of the SNr of 15- to 20-day-old mice by papain digestion. Spontaneously firing (>3Hz), medium-sized (15 to 25 μm) neurons, the major constituent of SNr cells, which had no hyperpolarization-activated current ($I_h$) in perforated whole-cell patch recordings (*24*), were analyzed. Membrane properties were similar to those reported for SNr GABAergic neurons (*27*, *33*, *34*) (see Web fig. 2) (*13*). This was verified by immunostaining with antibodies to glutamic acid decarboxylase in separate experiments. GABAergic SNr neurons of wild-type and KO mice had similar resting membrane potentials of $-52.3 \pm 0.9$ mV ($n = 38$) and $-51.4 \pm 1.2$ mV ($n = 26$), respectively, and membrane input resistance was $2.3 \pm 0.2$ gigaohms ($n = 33$) and $2.4 \pm 0.3$ gigaohms ($n = 23$), respectively.

21. See Web fig. 3 (*13*) for details of single-channel analyses.
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25. Hypoxia was introduced by exchanging the perfusion medium from that equilibrated with 100% $O_2$ to a solution containing 2 mM $Na_2S_2O_4$ bubbled with 100% $N_2$ (<1 torr, measured with a gas analyzer).
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# Requirement of DNase II for Definitive Erythropoiesis in the Mouse Fetal Liver

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**Mature erythrocytes in mammals have no nuclei, although they differentiate from nucleated precursor cells. The mechanism by which enucleation occurs is not well understood. Here we show that deoxyribonuclease II (DNase II) is indispensable for definitive erythropoiesis in mouse fetal liver. No live DNase II–null mice were born, owing to severe anemia. When mutant fetal liver cells were transferred into lethally irradiated wild-type mice, mature red blood cells were generated from the mutant cells, suggesting that DNase II functions in a non–cell-autonomous manner. Histochemical analyses indicated that the critical cellular sources of DNase II are macrophages present at the site of definitive erythropoiesis in the fetal liver. Thus, DNase II in macrophages appears to be responsible for destroying the nuclear DNA expelled from erythroid precursor cells.**

Apoptosis is usually accompanied by DNA degradation that is regulated by caspase-activated DNase (CAD) (*1*). We recently showed that apoptotic DNA fragmentation occurs in macrophages in the absence of CAD after the apoptotic cells are phagocytosed (*2*), suggesting that DNase II, a lysosomal DNase (*3*, *4*), degrades DNA of apoptotic cells. Nuc-1, which is responsible for the apoptotic DNA fragmentation in *Caenorhabditis elegans*, has recently been identified as a homolog of DNase II (*5*). To investigate the physiological roles of DNase II in mammals, we generated mice deficient in the enzyme.

The murine DNase II gene is encoded by six exons within 2.3 kb of genomic DNA (*6*). We constructed a targeting vector, in which exons 1 to 5 and part of exon 6 were replaced with the *neo* gene (Fig. 1A) (*7*). The vector was introduced into R1 mouse embryonic stem (ES) cells, and G-418–resistant transformants containing the mutation in one allele of the DNase II gene were established (Fig. 1B). The ES clones were aggregated with eight-cell embryos, and the embryos were implanted into foster mothers. Chimeric mice derived from four clones transmitted the mutation to their offspring ($F_1$). The DNase $II^{+/-}$ mice were healthy and fertile. However, no live DNase $II^{-/-}$ mice were found among the progeny ($F_2$) of the DNase $II^{+/-}$ intercrosses. Analysis of timed pregnancies revealed that DNase $II^{-/-}$ embryos, confirmed by Southern hybridization (Fig. 1C), were present at a frequency roughly consistent with Mendelian inheritance throughout the embryonal stages (*8*).

Northern hybridization analysis indicated that the DNase II gene was expressed in the fetal liver of the wild-type and DNase $II^{+/-}$ embryos, but not in the DNase $II^{-/-}$ liver (Fig. 2A). The gene encoding erythroid Krüppel-like factor (EKLF), a transcription factor that is indispensable for erythroid cell differentiation (*9*, *10*), is located 3.9 kb upstream of the DNase II gene (*6*) (Fig. 1A). EKLF and $\beta^{major}$-globin mRNAs at embryonic day 14.5 (E14.5) were expressed in the fetal liver of DNase $II^{-/-}$ embryos at levels similar to those in normal fetal liver (Fig. 2A), indicating that the expression and function of EKLF were not impaired in DNase $II^{-/-}$ embryos. Other DNases such as DLAD and Xib have properties (acidic pH optimum and no requirement for divalent cations) similar to those of DNase II (*11*, *12*). To examine whether these enzymes compensated for loss of DNase II, we assayed extracts from E14.5 fetal livers for DNase activity (*13*). As shown in Fig. 2B, cell extracts from normal or heterozygous fetal liver showed substantial DNase activity at pH 5.7, whereas little, if any, DNase activity was detected under these conditions in the extracts from DNase II fetal liver. In contrast, the DNase I–like activity determined at neutral pH in the presence of $Ca^{2+}$ and $Mg^{2+}$ was normal or slightly higher in the extracts from DNase II fetal liver.

Homozygous DNase II embryos were apparently normal until E12.5, when a normal level of vasculature was seen in the embryos and yolk sac (Fig. 3A). Histological studies revealed similar numbers of nucleated primitive erythrocytes in the peripheral blood of normal and DNase II embryos at E12.5 (*14*). However, some DNase II embryos became paler than the wild-type embryo at E14.5, and all mutant embryos were severely anemic by E17.5 (Fig. 3B). The number of mature erythrocytes in the peripheral blood of

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DNase II$^{-/-}$ embryos was less than 1/10th of that present in normal littermates at E17.5 (*14*). There was no apparent hemolysis in the mutant embryos. The peripheral blood of the wild-type embryos carried nucleated cells (Fig. 3C), which were neutrophils and monocytes. A similar number of neutrophils and monocytes was found in the peripheral blood of DNase II$^{-/-}$ embryos. In addition, the peripheral blood of DNase II$^{-/-}$ embryos contained nucleated erythrocytes, which were not erythrosin-positive primitive erythrocytes, but appeared to be erythroblasts that had not undergone enucleation (Fig. 3C). Except for the severe anemia, DNase II$^{-/-}$ embryos appeared normal at E17.5. Thus, loss of DNase II specifically impaired definitive erythropoiesis.

Definitive erythropoiesis in embryos takes place in the fetal liver and spleen. When blood-cell progenitors in the fetal liver were assayed in methylcellulose culture in the presence of lineage-specific cytokines, the numbers of CFU-E (colony-forming unit, erythroid) as well as BFU-E (burst-forming unit, erythroid), CFU-G (granulocyte), and CFU-M (macrophage) in the E12.5 fetal liver were similar in the normal and DNase II$^{-/-}$ embryos (*8*). The cells in BFU-E and CFU-E colonies could be stained with DAF (2,7-diaminofluorene), indicating that DNase II$^{-/-}$ erythroid cells could differentiate into hemoglobin-producing cells. In colonies of CFU-E generated with either wild-type or DNase II$^{-/-}$ fetal liver cells, about 10% of cells were enucleated (Fig. 3D).

To determine if the defect in erythropoiesis was cell autonomous, we transplanted fetal liver cells from $F_2$ embryos into wild-type B6.SJL mice that had been lethally irradiated (*15*). The control irradiated mice did not survive beyond 3 weeks, whereas mice that received the wild-type or DNase

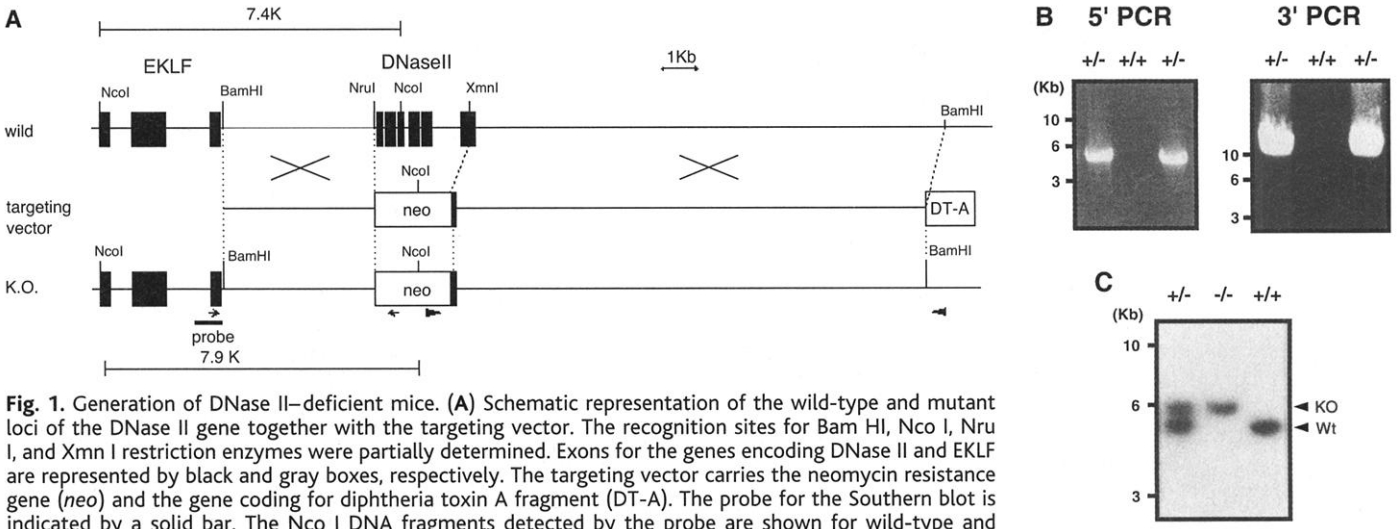


**Fig. 1.** Generation of DNase II–deficient mice. (**A**) Schematic representation of the wild-type and mutant loci of the DNase II gene together with the targeting vector. The recognition sites for Bam HI, Nco I, Nru I, and Xmn I restriction enzymes were partially determined. Exons for the genes encoding DNase II and EKLF are represented by black and gray boxes, respectively. The targeting vector carries the neomycin resistance gene (*neo*) and the gene coding for diphtheria toxin A fragment (DT-A). The probe for the Southern blot is indicated by a solid bar. The Nco I DNA fragments detected by the probe are shown for wild-type and targeted alleles. PCR primers used to detect the homologous recombination in the 5′ and 3′ regions are indicated by arrows and arrowheads, respectively. (**B**) PCR analysis of genomic DNA isolated from ES clones. PCR was carried out with the Expand Long Template PCR System (Roche Diagnostics). The primers used were 5′-TGCACATGAAGCGTCACCTCTGAGTGATCC and 5′-ACCTGCGTGCAATCCATCT-TGTTCAATGGC for the 5′ region, and 5′-GATTCGCAGCGCATCGCCTTCTATCG and 5′-CACCTAGAGTCAGAAGATGACACCAGCTAC for the 3′ region. (**C**) A representative Southern blot analysis of Nco I–digested DNA prepared from embryos generated by heterozygous pairs. Hybridization was carried out with a 0.7-kb DNA fragment located 3.9 kb upstream of the DNase II gene (Fig. 1A).

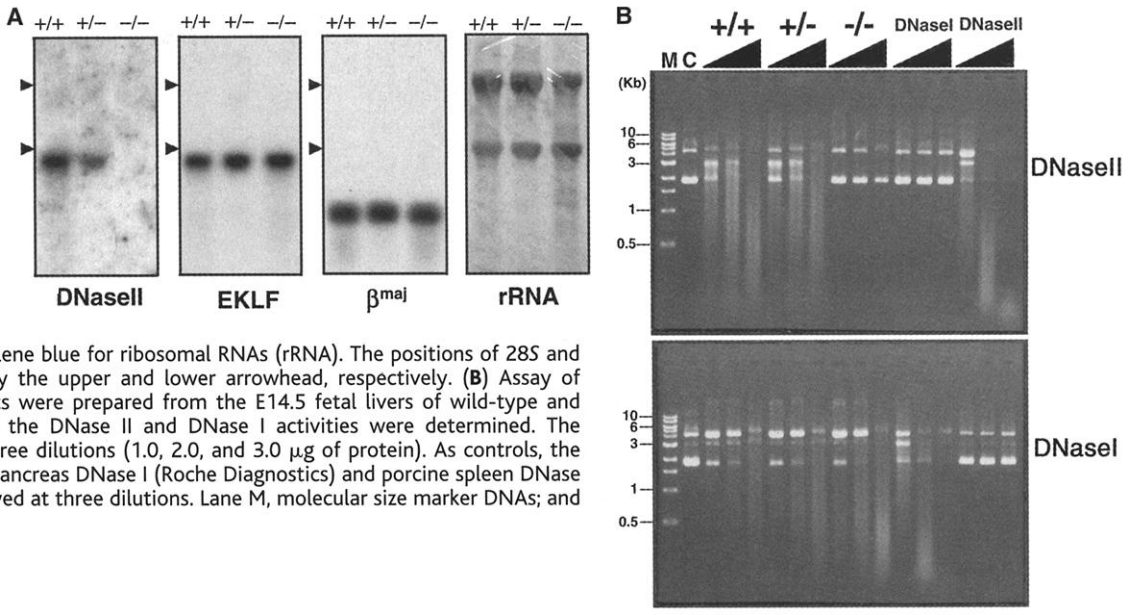


**Fig. 2.** Analysis of gene expression in the fetal liver of wild-type and mutant mice. (**A**) Northern hybridization. Total RNA (6 μg) from the E14.5 fetal liver of DNase II$^{+/+}$, DNase II$^{+/-}$, and DNase II$^{-/-}$ was analyzed by Northern hybridization with DNase II, EKLF, or mouse $\beta^{major}$-globin cDNA as probe. In the fourth panel, the filter was stained with methylene blue for ribosomal RNAs (rRNA). The positions of 28*S* and 18*S* rRNAs are indicated by the upper and lower arrowhead, respectively. (**B**) Assay of DNase activity. Cell extracts were prepared from the E14.5 fetal livers of wild-type and mutant embryos (*29*), and the DNase II and DNase I activities were determined. The extracts were assayed at three dilutions (1.0, 2.0, and 3.0 μg of protein). As controls, the DNase activities of bovine pancreas DNase I (Roche Diagnostics) and porcine spleen DNase II (Worthington) were assayed at three dilutions. Lane M, molecular size marker DNAs; and lane C, input plasmid.

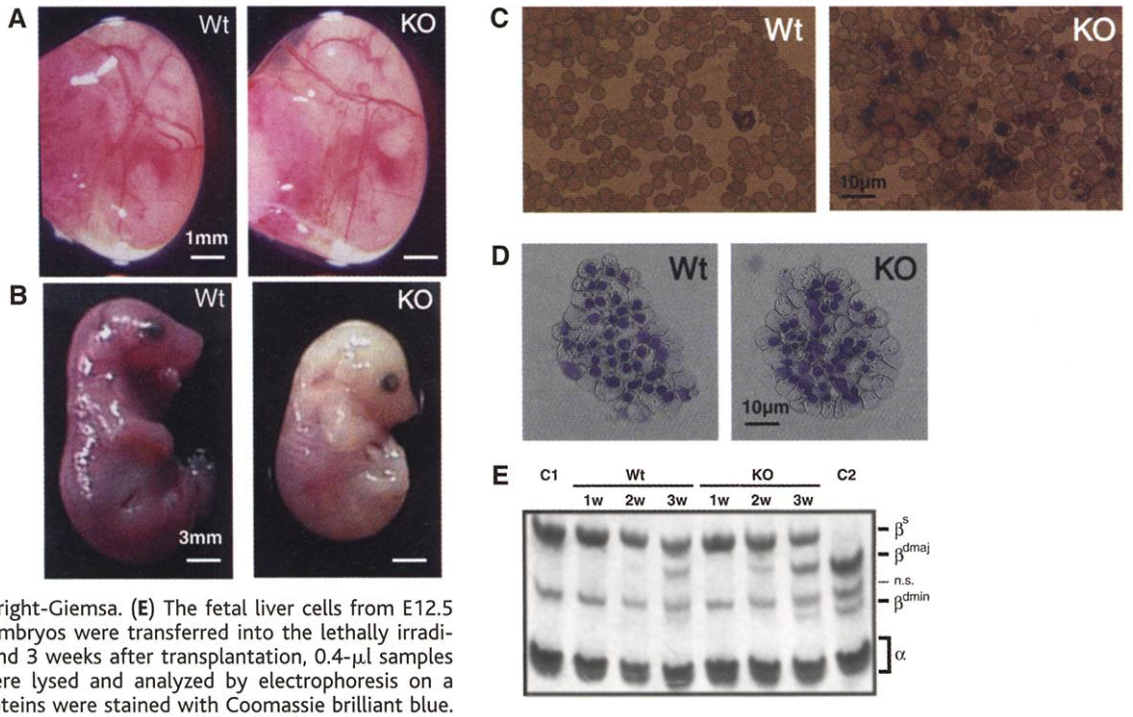


**Fig. 3.** Comparative morphology of wild-type and DNase $II^{-/-}$ embryos. (**A**) E12.5 wild-type and DNase $II^{-/-}$ knockout (KO) embryos within their yolk sacs. (**B**) E17.5 wild-type and DNase $II^{-/-}$ embryos dissected free of the yolk sacs. The DNase $II^{-/-}$ embryo was paler than the wild-type embryo, but otherwise developmentally normal. (**C**) Peripheral blood cells collected from E17.5 wild-type and DNase $II^{-/-}$ embryos were cytospun and stained with Wright-Giemsa. (**D**) The CFU-E colonies developed with fetal liver cells from E12.5 wild-type and DNase $II^{-/-}$ embryos were cytospun and stained with Wright-Giemsa. (**E**) The fetal liver cells from E12.5 wild-type or DNase $II^{-/-}$ embryos were transferred into the lethally irradiated B6.SJL mice. At 1, 2, and 3 weeks after transplantation, 0.4-μl samples of the peripheral blood were lysed and analyzed by electrophoresis on a polyacrylamide gel (*30*). Proteins were stained with Coomassie brilliant blue. In lanes C1 and C2, hemoglobin carrying $Hbb^s$ or $Hbb^d$ haplotype was subjected to electrophoresis. The bands for $\beta^s$-, $\beta^{dmajor}$-, $\beta^{dminor}$-, and α-globin are indicated. n.s., nonspecific band.

$II^{-/-}$ fetal liver cells survived for at least 4 weeks. The recipient B6.SJL strain is homozygous for the $Hbb^s$ haplotype, whereas ES cells of 129/Sv are homozygous for the $Hbb^d$ haplotype (*16*). As shown in Fig. 3E, 30 to 50% of β-globin in mice transplanted with either wild-type or DNase $II^{-/-}$ fetal liver cells had the $Hbb^d$ haplotype at 3 weeks after transplantation. These results indicated that the DNase $II^{-/-}$ erythroid cells can develop to mature cells, and the failure of definitive erythropoiesis in the DNase $II^{-/-}$ mice is due to a non–cell-autonomous effect.

We next performed a histochemical analysis of the fetal livers. The normal E12.5 to E17.5 fetal liver consisted largely of erythroid cells at various stages of differentiation (Fig. 4A). The cellularity of the DNase $II^{-/-}$ liver at E14.5 was comparable to or slightly less than that of the normal liver. However, many abnormal foci of various sizes were observed throughout the liver of the DNase $II^{-/-}$ embryos. The foci carried many small particles that were positive in the Feulgen reaction or DAPI (4′,6′-diamidino-2-phenylindole), but did not stain as well with hematoxylin-eosin, suggesting that the inclusions in the abnormal foci were composed mainly of DNA. Similar Feulgen-positive foci were also observed in the spleen of E17.5 embryos (*14*). The E14.5 fetal liver sections from DNase $II^{-/-}$ embryos were then analyzed by electron transmission microscopy. A macrophage-like cell was apparent in the middle of each focus, which contained many loosely packed nucleus-like structures (Fig. 4B). The nucleus-like structures contained fibrous material that may have been DNA. In some cases, the cells contained condensed material that probably consisted of newly phagocytosed nuclei. The macrophage-like cells were often surrounded by erythroid cells at various stages of differentiation (erythroblasts, reticulocytes, and mature erythrocytes). The E12.5 fetal liver sections were then stained with the macrophage-specific F4/80 antigen (*17*) and for megakaryocytes-specific acetylcholinesterase (AChE) activity. Numerous F4/80-positive macrophages were present throughout the liver of normal embryos (Fig. 4C). These macrophages were encircled by erythroblasts, suggesting that they were the central macrophages of the blood islands (*18*). Compared with the normal liver, the number of F4/80-positive macrophages in DNase $II^{-/-}$ fetal liver was substantially reduced, and all the Feulgen-positive abnormal foci were inside F4/80-positive macrophages. The AChE-positive megakaryocytes were also scattered in the fetal liver, but none of the megakaryocytes were associated with nucleus-like structures in DNase $II^{-/-}$ fetal liver (Fig. 4D).

On the basis of our results, we propose that DNase II in macrophage lysosomes is responsible for digesting nuclear DNA expelled from erythroid precursor cells. The presence of DNase II transcripts in macrophage cell lines (*14*) is consistent with this proposal. Although no live DNase $II^{-/-}$ mice were born, the embryos developed almost to the final stage, and low but appreciable numbers of mature erythrocytes were present in the peripheral blood of the embryos. The presence of macrophages in the fetal liver of DNase $II^{-/-}$ embryos probably supports definitive erythropoiesis at the initial stage. When the macrophages cannot digest the nuclear DNA, they may lose their ability to support the definitive erythropoiesis. Because the profile of cytokines produced by macrophages changes after phagocytosis (*19*, *20*), it is also possible that macrophages containing undigested DNA produce cytokines that inhibit definitive erythropoiesis. Mice deficient in the transcription factor PU.1 and *op/op* mice with mutations in the M-CSF gene have a reduced number of macrophages (*21–24*). With the exception of one study (*23*), erythropoiesis in these mutant mice seems to be normal. The existence of phagocytic cells in PU.1-null embryos (*25*), and the normal number of macrophages in the liver of neonate *op/op* mice (*26*) may explain the development of erythroid cells in these mutant mice.

In summary, our results show that DNase II in macrophages is indispensable for definitive erythropoiesis. The role of macrophages in removing apoptotic cells is well established (*27*). Whether DNase II works as an auxiliary mechanism for apoptotic DNA fragmentation can now be addressed by intercrossing DNase $II^{-/-}$ mice with mice that lack caspase-activated DNase.

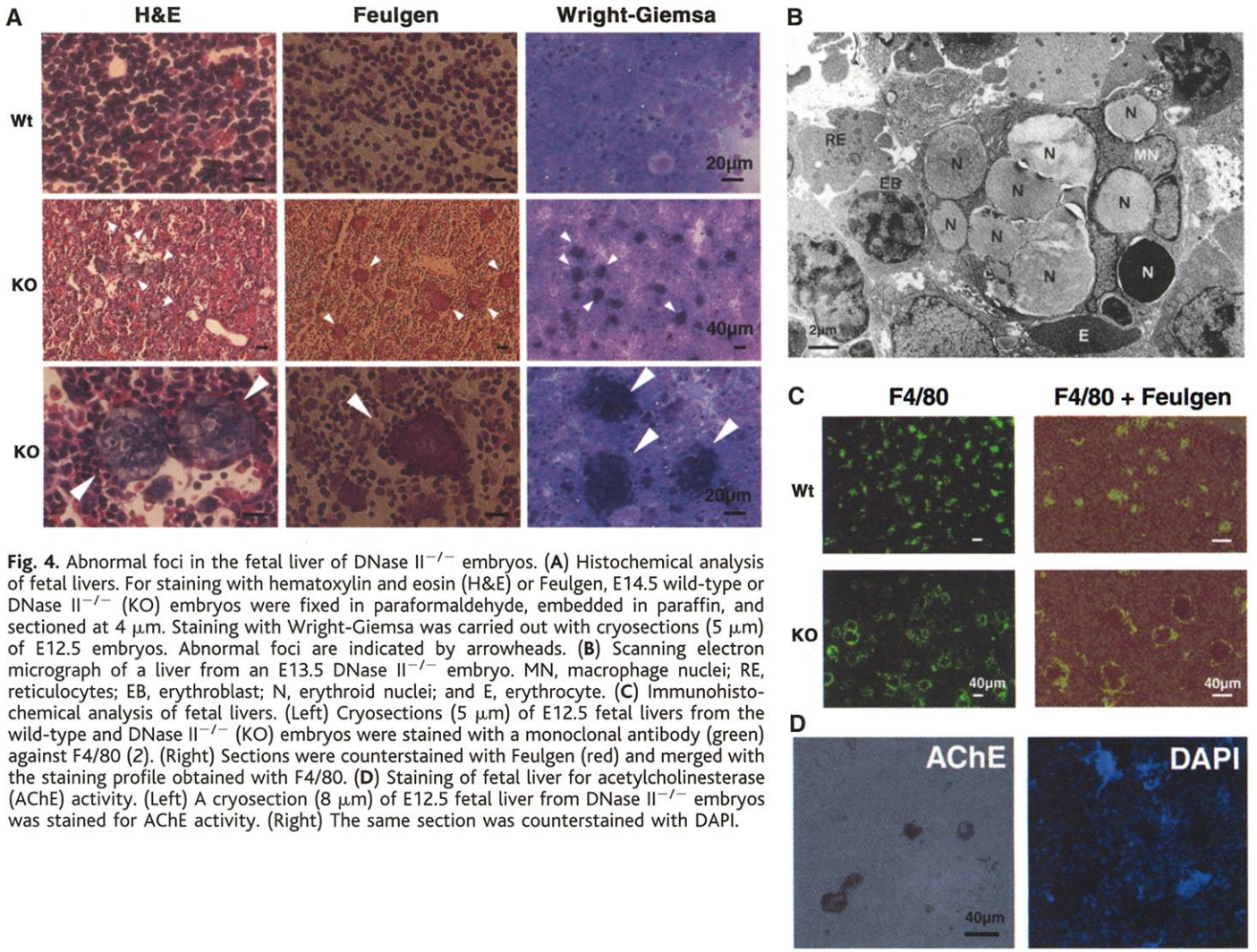


**Fig. 4.** Abnormal foci in the fetal liver of DNase II$^{-/-}$ embryos. (**A**) Histochemical analysis of fetal livers. For staining with hematoxylin and eosin (H&E) or Feulgen, E14.5 wild-type or DNase II$^{-/-}$ (KO) embryos were fixed in paraformaldehyde, embedded in paraffin, and sectioned at 4 μm. Staining with Wright-Giemsa was carried out with cryosections (5 μm) of E12.5 embryos. Abnormal foci are indicated by arrowheads. (**B**) Scanning electron micrograph of a liver from an E13.5 DNase II$^{-/-}$ embryo. MN, macrophage nuclei; RE, reticulocytes; EB, erythroblast; N, erythroid nuclei; and E, erythrocyte. (**C**) Immunohistochemical analysis of fetal livers. (Left) Cryosections (5 μm) of E12.5 fetal livers from the wild-type and DNase II$^{-/-}$ (KO) embryos were stained with a monoclonal antibody (green) against F4/80 (*2*). (Right) Sections were counterstained with Feulgen (red) and merged with the staining profile obtained with F4/80. (**D**) Staining of fetal liver for acetylcholinesterase (AChE) activity. (Left) A cryosection (8 μm) of E12.5 fetal liver from DNase II$^{-/-}$ embryos was stained for AChE activity. (Right) The same section was counterstained with DAPI.


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7. A mouse DNase II cDNA (*3*) was used to screen a genomic library of mouse strain 129/Sv (Stratagene). A DNA fragment carrying the mouse DNase II locus was subcloned, and the DNase II–targeting vector was constructed by replacing the 2.3-kb Nru I–Xmn I DNA fragment containing the DNase II gene with the 1.9-kb *neo* gene driven by the phosphoglycerate kinase–1 gene promoter (Fig. 1A). A DNA fragment encoding diphtheria toxin A fragment under control of the polyoma virus enhancer was inserted downstream of the DNase II gene to generate pKODNII. The pKODNII plasmid (19 μg) was digested with Not I and was introduced into $7.7 \times 10^6$ R1 ES cells by electroporation. The cells were selected in the presence of 150 μg/ml G-418 for 7 days. G-418–resistant clones were screened for the homologous recombination by PCR, and the ES clones carrying the DNase II–mutant allele were introduced into the host embryos by aggregation (*28*). Embryos that had developed to the blastocyst stage were transplanted into pseudopregnant foster mothers. Chimeric mice were crossed with C57BL/6 mice. Germ-line transmission of the ES cells to their offspring ($F_1$) was judged by the presence of agouti pups, and the disrupted alleles were identified by PCR.
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13. To measure the DNase II activity, we incubated 2 μg of plasmid DNA (pBluescript) at 37°C for 1 hour with the cell extracts in 300 μl of 10 mM tris-HCl buffer (pH 5.7) containing 10 mM EDTA. After phenol extraction, DNA was subjected to electrophoresis on a 1% agarose gel. The assay for DNase I activity was similar to that described above for DNase II, except that the reaction mixture contained 10 mM tris-HCl buffer (pH 7.4), 10 mM $CaCl_2$, and 10 mM $MgCl_2$.
14. K. Kawane, H. Fukuyama, S. Nagata, data not shown.
15. For transplantation of the fetal liver cells, single-cell suspensions prepared from E12.5 fetal liver of the wild-type or DNase II$^{-/-}$ embryos (seven to eight embryos each) were pooled. Three-month-old female B6.SJL mice (Taconic) were lethally irradiated (900 cGy) by a $^{137}Cs$ source and were intravenously injected with $1 \times 10^6$ fetal liver cells. The mice were maintained on aqueous antibiotics (1000 U/ml polymixin B sulfate and 1.1 mg/ml neomycin sulfate).
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