of the cDNA labeled with Cy-3 or Cy-5 to a DNA chip (mouse 10 K DNA chip, KRIBB, Daejeon, Korea) was performed respectively, according to the manufacturers protocol (3DNA Array 50, Genisphere, Inc., Watertown, MA). The hybridized microarray was scanned using a Scan array 5000 at 635 nm (Cy-5, green) and 532 nm (Cy-3, red). The scanned DNA chip was analyzed using Gene Pix Pro 3.0 analysis software. This array of analysis software included image processing and data normalization. Significance Analysis of Microarrays (SAM) was used as a statistical technique for identifying the significant genes among experiments of three times.

Data Analysis

Normalization of cDNA concentrations for all samples was performed using the R package program which is downloadable from the website, http://www.braju.com/r/com.braju.sma/. Hierarchical clustering was applied to both genes and samples using web available software Cluster and TreeView (http://www.microarrays.org/software). This program clustered gene expression profiles by 'Cluster' software (Version 2.11), which was visualized by the 'Tree view' software program (Version 1.50). The pairwise log ratios were analyzed using the SAM (significance analysis of microarray) method, which provides the serial lists of both up-regulated and down-regulated genes at a given false significance rate of choice. The pooled mice samples were also analyzed using SAM at the similar threshold level as the pairwise analysis to compare the efficiency of detection of differentially expressed genes [Kim et al., 2003].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Real-Time PCR

RNA isolation was performed using Trizol (MRC, Inc., Cincinnati, OH) and Oligo dTs were used to prime the cDNA synthesis. The total RNA of the mouse BMCs and human $CD34^+$ cells was reverse-transcribed into cDNA using AMV reverse transcriptase (iNtRON Biotechnology, Daejeon, Korea). Supplemental Table 2 lists the mouse primers used for RT-PCR of the mouse BMCs. The total RNA of in vitro differentiated human $CD34^+$ cells was collected

on day 0, 3, 7, 9, and 12. RT-PCR was carried out in 40 μl reaction mixtures using the TaqMan Reverse Transcription Reagents (Applied Biosystems), and quantitative real-time PCR (50°C/2 min; 95°C/10 min; 95°C/15 s and 60°C/1 min, 45 cycles) was performed using the SYBR GREEN PCR Master Mix (Applied Biosystems). Quantitative real-time PCR was performed using an ABI Prism 7300 Real-Time PCR System (Applied Biosystems). Supplemental Table 3 lists the human primers used for quantitative real-time PCR.

Statistical Analysis

Data are shown as averages and standard deviations. *P* values were calculated by unpaired Student's *t*-test. All statistical analyses were analyzed with SigmaPlot 9.0.

RESULTS

Erythropoietic Compensation in Prdx II$^{-/-}$ Mice

We performed FACS analysis in order to measure the erythropoietic differentiation by using mouse HSC markers, Lin, c-Kit, and Sca-1, or erythrocyte markers, Ter-119 and CD71. The population of Lin$^-$Sca-1$^+$c-Kit$^+$ cells at 8 weeks of age was slightly decreased in Prdx II$^{-/-}$ mice than in Prdx II$^{+/+}$ mice. At 6 months, Lin$^-$Sca-1$^+$c-Kit$^+$ cells of the Prdx II$^{-/-}$ mice were recovered (Fig. 1A). However, Ter-119$^+$ and CD71$^+$ cells of Prdx II$^{-/-}$ mice were 8% higher than Prdx II$^{+/+}$ mice at 8 weeks of age (Fig. 1B,C).

Differential Gene Expression Between BMCs of Prdx II$^{-/-}$ and Prdx II$^{+/+}$ Mice

We accomplished cDNA microarray to compare differentially expressed transcripts between BMCs of Prdx II$^{-/-}$ and Prdx II$^{+/+}$ mice. We obtained a total of 560 differentially expressed transcripts between Prdx II$^{-/-}$ mice and Prdx II$^{+/+}$ mice (Supplemental Fig. 1 and Supplemental Table 1). Among them, we selected the 136 transcripts in which were differentially expressed twofold increase or decrease greater than EPO receptor gene in Prdx II$^{+/+}$ mice (Fig. 2A). Of 136 transcripts, the 70 known transcripts contained 48 up- and 22 down-regulated transcripts and 66 uncharacterized transcripts or EST included 43 up- and 23 down-regulated transcripts in Prdx II$^{-/-}$ mice (Fig. 2A). The 70 known transcripts were classified according to diverse cellular functions (Fig. 2B and Table I). To confirm microarray data, we performed RT-PCR for these transcripts. As a result of RT-PCR, the 49 transcripts were identified with microarray data (Supplemental Fig. 2A,B). However, the 21 transcripts were not matched to our cDNA microarray results.

Expression of NBPs During In Vitro Erythropoiesis

Among the 49 transcripts, we confirmed whether the overexpressed transcripts of NBPs related to erythropoiesis. Based on in vitro human CD34$^+$ stem cell differentiation system [Yang et al., 2007], we ascertained the expression pattern of the NBPs. Six NBPs, *eIF-p44*, *hnRNPH1*, *G3bp*, *Zfpm-1*, *DJ-1*, and *Rbm3*, were expressed at high level in Prdx II$^{-/-}$ mice BMCs (Fig. 3A,B). As shown in Figure 3C, *eIF3-p44*, *hnRNPH1*, *G3bp*, and *Zfpm-1* were dramatically increased at day 7, and then decreased till day 12. *eIF3-p44* and *Zfpm-1* were more expressed at day 7 than at day 3, as above 40-fold. Moreover, the expression of *hnRNPH1* and *G3bp* were more than 10-fold increased at day 7. However, *DJ-1* and *Rbm3* showed the different expression pattern to the other NBPs. The expressions of these were sustained till day 7, and then decreased at day 9, but increased at day 14 (2.6-fold and 5.4-fold, respectively). Therefore, NBPs may contribute to determine cell fate in a specific stage of erythropoiesis.

DISCUSSION

Anemia, usually mild, is one of the more common problems of the aged, especially in men [Carmel, 2001]. Previous report utilized α- and β-globin knockout mouse models in order to comprehend the mechanism of erythropoiesis [Ryan et al., 1997]. Although these models possessed severe anemia, the erythropoietic compensation against it did not appear. From the erythropoietic point of view, Prdx II$^{-/-}$ mice offered the in vitro model system suitable to clarify erythrocyte differentiation [Lee et al., 2003], because these mice show the increased EPO level and the number of reticulocyte in peripheral blood and pronormoblasts in BM at 8 weeks of age. In addition, our personalized data suggest that the life span and reproduction rate of Prdx II$^{-/-}$ mice was similar to those of wild type littermate in experiments for 2 years after birth (data not shown).

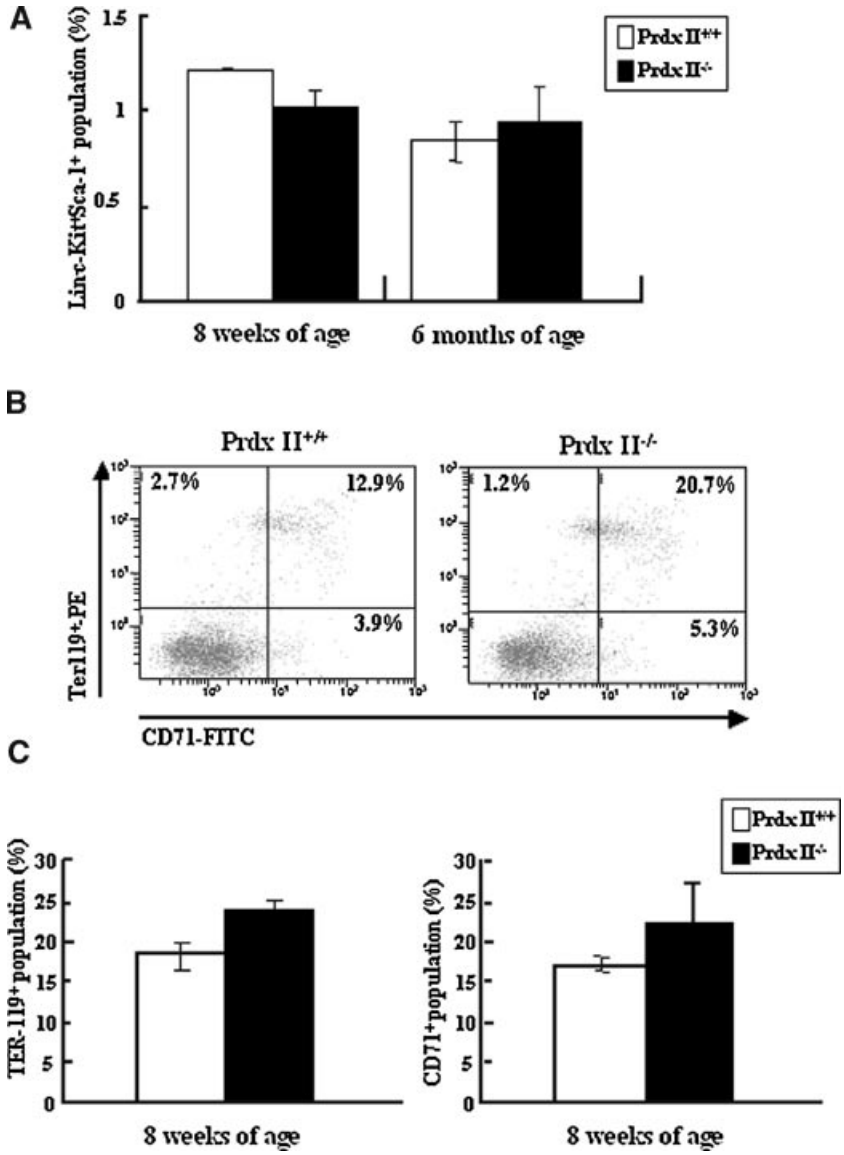

Fig. 1. Analysis of erythropoietic compensation in BMCs of Prdx II$^{-/-}$ mice by FACS. **A**: Hematopoietic stem cell in Prdx II$^{-/-}$ and Prdx II$^{+/+}$ mice at 8 weeks or 6 months of age. **B**: Proerythrocyte in Prdx II$^{-/-}$ and Prdx II$^{+/+}$ mice at 8 weeks of age. (**C**) The population of Ter-119 and CD71 positive cell resulted from (B).

In this study, we compared the cell population between in Prdx II$^{-/-}$ and Prdx II$^{+/+}$ mouse BMCs. The Lin^{-}Sca-1^{+}c-Kit^{+} cell population slightly decreased, but Ter-119^{+} and CD71^{+} cell populations were expanded in Prdx II$^{-/-}$ mice (Fig. 1). Ter-119 was highly specific for erythroid cells differentiating from early proerythroblast to mature erythrocyte [Kina et al., 2000; Asari et al., 2005]. The transferrin receptor (CD71), which is not erythroid-specific, is also detected on early erythroid precursors at very high levels, and its levels decreased with erythroid maturation [Asari et al., 2005]. Taken together, these data indicate that erythropoietic compensation actively occurs in Prdx II$^{-/-}$ mice BM.

Among the identified 560 genes, 70 genes were known the name and function. They take part in diverse cellular appearance, such as transcription, phosphorylation, dephosphorylation, ribosome biosynthesis, nucleotide binding, cell cycle, and ROS-related processes (Fig. 2B and Table I). All of the 70 genes predicted with microarray data were confirmed by RT-PCR. As a result of RT-PCR, the 49 candidates were identified (Supplemental Fig. 2A,B). So far,

TABLE I. Differential Expression of Transcripts Between Prdx II$^{-/-}$ and Prdx II$^{+/+}$ Mice

Function	UniGene	Gene	Fold ratio
Transcription factor	Mm.29590	*E2F*	+3.23
	Mm.29891	*Foxo1*	+2.40
	Mm.2444	*Myc*	+1.93
	Mm.24102	*E2F-4*	+1.79
	Mm. 5043	*Fos*	−6.31
	Mm.12926	*Pparbp*	−2.81
	Mm.34530	*Nfat5*	−2.55
	Mm.22216	*TSC-22*	−2.13
	Mm.9394	*Nfix*	−1.94
	Mm.1273	*Rarg*	−1.92
Kinase	Mm.2661	*TK1*	+3.00
	Mm.43737	*Csnk1a1*	−2.37
	Mm.28881	*Csnk2a2*	−2.02
	Mm.88628	*JAK3*	−1.96
	Mm.1881	Serine/Threonine protein kinase	−1.89
	Mm.21495	*Mapk8*	−1.87
Phosphatase	Mm.41375	*Ptpr*	+1.83
	Mm.2404	*Ptpn16*	−3.97
	Mm.40777	PTP 35 protein	−1.89
Protease	Mm.29352	*Ube 2g2*	+2.77
	Mm.29582	*Psmc4*	+1.88
	Mm.19945	*MMP14*	−1.93
Transport	Mm.658	*Slc25a5*	+2.39
	Mm.30155	ATPase-like vacuolar-proton channel	+2.04
	Mm.18625	*Aqp1*	+1.87
Nucleotide binding	Mm.30080	*eIF3-p44*	+2.26
	Mm.21740	*hnRNPH1*	+2.20
	Mm.2038	*G3bp*	+2.23
	Mm.3105	*Zfpm-1*	+2.03
	Mm.2507	*DJ-1*	+1.93
	Mm.2591	*Rbm3*	+1.72
	Mm.146	*Zfp-127*	−2.86
Ribosome biosynthesis	Mm.5291	*Rps5*	+2.09
	Mm.29896	*Rpl21*	+1.98
	Mm.3486	*Rpl3*	+1.79
Cell adhesion and ECM	Mm.321	*Spp1*	+2.04
	Mm.22194	*Prg*	+1.84
Surface molecule and receptor	Mm.41359	Thymic epithelial cell surface antigen	+1.81
	Mm.2395	*Mea1*	+1.78
	Mm.30246	*CD151*	+1.72
	Mm.4603	*Srb1*	+1.88
	Mm.4519	*Ryr1*	−2.44
Structure molecule	Mm.14245	Keratin complex 2, basic, gene 1	+1.78
	Mm.20936	*Syn1*	+1.87
Defense/Immunity	Mm.10225	*Ppl*	+2.21
	Mm.28155	MHC class III region RD region	+2.07
	Mm.6461	*Hrb*	+1.98
	Mm.830	*Psme1*	+1.91
	Mm.163	*B2m*	+1.69
Cell cycle and proliferation regulator	Mm.43444	*Mad2*	+2.76
	Mm.17898	*Cirbp*	+2.25
	Mm.27872	*D52*	+2.34
RNA helicase	Mm.29794	*DbpA*	+2.03
ROS-related	Mm.20905	*Keap1*	+3.36
	Mm.4668	*MPO*	+2.05
	Mm.42948	*Prdx II*	−10.75
	Mm.3043	Xanthine dehydrogenase	−1.98
Enzyme	Mm.19027	*Mocs2*	+2.58
	Mm.2942	*Asns*	+2.18
	Mm.22001	*Dpm2*	+2.16
	Mm.589	*Gpi1*	+2.03
	Mm.2338	*Glns*	+1.81
	Mm.27162	*P540ASL*	−2.25
Etc.	Mm20908	*SHD*	+2.40
	Mm.28438	*Plunc*	+2.14
	Mm.24186	*SH3P9*	+1.85
	Mm.4877	*P16k* gene for 16 kDa protein	−2.29
	Mm.17224	*Spop*	−1.98
	Mm.1813	*Cct5*	−1.98

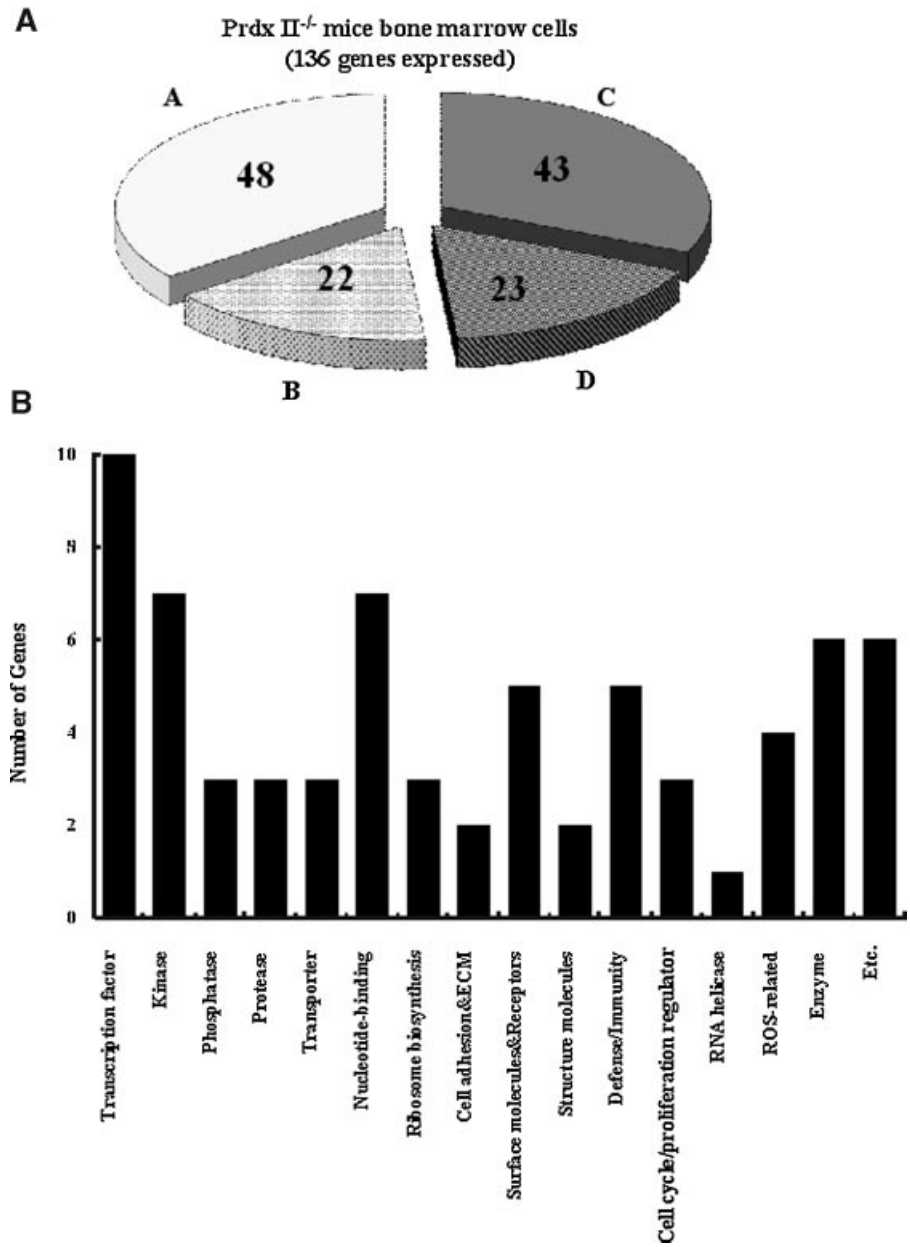

Fig. 2. The differentially expressed transcripts in BMCs of Prdx II$^{-/-}$ mice. **A**: Distribution of all differentially expressed transcripts of microarray data in BMCs of Prdx II$^{-/-}$ mice. A: The increased 48 transcripts for known function; (**B**) the decreased 22 transcripts for known function; (**C**) the increased 43 transcripts for EST; (**D**) the decreased 23 transcripts for EST. B: The functional categorization of 70 up- and down- regulated transcripts for characterized functions.

many studies reported on the expression pattern of diverse genes, such as transcription factors, receptors, and structural proteins related to erythrocyte, during lineage commitment to erythrocyte [Cantor and Orkin, 2002; Welch et al., 2004].

Several factors identified from our experiment, such as *E2F*, *E2F4*, *thymidine kinase 1* (*TK1*), and *nuclear factor 1 family* (*nfix1*), were already reported in previous microarray studies [Hackney et al., 2002; Park et al., 2002; Steidl et al., 2002; Georgantas et al., 2004]. For example, *E2F*, one of gene family related with cell cycle, is a key regulator of cell cycle progression that controls the expression of genes required for the G1/S transition [Polager et al., 2002]. In particular, *E2F4* is essential for end stage maturation of erythroid cells,

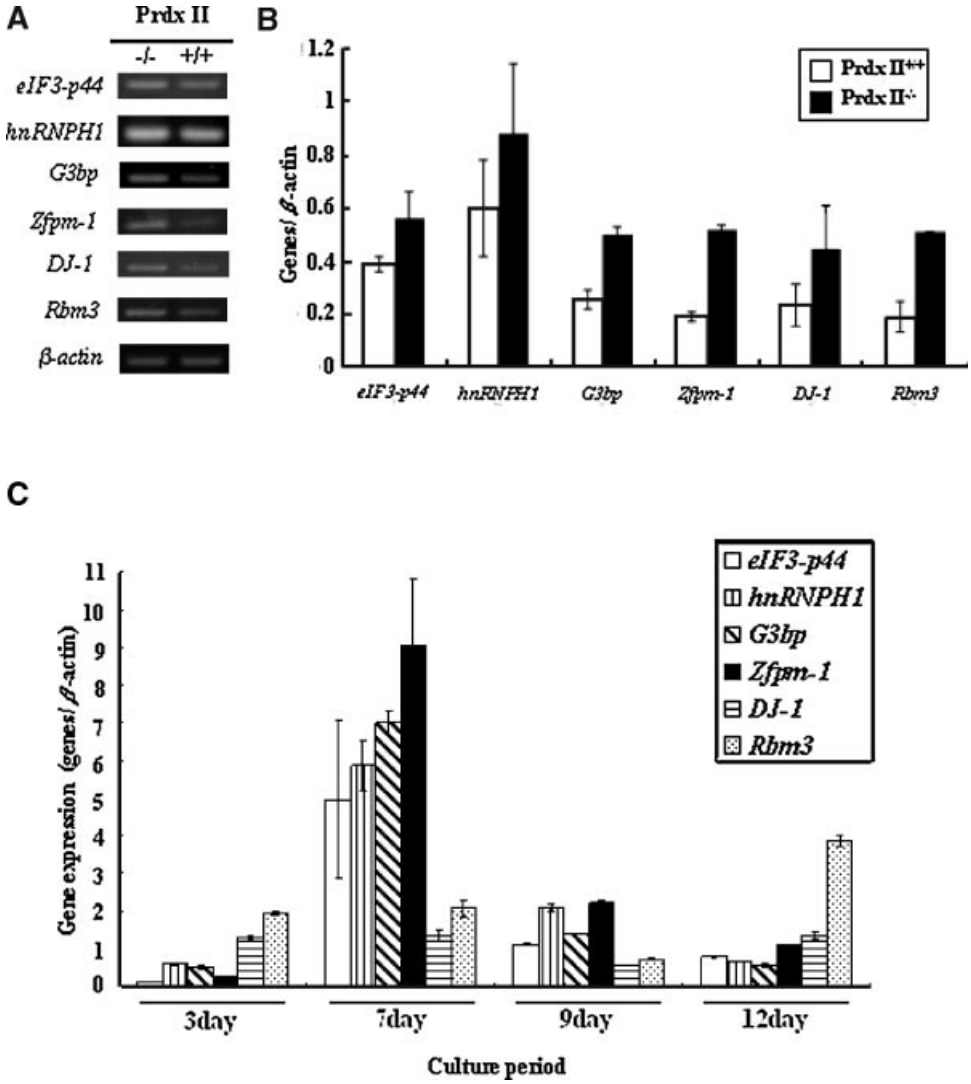

Fig. 3. Expression analysis of NBPs in BMCs of Prdx II$^{-/-}$ mice and during human CD34^{+} cells differentiation. **A**: RT-PCR data coincides with the results of microarray on BMCs of Prdx II$^{-/-}$ mice. **B**: The densitometric analysis to express quantatively result of (A) as each of gene/β-actin ratio. One of three experiments with similar results is shown. **C**: Transcriptional expression pattern of the six genes, *eIF-p44, hnRNPH1, G3bp, Zfpm-1, DJ-1,* and *Rbm3,* belonged to NBPs during erythroid differentiation of human CD34^{+} cells. The results are expressed as the ratio of gene/β-actin by quantitative Real-Time PCR analysis.

highlighting the importance of the pRB/E2F pathway in the control of erythropoiesis in vivo. *E2F4* may contribute to the regulation of erythropoietic system development by the RB tumor suppressor [Humbert et al., 2000]. And, *TK 1*, a cytosolic form of thymidine kinase (TK), is present in proliferating normal and malignant cells. It is activated at late G1 of cell cycle, strongly expressed in the cells in S + G2 period, raised at late G1 and decreased during mitosis [He et al., 2000; Wang et al., 2001]. Dou et al. [1994] have provided evidence for an S phase-dependent mechanism of *TK 1* expression based on the transcription factor *E2F* [Dou et al., 1994; Arner and Eriksson, 1995]. Therefore, it is suggested that activation of *TK 1* by *E2F* may be involved in erythropoietic system. In addition, *nfix 1* was decreased in Rh^{hi}Sca-1^{+} c-kit^{+}lin$^{-/lo}$ cells, hematopoietic multipotent progenitors (MPP), which have the highly differentiated activity [Park et al., 2002].

NBPs, which formed in various complexes with transcription factors, have an effect on expression pattern of specific genes. And they

may regulate specific cell fates. However, the expression of NBPs is not clear during erythropoietic differentiation. Herein, we identified the expression of the increased six NBPs in the BMCs of Prdx II$^{-/-}$ mice and also on in vitro differentiation of human CD34^{+} cells. As showed in Figure 3, four NBPs, *eIF3-p44*, *hnRNPH1*, *G3bp*, *Zfpm-1*, were increased not only in Prdx II$^{-/-}$ mice at 8 weeks of age but also on in vitro erythropoiesis of human CD34^{+} cell at day 7. In addition, *DJ-1* and *Rbm3* were slightly increased only at day 12. In previous report, day 7 is an important period to commit cell fate to erythrocyte, because of high expression of genes related to erythropoiesis [Yang et al., 2007]. Therefore, it is estimated that much of cellular phenomenon is changed in proportion to cell commitment at day 7. For instance, *Zfpm-1* (*FOG-1*) is rapidly expressed from the erythroid colony-forming unit (CFU-E) stage to early proerythroblast during erythroid differentiation, which may serve as a compass for the direction of cell fate [Fox et al., 1999; Deconinck et al., 2000; Querfurth et al., 2000; Katz et al., 2002; Letting et al., 2004]. We also verified the behavior of *Zfpm-1* during in vitro erythropoietic differentiation [Yang et al., 2007]. As seen in Table I and Figure 3A, the transcriptional expression of *Zfpm-1* was augmented in Prdx II$^{-/-}$ mice because the number of proerythroblast was increased in Prdx II$^{-/-}$ mice more than in Prdx II$^{+/+}$ mice, but there was no difference in the number of megakaryoctic progenitor cells in Prdx II$^{-/-}$ mice (data not shown) [Cantor et al., 2002; Tanaka et al., 2004]. *Rbm3* is up-regulated by granulocyte macrophage-colony-stimulating factor (GM-CSF). This gene was also found to be up-regulated in purified CD34^{+} cells, suggesting their involvement in proliferative process during hematopoiesis [Park et al., 2002]. *Rbm3* may be important in hematopoiesis because the process that governs cell proliferation/differentiation is undoubtedly complex and probably involved in a combination of cell-specific and widely expressed proteins [Baghdoyan et al., 2000]. In our result, *Rbm3* was highly increased at day 12, even though sustained days 3–7. We suggested that *Rbm3* might important to the late stage of in vitro erythropoietic differentiation. We also showed the expression pattern of *eIF3-p44*, *hnRNPH1*, *G3bp*, and *DJ-1* during in vitro erythropoiesis. Therefore, we are convinced that this study would contribute that candidates derived from microarray result to assist in understanding hematopoietic mechanism, especially erythropoiesis.

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