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Ablation of Nrf2 Function Does Not Increase the Erythroid or Megakaryocytic Cell Lineage Dysfunction Caused by p45 NF-E2 Gene Disruption[1]

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Maf recognition elements (MAREs or NF-E2 binding sites) have been shown to be vital for erythroid- and megakaryocyte-specific gene expression. Transcription factor NF-E2 is composed of p45, a large subunit belonging to the CNC family proteins, and a small Maf subunit, and is thought to activate transcription through its binding to MAREs in both the erythroid and megakaryocytic cell lineages. While *p45* gene knockout mice exhibit thrombocytopenia due to abnormal terminal differentiation of megakaryocytes, and the mutant mice die of massive bleeding within a week after birth, anemia is not apparent in these animals. Disruption of the *nrf2* gene, encoding another CNC family protein, results in no hematological abnormalities. We have therefore tested the hypothesis that Nrf2 might compensate for the p45 deficiency in erythroid lineage cells of *p45*-knockout mice, thereby masking the anticipated anemia. However, we failed to detect any greater failure in either erythroid or megakaryocytic cell development in Nrf2 plus p45 compound mutant mice as compared to with either individual homozygous mutation. These data suggest that p45 and Nrf2 may both be dispensable for hematopoietic cell development, and that other factors regulate erythroid- and megakaryocyte-specific gene expression through their required MAREs.

Key words: double knockout mice, erythropoiesis, NF-E2 p45, Nrf2, thrombopoiesis.

The vertebrate β-globin gene loci are all regulated by a cooperative regulatory element referred to as the locus control region (LCR) (*1-3*). The importance of LCR in globin gene regulation has been quite thoroughly documented. The human β-globin LCR contains five widely spaced DNase I-hypersensitive (HS) sites, of which HS2, HS3, and HS4 are believed, or have been demonstrated, to be necessary for LCR function. Three transcription factor binding sites, *i.e.*, GATA, CACC, and MARE (Maf recognition element or NF-E2 binding sequence), are found in common within these HS elements (*1, 4*). Of these three *cis*-motifs, MARE has been shown to contribute to tissue-specific enhancer activity exhibited by individual sites and is thought to be essential for LCR function (*5-11*). Therefore, the identification of transcription factors that bind to MAREs, both in LCR as well as other erythroid-specific gene regulatory regions, is very important for further understanding of erythroid gene regulation.

In this regard, a number of CNC family transcription factors have already been identified which can bind to MAREs by forming heterodimers with small Maf family proteins. These include p45 NF-E2, Nrf1, Nrf2, Bach1, and Bach2 (*12-17*). Of these factors, p45 was originally the favored candidate as the principal activator of MAREs in the erythroid lineage (*18*), since the expression of p45 is restricted to erythroid cells, megakaryocytes, and intestinal epithelia. However, gene targeting revealed that the loss of p45 activity does not affect globin gene expression nor erythroid cell development *in vivo* (*19, 20*). Targeted disruption of p45 affected only the terminal differentiation of megakaryocytes, so that mice bearing the mutation died after birth of severe hemorrhage. Other recent studies have also shown that the loss of either Nrf1 (*21*) or Nrf2 (*22, 23*) functions similarly does not affect erythropoiesis *in vivo*.

Two possible mechanisms could account for this lack of a discernible phenotype in erythroid cells that are known to require transcriptional activation through MARE regulatory sites. One possibility is that the loss of any one factor is not sufficient to cause a phenotype because of the compensating effects of related proteins. The other possibility is that another erythroid regulatory protein, differing from p45, exists, which is the genuine activator of erythroid MAREs. To address these possibilities, we generated p45 and Nrf2 compound knockout mice and examined their

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Abbreviations: CFU-Meg, colony forming unit-megakaryocyte; CNC, Cap'n'coller; HS, hypersensitive site; LCR, locus control region; MARE, Maf recognition element; MCV, mean red cell corpuscular volume; PCR, polymerase chain reaction.

phenotypic characteristics.

To generate p45 and Nrf2 compound homozygous mutant animals, p45 heterozygous mutant mice (*19, 20*) were obtained from the Jackson Laboratory, and were mated with Nrf2 homozygous knockout mice (*23*). We recovered double heterozygous mutant mice (*nrf2*−/+ and *p45*−/+) in this intercross experiment. These mice were then intercrossed to generate either compound homozygous mutant mice or ones with various other combinations of mutant and wild type alleles: polymerase chain reaction (PCR) analysis was performed to determine the genotype of the mice. High molecular weight DNA was prepared from the tails of newborn mice. For p45 screening, PCR was carried out under conditions of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 2 min at 72°C. Amplification (35 cycles) was performed using a set of primers, TS104 (5′-GTTAACTTGCCGGTAGATGACTTT-3′) and TS105 (5′-AGACCAGCTCAATCTGTAGCCTCC-3′). For Nrf2, genotyping was performed as previously described (*23*).

Newborn mice exhibiting all nine possible genotypes were obtained in these compound heterozygous intercross mating experiments (Fig. 1), of which five are shown: *nrf2*−/+::*p45*+/+ (lanes 1-3), *nrf2*−/+::*p45*−/− (lanes 4 and 5), *nrf2*−/−::*p45*+/+ (lanes 6 and 8) or −/+ (lane 7), and *nrf2*−/−::*p45*−/− (lanes 9-11). We also performed RT-PCR analysis to determine mRNA expression for these transcription factors. To this end, total cellular RNA was prepared from the spleens of newborn mice and cDNA was obtained according to standard procedures (*24*). The PCR conditions for p45 amplification were as described above, except that 25 cycles were performed using primers TS103 (5′-ACAGTAGGATGCCCCCGTGTCCTC-3′) and TS105. Reverse transcribed Nrf2 cDNA was detected by denaturation for 30 s at 94°C, 30 s at 60°C, and then 2 min at 72°C, for 25 cycles, using primers 313 (5′-CGGCTTTTGGCAGAGACATT-3′) and 1706r (5′-AGGAACACATTGCCATCTCT-3′). The mRNA expression patterns on RT-PCR analysis were consistent with the genotypes of these mice (Fig. 1).

The p45 homozygous mutant mice were readily distinguishable from the heterozygous mutant or wild type mice due to the bleeding spots (see arrows in Fig. 2A) and pallor, which were typical signs of severe bleeding (*20*). The p45 homozygous mutant mice usually started bleeding after birth. Therefore, since we analyzed these mice soon after birth, the hemorrhage and anemia was not so severe at this time. Importantly, we found that there was no clear difference in the appearance of Nrf2 homozygous mutant mice *versus* Nrf2 heterozygous mutant or wild type mice, all of which carry the p45 homozygous mutant alleles (Fig. 2A). Thus, further loss of Nrf2 function does not embellish the phenotype caused by the simple loss of p45 in these animals.

Peripheral blood samples were collected from newborn mice in heparinized microtubes, and then the hematocrit and platelet numbers were determined. Although there were varying degrees of anemia in the p45 homozygous mutant mice, it was mainly due to bleeding, because several mice with normal hematocrits were found among the *p45*−/− pups (Fig. 2, A and B). The mean red cell corpuscular volume (MCV) was slightly low in the p45 homozygous mutant mice as compared to in the wild type and heterozygous mutant mice. MCV was 57.5 ± 3.7 fl in the wild type mice and p45 heterozygous mutant mice, but 55.1 ± 1.6 fl in the p45 homozygous mutant mice. This decrease might be due to the hemorrhage. On the other hand, the red blood cell number was not further affected by disruption of the *nrf2* gene in the p45 homozygous mutant mice ($675 \pm 40 \times 10^6/\mu$l in single mutant mice *versus* $695 \pm 50 \times 10^6/\mu$l in double mutant mice).

We next examined peripheral blood smears and stamp samples of newborn spleens by Wright-Giemsa staining. Newborn mice were fixed in 10% buffered formalin and then embedded in paraffin for histological analysis. Sections were stained with hematoxylin and eosin. Cytological and histological analysis revealed normal erythroid and myeloid cell differentiation in the double homozygous mutant mice. No abnormality was observed in peripheral blood smears except for the absence of platelets in the *p45*−/− and *nrf2*−/− mice (data not shown). As seen in paraffin sections, erythropoiesis in the liver and spleen of the doubly mutant newborn mice was apparently normal (data not shown). Megakaryocytes were present in a slight excess in the liver and spleen of the p45 and Nrf2 double mutant mice, and megakaryocytes in the *p45*−/− mice appeared larger in size than those in wild type and p45 heterozygous mutant littermates (Fig. 3, arrows). These morphological abnormalities suggest the arrest of megakaryocytic terminal differentiation in p45 homozygous knockout mice.

Colony assaying of hematopoietic cells was also performed with newborn liver cells as described (*25, 26*). Colonies were scored visually on day 10 of incubation. Although platelets were absent in the *p45*−/− mice, the number of CFU-Meg colonies recovered from the livers of

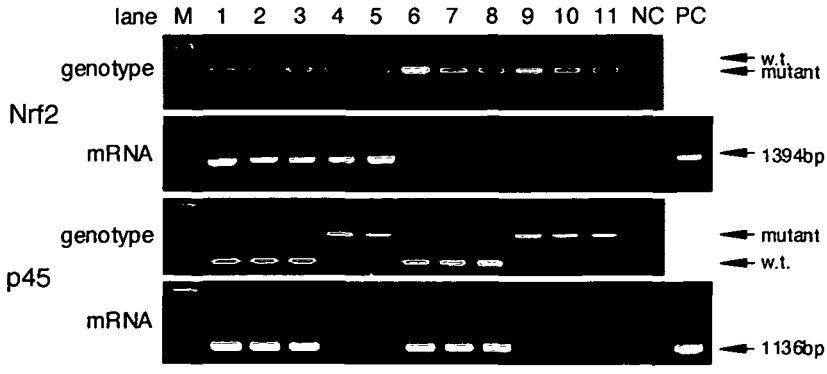

Fig. 1. **Genotype and mRNA expression analysis of p45 and/or Nrf2 knockout newborn mice.** High molecular weight DNA was prepared from the tails of eleven newborn mice (corresponding to the lane numbers), and PCR analysis was performed to determine the genotype. Arrows indicate the amplified bands of the wild type (wt) or mutant allele. Total RNA was prepared from the spleens of newborn mice, and then RT-PCR analysis was performed to examine the expression of Nrf2 and p45 mRNAs. Arrows indicate the amplified bands with the expected sizes. Note that the genotypes of the newborn mice are consistent with the mRNA expression profile.

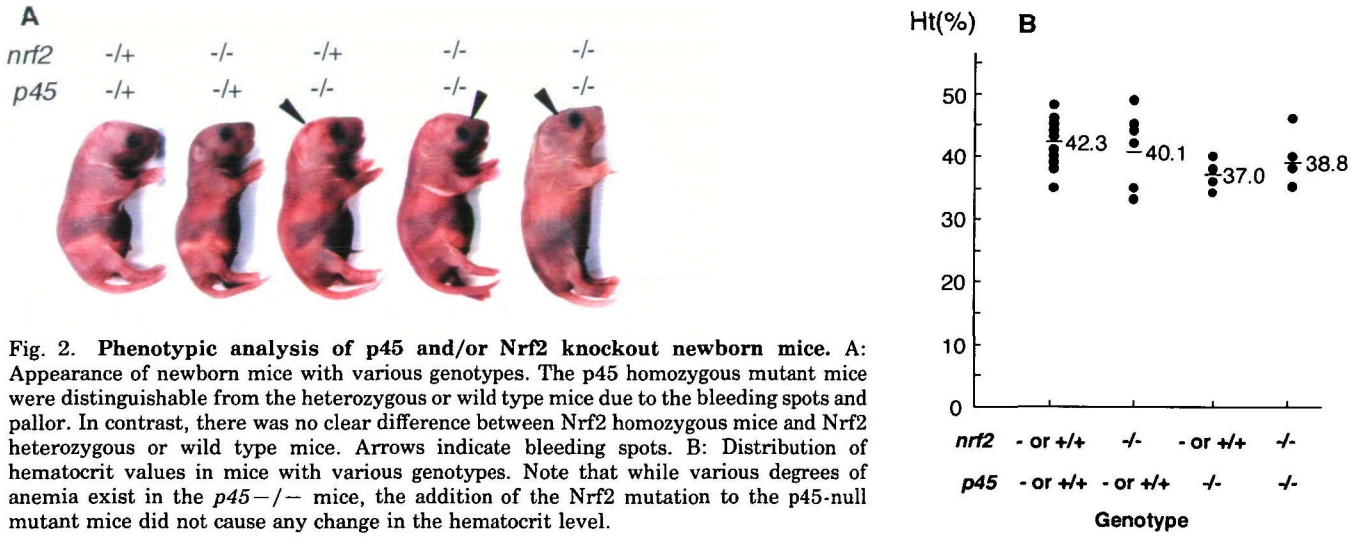

Fig. 2. **Phenotypic analysis of p45 and/or Nrf2 knockout newborn mice.** A: Appearance of newborn mice with various genotypes. The p45 homozygous mutant mice were distinguishable from the heterozygous or wild type mice due to the bleeding spots and pallor. In contrast, there was no clear difference between Nrf2 homozygous mice and Nrf2 heterozygous or wild type mice. Arrows indicate bleeding spots. B: Distribution of hematocrit values in mice with various genotypes. Note that while various degrees of anemia exist in the *p45*−/− mice, the addition of the Nrf2 mutation to the p45-null mutant mice did not cause any change in the hematocrit level.

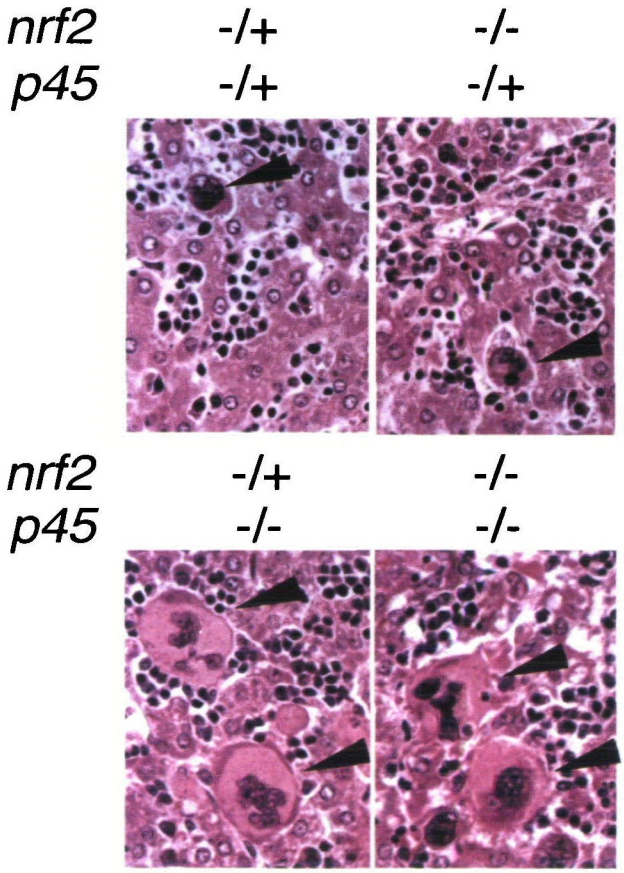

Fig. 3. **Histological analysis of the livers from p45 and/or Nrf2 knockout newborn mice.** Thin sections of the livers of four types of mice were stained with hematoxilin and eosin. Arrows indicate megakaryocytes. Original magnification, ×400.

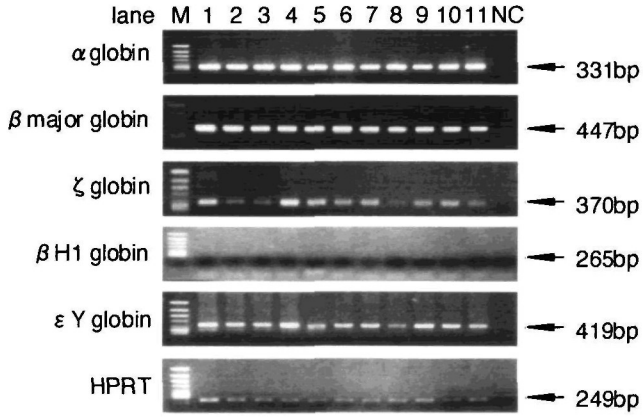

Fig. 4. **RT-PCR analysis of embryonic and adult globin mRNAs.** The expression levels of various globin mRNAs were examined by RT-PCR. Hypoxanthine phosphoribosyl transferase (HPRT) primers were used as an internal control of the PCR reaction. The lane numbers correspond to the mouse numbers in Fig. 1.

double or single mutant newborn mice appeared to be the same as for wild type animals ($11.5 \pm 0.5/5 \times 10^4$ newborn liver cells in wild type mice *versus* 10.5 ± 1.5 or $10/5 \times 10^4$ newborn liver cells in double or single p45 mutant mice). These data are therefore in excellent agreement with the previous conclusion (*20*) that the thrombocytopenia detected in p45 deficient mice is due to the arrest of megakaryocyte differentiation at the terminal stage of maturation.

We then analyzed the expression levels of embryonic and adult globin mRNAs in these mutant mice. The PCR primers and conditions for adult and embryonic globin mRNAs were as previously described (*24*). mRNAs for embryonic globins (εy, βH1, and ζ) were expected to be expressed at low levels in newborn mice. Indeed, these mRNAs were detected faintly in our p45 and Nrf2 double homozygous mutant and wild type mice, and there was no significant difference in the expression of these globin mRNAs. In addition, mRNAs for the adult globins (α and β major) were expressed abundantly in the wild type and p45 single knockout mice, as well as in the p45 plus Nrf2 compound homozygous mutant mice. The expression levels of globin mRNAs in the double mutants (lanes 9 to 11) was similar to that of the other genotypes (lanes 1 to 8), as judged on RT-PCR analysis (Fig. 4). These results indicate that embryonic and adult globin gene expression is not affected by the absence of p45 plus Nrf2 proteins.

In the present study, we examined the functional contri-

bution of p45 and Nrf2 to the differentiation of erythroid and megakaryocytic lineages by examination of compound mutant mice. We found that the addition of the Nrf2-null mutation to the p45 deficiency did not cause any enhanced alteration in either the erythroid or megakaryocytic lineages *in vivo*. p45 has been assumed to be an important regulator of globin gene expression (*18*). However, the present and previous (*20*) analyses revealed p45 to be rather an important regulator of the terminal differentiation of megakaryocytes. We recently presented evidence demonstrating that Nrf2 functions directly as the general transcription regulator of phase II-detoxifying enzyme genes (*23*). Since MAREs (or NF-E2 sites) have been shown to be essential *cis*-acting elements in many erythroid genes (*18*), this situation raises the question as to what is the genuine MARE binding factor in the hematopoietic lineage? While recent analysis of Nrf1 homozygous mutant embryonic stem cells in chimeric mice indicated that Nrf1-null cells contribute normally to the mature definitive erythroid cell population (*21*), the possibility still remains that compound mutations of Nrf1 and p45 (and/or Nrf2) may affect the erythroid differentiation program. However, the results of the present study seem rather to argue that the activity of other known and/or unknown factors may regulate gene expression from erythroid MAREs.

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