MafB Is Essential for Renal Development and F4/80 Expression in Macrophages

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MafB is a member of the large Maf family of transcription factors that share similar basic region/leucine zipper DNA binding motifs and N-terminal activation domains. Although it is well known that MafB is specifically expressed in glomerular epithelial cells (podocytes) and macrophages, characterization of the null mutant phenotype in these tissues has not been previously reported. To investigate suspected MafB functions in the kidney and in macrophages, we generated mafB/green fluorescent protein (GFP) knock-in null mutant mice. mafB homozygous mutants displayed renal dysgenesis with abnormal podocyte differentiation as well as tubular apoptosis. Interestingly, these kidney phenotypes were associated with diminished expression of several kidney disease-related genes. In hematopoietic cells, GFP fluorescence was observed in both Mac-1- and F4/80-expressing macrophages in the fetal liver. Interestingly, F4/80 expression in macrophages was suppressed in the homozygous mutant, although development of the Mac-1-positive macrophage population was unaffected. In primary cultures of fetal liver hematopoietic cells, MafB deficiency was found to dramatically suppress F4/80 expression in nonadherent macrophages, whereas the Mac-1-positive macrophage population developed normally. These results demonstrate that MafB is essential for podocyte differentiation, renal tubule survival, and F4/80 maturation in a distinct subpopulation of nonadherent mature macrophages.

The product of the c-maf proto-oncogene and its relatives (the large Maf family of transcription factors) share conserved basic regions and amphipathic helices (bZip) that mediate DNA binding to Maf recognition elements (MAREs) (20, 21) and dimer formation, respectively. The large Maf proteins MafA/L-Maf/SMAF, MafB, c-Maf, and NRL each contain an acidic domain that promotes transcriptional activation, and each protein has been shown to play key roles in cellular differentiation (19–22, 33, 44, 46, 50).

MafB deficiencies were originally reported to be responsible for the phenotypes observed in the Kreisler mutant mouse. The Kreisler mutation was an X-ray-induced chromosomal inversion that led to abrogated MafB expression, specifically in the fifth and sixth rhombomeres of the embryonic hindbrain (8). Hindbrain-specific mafB loss of function in the Kreisler mutant led to aberrant segmentation and subsequent hyperactive behavior (characterized by head tossing and running in circles as a consequence of an inner ear defect). In all other tissues and organs (in embryos at 8.5 days postcoitum through adulthood), MafB expression was conserved and equivalent in wild-type and Kreisler mutant animals (13). Analysis of mice homozygous for a second mutant allele of mafB (called krENU) showed that it is also expressed in glomerular visceral epithelial cells (podocytes) and is required for renal foot process formation (39). The krENU mutation, an Asn-to-Ser substitution at residue 248 within the MafB DNA binding domain, reduced MafB binding activity without affecting the stability of the MafB protein (39). The less severe hindbrain phenotype observed in krENU mice suggested that it was a mild hypomorphic allele of the original mafB gene (39).

MafB is expressed selectively in monocytes and macrophages but not in other hematopoietic lineages distinct from many other myeloid transcription factors (13, 44). During macrophage differentiation, MafB is not expressed in multipotent progenitors, but it is expressed at moderate levels in myeloblasts and is strongly induced in mature monocytes and macrophages (44). It has previously been reported that MafB is both necessary and sufficient to induce myelomonocytic differentiation of transformed avian myeloblast cells in vitro (23). MafB is known to interact with Ets-1, a helix-turn-helix transcription factor, and to inhibit Ets-1 trans-activation activity, thereby interfering with erythroid differentiation (44). These results strongly suggested that MafB could be a key regulator of lineage determination during myelopoiesis, but in vivo verification of this hypothesis has not been forthcoming.

Since both the Kreisler and krENU mutants retain residual MafB function, the consequences of complete MafB inactivation are unknown. Recently, Blanchi et al. reported that mafB
null mutant mice displayed defective respiratory rhythmogenesis (4), while the biological consequences of the null mutation in other tissues and organs were not addressed. Furthermore, the expression of MafB within the panoply of hematopoietic cells has not yet been fully documented. To address these issues, we generated mafB mutant mice in which the green fluorescent protein (GFP) gene was inserted into the mafB locus by homologous recombination. GFP expression recapitulates the expression of endogenous MafB, thus facilitating the analysis of MafB-expressing cell lineages and the identification of functional abnormalities that might result as a consequence of MafB loss of function. We report here that mafB homozygous mutant mice display severe renal dysgenesis, characterized by abnormal glomerular differentiation and nephric tubular apoptosis associated with the diminished expression of several kidney disease-related genes. In addition, MafB expression was observed in specific subpopulations of differentiated macrophages in vivo, and its deficiency significantly suppressed F4/80 transcription in nonadherent mature macrophages.

MATERIALS AND METHODS

Construction of the mafB targeting vector. To disrupt the mafB gene and simultaneously examine the tissue distribution of MafB expression, mice in which the GFP gene was inserted into the chromosomal mafB locus were generated. The targeting vector was constructed using genomic DNA from a 129SV/J mouse library (Stratagene, La Jolla, CA) recovered by screening with a mouse MafB cDNA probe. We subcloned an 8.2-kbp genomic SacI fragment spanning from −7.5 kb to +3.64 bp relative to the transcription initiation site of the gene that was ligated into the GFP gene in pCMX-SAH/Y145F (30). The 3’ end of the GFP expression cassette was joined to a neo gene cassette flanked by loxP sequences, followed by a 2.5-kbp XhoI/EcoRI fragment of 129SV/J mafB DNA (Fig. 1A). This construct was then joined to a thymidine kinase expression cassette at the 3’ end and inserted between the XhoI and NotI sites of pBlue-script (Stratagene, La Jolla, CA). Generation of mafB null mutant mice. The targeting vector was linearized by NotI digestion and electrointroduced into E14 embryonic stem cells (17). Colonies resistant to double selection with G418 and ganciclovir were picked, expanded, and analyzed by PCR. A pair of primers (sense, 5’-AGTGACCAAGGCGCTTCCACGCTCTTCAATC-3’; antisense, 5’-AGCAGGATTGTTGTGCCCTGATCCGATCATC-3’) that amplify a 3.0-kbp fragment composed of part of the 129SV/J mafB 3’-flanking region and the neo gene cassette was used for screening. PCR-positive clones were subsequently verified by Southern blotting. Homologous recombinant clones were injected into blastocysts of C57BL/6 mice. Male mice chimeric for the targeted allele were mated with C57BL/6 wild-type females, and heterozygous F1 (+/−Neo) mice were then crossed to Ayu-1 Cre mice, a general deleter Cre recombinase-expressing transgene line (34), to remove the neo cassette. The resultant heterozygotes bearing the targeted allele were subsequently interbred to yield wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mafB null mutant mice. Genotypes were determined by genomic Southern blotting (Fig. 1B and C) and PCR analysis of tail DNA. For amplification of the wild-type allele, sense and anti-sense primer pairs within the coding region of the mouse mafB gene were used; a primer pair specific for enhanced GFP cDNA was used to detect the targeted allele (Table 1). All experiments were performed according to the guide for the care and use of laboratory animals of the University of Tsukuba.

Histological analysis. Tissues and embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight and then processed for paraffin or frozen sections. Paraffin sections (8 to 12 μm) were cut with a microtome and processed for either hematoxylin and eosin or periodic acid-Schiff staining. For hygiene in phosphate-buffered saline overnight and then processed for paraffin or frozen sections were incubated at a 1:1,000 dilution of rabbit polyclonal anti-GFP antibody (A-6455; Molecular Probes, Eugene, OR), anti-MafB (BL658; Chemicon, Temecula, CA), and anti-β-galactosidase antibody (100 units/ml) was centrifuged at 1,200 rpm for 5 min, and the cell pellet was resuspended in Dulbecco’s modified Eagle’s medium (high glucose, endotoxin tested; Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (heat inactivated), streptomycin and penicillin (100 units/ml), and macrophage colony-stimulating factor (M-CSF) (10 ng/ml) and then seeded either onto an adhesive tissue culture dish (catalog no. 353803; Falcon) or onto nonadhesive dishes coated with hydrophile polymers (Hydrosell; Cell Seed, Tokyo). The culture medium was not changed during the experiment. M-CSF (final concentration, 10 ng/ml) was added every day from day 4 onwards. One, 2, 4, and 6 days after seeding, the cells were harvested and analyzed by flow cytometry. For the semisolid medium culture, 104 cells were pelleted and then resuspended in Methocult M3231 (StemCell Technologies Inc.) supplemented with M-CSF (100 ng/ml). Fourteen days after seeding, the cells were washed away from the methylcellulose and analyzed by fluorescence-activated cell sorter analysis.

Mammalian cell culture and transient transfection assays. The pGL3 Basic-based 668-bp human F4/80 promoter luciferase construct was reported previously (37). A full-length MafB cDNA was subcloned into the pCDNA3.1 neo eukaryotic expression plasmid (Invitrogen). Murine macrophage cell line
RAW264.7 was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were transfected by lipofection using FuGENE6 transfection regent (Roche) and then harvested 24 h posttransfection. Luciferase assays were performed using the dual-luciferase reporter assay system (Promega). Transfection efficiencies were normalized to cotransfected, coexpressed Renilla luciferase activity.

**FIG. 1.** Generation of mafB null mutant mice. (A) Strategy for GFP reporter gene knock-in to the mafB locus by homologous recombination. H and S are abbreviations for restriction enzyme cleavage sites for HindIII and SacI, respectively. Neo indicates the neomycin resistance gene flanked by loxp elements. The positions of the 3’ genomic probe 1 and internal probe 2 used for Southern blot analysis are indicated by the horizontal bar. (B) The HindIII restriction fragments detected with internal probe 2 in the wild type (WT) (4.6 kbp), knockout (KO) Neo allele (4.0 kbp), and KO allele (2.9 kbp) are indicated. (C) Genotyping by Southern blotting. HindIII digestion of genomic DNA extracted from the tails of sibling pups of each genotype generated 4.6-kbp and 3.0-kbp bands for the wild-type and KO alleles, respectively, using 3’ genomic probe 1. (D) RT-PCR analysis of MafB mRNA. RNA was prepared from the kidneys of wild-type and heterozygous and homozygous mutant mafB neonates. (E and F) GFP immunoreactivity in podocytes of mafB+/− and mafB−/− neonates. (G and H) Endogenous MafB mRNA expression was specifically detected in the podocytes of mafB+/− kidneys, whereas mafB−/− neonatal kidneys had no MafB mRNA accumulation. Scale bars represent 20 μm in all panels.

**RESULTS**

Generation and molecular characterization of mafB−/− mice. In order to both disrupt the mafB gene in embryonic stem cells and enable the examination of its sites of expression in mice, a
targeting construct that would replace the coding sequence of mafB with GFP was generated (Fig. 1A). Homologous recombination was confirmed by genomic Southern blotting (data not shown). Three independent clones were injected into C57BL/6J blastocysts to generate chimeric offspring. Male chimera were mated with C57BL/6J females to obtain heterozygous animals, and the heterozygous animals were then crossed with Ayu-1 Cre mice that ubiquitously express Cre recombinase (34) to remove the neomycin resistance cassette (that was flanked by loxP sites). These heterozygotes (+/−) were interbred to generate homozygous mafB−/− mice. Neo gene excision and progeny genotypes were confirmed by Southern analysis of tail DNA (Fig. 1B and C).

To verify that the targeted mutagenesis resulted in the generation of a genuine null allele, we performed RT-PCR analyses of kidney mRNA recovered from neonates generated from heterozygous intercrosses. Unlike wild-type or heterozygous mutant kidneys that express high levels of MafB mRNA, mafB−/− neonatal kidneys lacked detectable MafB transcript (Fig. 1D). We observed the expected Mendelian ratios of wild-type, heterozygous, and homozygous mutant progeny at all (Fig. 1D). RT-PCR analysis of kidney mRNA recovered from neonates generated from heterozygous intercrosses. Unlike wild-type or heterozygous mutant kidneys that express high levels of MafB mRNA, mafB−/− neonatal kidneys lacked detectable MafB transcript (Fig. 1D). We observed the expected Mendelian ratios of wild-type, heterozygous, and homozygous mutant progeny at all

Renal dysgenesis in mafB−/− mice. The renal vesicle, from which the nephron arises, proceeds through four morphologically well-defined stages: the comma-shaped body, the S-shaped body, the capillary loop, and the mature stages (1). Previous studies reported that MafB mRNA is expressed exclusively in the podocytes at the capillary loop stage and in the mature glomerulus, which are located more internally relative to the immature renal vesicles formed in the nephrogenic zone at the outer margin of the cortex (39).

Neonatal kidneys from homozygous mafB−/− mutant mice displayed punctuate surface hemorrhages and a dystrophic appearance, unlike heterozygous mutant neonatal kidneys (Fig. 2A). Histological analysis revealed that the newborn homozygous mutant kidneys had relatively fewer differentiated mature glomeruli and displayed tubular dysgenesis accompanied by cyst formation, primarily in the area of proximal tubuli, in comparison to heterozygous mutants (Fig. 2C to F). In electron microscopic analysis of mature glomeruli, the podocytes of homozygous mafB−/− mutant mice displayed a dearth of normal foot processes (Fig. 2H). The podocyte foot processes in the kidneys of the homozygous mutants were fused and did not interdigitate, whereas discrete foot processes were observed in the glomeruli of kidneys recovered from heterozygous littermates (Fig. 2G and H). Furthermore, we observed no urine collection in the bladder of homozygous mutant newborns, likely as a consequence of this tubular dysgenesis (data not shown).

To further evaluate renal function in mafB−/− homozygous mutants, we carried out serum creatinine analysis. The mean serum creatinine concentration in homozygous mutant neonates (0.567 ± 0.082 mg/dl) was significantly higher than that in their heterozygous littermates (0.400 ± 0.063 mg/dl; P = 0.0082, Mann-Whitney U test) (Fig. 2B). Thus, mafB−/− homozygous mutant newborns displayed clear evidence of renal dysfunction. These observations demonstrate that MafB is required for normal glomerular and tubular development and function.

Repression of kidney disease genes in mafB−/− homozygous mutant mice. Recent advances in the study of human kidney disease have identified several key molecules that are required for podocyte development and glomerular function. The slit diaphragm, which laterally joins podocyte foot processes, is a...
FIG. 2. Kidney abnormalities in mafB<sup>−/−</sup> neonatal mice. (A) Gross appearance of a neonatal kidney from a mafB<sup>−/−</sup> mouse. The mafB<sup>−/−</sup> kidney (right) has a punctate surface hemorrhage and a dystrophic general appearance, unlike kidneys recovered from a mafB<sup>+/−</sup> littermate (left). (B) mafB<sup>−/−</sup> mice display a significant difference from mafB<sup>+/−</sup> littermates in mean serum creatinine concentration (P = 0.0082, Mann-Whitney U test). (C and D) Periodic acid-Schiff staining of mafB<sup>−/−</sup> and mafB<sup>+/−</sup> neonatal kidneys. (C) Tissue sections of mafB<sup>−/−</sup> kidneys showed numerous proximal and distal tubules. (D) Kidneys from mafB<sup>−/−</sup> mice have a reduced number of proximal and distal tubules accompanied by numerous cysts, primarily in the area of proximal tubuli (arrows in panels D and F). (E and F) mafB<sup>−/−</sup> kidneys had mature glomeruli, whereas kidneys from mafB<sup>+/−</sup> animals had fewer mature glomeruli (outlined by dotted lines). (G and H) Lack of normal podocyte foot processes in mafB<sup>−/−</sup> glomeruli. Electron microscopy revealed that mafB<sup>−/−</sup> podocyte foot processes were fused and did not interdigitate (H), whereas normal discrete foot processes were observed in the glomeruli of mafB<sup>+/−</sup> kidneys (G). pc, podocyte; fp, foot process; GBM, glomerular basement membrane. The scale bars in panels C, D, E, and F are 60 μm, whereas those in panels G and H represent 1 μm.
FIG. 3. Expression of kidney disease-related genes in mafB mutant mice. (A) mafB homozygous mutant kidneys displayed a significant reduction in the accumulation of kidney disease-related mRNAs (nephrin, podocin, and CD2AP) on Northern blots. The proximal/distal tubular marker genes (mFuc-TIX and uromodulin) were also reduced. In contrast, expression of NEPH1 and c-Myc was significantly elevated in the mafB+/H11002/H11002 kidney. Pod1, a putative upstream regulator of mafB, was unchanged. The podocalyxin gene was unaffected (see Discussion). (B) Nephrin and podocin immunoreactivities were significantly reduced in the glomeruli of mafB homozygous mutant kidneys in comparison to their heterozygous littermates. The scale bars represent 20 μm. (C) Quantification of the RNA blotting analysis in panel A. The band intensities were quantified with ImageQuant version 5.2 (Molecular Dynamics) software and then normalized to GAPDH mRNA abundance. Three independent animals of each genotype were analyzed. Data are presented as the means ± SD. The statistical significances of the differences are indicated by asterisks (*, P < 0.05; **, P < 0.01 [Student’s t test]).
unique feature of podocytes. Three major components (nephrin, podocin, and CD2 adaptor protein (CD2AP)) are required for slit diaphragm differentiation and function. Nephrin, the gene mutated in congenital nephrotic syndrome of the Finnish type (NPHS1), encodes an immunoglobulin superfamily transmembrane protein that localizes to the slit diaphragm and joins the interdigitated pedicles (24). Podocin was identified as the gene mutated in autosomal recessive steroid-resistant nephritisc syndrome (NPHS2) and is a membrane-integral protein belonging to the stomatin family (5). CD2AP is an Src homology 3 domain-containing protein that interacts directly with nephrin and is required for the assembly of the slit diaphragm (26, 27).

Given the kidney pathophysiology detected in the mafB null mutant mice, we asked whether MafB might lie in a common regulatory pathway leading to the expression of any of these kidney disease-related genes. We did so by comparing their expression in the neonatal kidneys of mafB heterozygous and homozygous mutant mice by RNA blot analysis. The results of these experiments demonstrated a significant reduction of nephrin, podocin, and CD2AP mRNAs in the homozygous mutant kidneys (Fig. 3A and C). In the glomeruli of mafB homozygous mutant kidneys, both nephrin and podocin immunoactivities were significantly reduced in comparison to that of sibling heterozygotes (Fig. 3B). Since each of these genes is required for the normal development and function of the glomerulus, these data are fully congruent with the histological observations showing that podocytes in the homozygous mutant kidneys appear to be incompletely differentiated. We also observed an unexpected increase in NEPH1 expression in the homozygous neonatal mutant kidneys. NEPH1 is structurally related to nephrin and is known to interact with the C-terminal domain of podocin and nephrin (Fig. 3A and C and see Discussion) (3, 11, 43).

Given the perinatal anuria of mafB mutant newborns, we assayed the same kidneys for expression of podocalyxin, a podocyte membrane protein essential for foot process formation and maintaining the permeability of the glomerular filter (41). Podocalyxin mutant mice display a similar perinatal anuric phenotype due to lowered glomerular permeability (12). However, despite the similar anuric phenotype in the two mutants, podocalyxin expression was essentially unaffected by the homozygous mafB mutation (Fig. 3A and see Discussion). Taken together, these data show that MafB differentially regulates, directly or indirectly, the transcription of podocin, nephrin, and CD2AP in podocytes and that this regulation is essential for normal podocyte differentiation.

Renal tubular dysgenesis is accompanied by tubular epithelial cell death. We observed both proximal and distal tubular dysgenesis as well as renal cysts in mafB homozygous mutant kidneys (Fig. 2C to F), consistent with diminished expression of mFuc-TIX (CD15 synthase) and uromodulin (uromucoid, Tamm-Horsfall glycoprotein), which are proximal and distal tubule-specific markers, respectively (Fig. 3A and C) (25, 35, 48). To test the hypothesis that this tubular dysgenesis was caused by apoptotic tubular epithelial cell death, we carried out TUNEL assays to detect fragmented nuclear DNA in the kidneys of heterozygous and homozygous mutant neonatal mice.

The number of TUNEL-positive nuclei was significantly higher in mafB homozygous mutant kidneys than in those of their heterozygous littermates. The number of apoptotic cells increased predominantly in the tubule epithelium rather than in the glomerular region (Fig. 4A, B, and C). This observation indicates that a massive wave of cell death occurred in the renal tubuli of newborn homozygous mutant animals. As indicated above, MafB expression is detected specifically in podocytes but never in renal tubules (Fig. 1E to G), indicating that the increased apoptotic cell death of the tubular epithelial cells is not due to a cell-autonomous effect of MafB deficiency. We also observed significantly increased expression of c-Myc, a well-known apoptosis inducer, in the mafB-/- kidney (Fig. 3A) (9, 47), suggesting that tubular cell death may be induced through a c-Myc-dependent signaling pathway.

MafB is expressed in a subpopulation of mature macrophages and is required for expression of F4/80. It was previously reported that MafB is expressed specifically in myelomonocytic lineage hematopoietic cells and was both necessary and sufficient to induce monocytic differentiation of transformed avian myeloblast cells in culture (23, 44). To examine hematopoietic lineage-specific MafB expression in vivo, we first monitored GFP expression in fetal liver cells recovered...
from *mafB* heterozygous embryos at embryonic day 14.5 (e14.5) by flow cytometry in parallel with several hematopoietic lineage-specific markers. Interestingly, most of the GFP-positive cells in the *mafB*^−/−^ e14.5 fetal liver were stained with both terminally differentiated macrophage-specific antigen F4/80 and myelomonocyte-specific antigen Mac-1 (Fig. 5, middle column), whereas GFP-positive cells in the heterozygotes were largely negative for granulocyte-specific antigen Gr-1, the myeloid-related dendritic cell marker CD11c, Ter119-positive erythroid cells, and CD4-, CD8-, and CD19-positive lymphoid lineages (Fig. 5A and data not shown). GFP-positive cells were observed in both negative and positive fractions for antigen-presenting cell marker MHC class II (data not shown). Immunohistochemical analysis of MafB plus F4/80 showed that endogenous MafB expression colocalized with the macrophage-specific antigen F4/80 in the e14.5 heterozygous fetal liver (Fig. 5B). Thus, these observations suggest that MafB is most specifically expressed in F4/80- and Mac-1-positive macrophages in vivo.

We next asked if hematopoietic deficiencies are observed in *mafB* null mutant hematopoietic cells. When homozygous mutant e14.5 fetal livers were examined simultaneously for MafB and F4/80, although MafB immunoreactivity was absent, as expected, F4/80 staining persisted (Fig. 5B). Interestingly, in flow cytometric analysis of e14.5 homozygous mutant fetal liver cells, the absolute number of F4/80-expressing cells (8% ± 0.45% [n = 8]) was slightly but reproducibly reduced in comparison to that of the wild type (10% ± 0.42% [n = 9]) (Fig. 5A and C). Furthermore, in the mutants, we observed a significant increase in the number of F4/80^-/^ GFP^hi^ and F4/80^-/^ GFP^- cells in a continuum with F4/80^-/^ GFP^hi^ cells (Fig. 5A, blue gate). Those *MafB*-deficient cells are Mac-1^-/CD11c^- macrophages (Fig. 5A, Mac-1 and CD11c plot), and thus, these data suggest that the MafB deficiency causes the suppression of F4/80 expression in Mac-1^-^ macrophages (Fig. 5A). To attempt to determine the fate of MafB-deficient macrophages, we analyzed the F4/80 and Mac-1 expression profiles among the GFP-bright (highly MafB-expressing) macrophages in both *mafB*^−/−^ and *mafB*^+/−^ e14.5 fetal liver cells (Fig. 5A, blue gates). In the homozygous mutant, F4/80^-/^ Mac-1^-^ immature macrophages are significantly more abundant (up to 14.5% of the GFP^-^ fraction), whereas comparable heterozygous fetal livers had only 2.3% of fetal liver cells in the same fraction, confirming the hypothesis that MafB deficiency suppresses F4/80 expression in Mac-1^-^ macrophages (Fig. 5D). At the same time, the Mac-1^-^ total macrophage population as well as all other lineage markers tested (Gr-1, CD11c, CD4, CD8, CD19, Ter-119, and MHC class II) displayed no difference between genotypes. In the *mafB*^−/−^ control, MafB and F4/80 expression is nicely colocalized, whereas MafB immunoreactivity was absent in *mafB*^+/−^ fetal livers. (C) In e14.5 fetal liver cells from *mafB*^+/−^ mice, the total population of F4/80-positive cells was slightly reduced in comparison to the number recovered in either *mafB*^−/−^ or wild-type mice. (D) F4/80 and Mac-1 expression profile in GFP-positive (MafB-expressing) macrophages. A total of 1.1% of GFP-positive cells (blue gates in panel A) from each genotype were analyzed. In the *mafB*^+/−^ cells, an increase of F4/80^-/^ Mac-1^-^ premature macrophages and a reduction of F4/80^-/^ Mac-1^-^ mature macrophages were observed.
ences (Fig. 5A and data not shown), suggesting that MafB influences F4/80 expression in MafB-expressing macrophages in a cell-autonomous manner. The reduction in the F4/80-positive population and concurrent increase of F4/80-negative GFP⁺ cells in the mafB homoygotes was even more prominent in newborn peripheral blood and spleens (Fig. 6A and C), while the total Mac-1⁺ macrophage population was hardly affected (Fig. 6C). As expected, the accumulation of F4/80⁻ Mac-1⁺ antigen in the GFP⁺ (MafB-expressing) macrophages significantly increased in the homozygous mutant spleen cells (Fig. 6B) and peripheral blood (data not shown).

We next assessed whether e14.5 fetal liver cells in the mafB mutants were altered in the normal spectrum of erythroid and myeloid CFU by in vitro colony assays. Only few differences were observed in CFU-erythrocyte, CFU-granulocyte/macrophage, and CFU-granulocyte/erythrocyte/macrophage/megakaryocyte in erythropoietin-, stem cell factor-, interleukin-3 (IL-3)-, and granulocyte colony-stimulating factor-supplemented media (data not shown). These data suggest that while MafB is important for the presentation of the F4/80 terminal differentiation marker and its expression in MafB-expressing mature macrophages, MafB is not necessary for the development of macrophages.

MafB is indispensable for F4/80 expression in nonadherent macrophages. We next examined the differentiation potential of fetal liver macrophages in primary cultures. The traditional view of the mononuclear phagocyte system suggests that circulating monocytes enter peripheral tissues to become macrophages, implying that their direct attachment to extracellular substrates could be a critical cue for macrophage differentiation. It is well established that cell-adhesive interactions with the extracellular matrix transmit differentiation signals through integrin-mediated pathways in monocytes (7). In contrast, cell adherence to plastic tissue culture dishes results in a rapid induction of multiple mature macrophage-specific genes, suggesting that in an adhesive plastic petri dish, macrophage progenitor cells might be induced to undergo differentiation (29). Since blood cells in hematopoietic tissues are exposed to constant blood flow-mediated shear stress, this dynamic process could normally prevent tight interactions between myelomonocytic cells and the extracellular matrix (10). We therefore attempted to exclude such external differentiation signals arising from cell adhesion and to reconstitute a physiological hematopoietic environment by culturing e14.5 fetal liver cells on nonadhesive dishes coated with hydrophilic polymers (Hydrocell; Cell Seed, Tokyo, Japan). Cell adherence is completely inhibited on the hydrophilic surface of these dishes. We then compared the differentiation profiles of these cells to those of normal (adhesive dish-cultured) macrophages.

Interestingly, in the mafB mutant fetal liver cells grown under nonadhesive conditions (Fig. 7A, right panel, blue line), most failed to express F4/80, although 90% of the wild-type cells under identical conditions produced F4/80⁺ mature macrophages (Fig. 7A, left panel, blue line). Conversely, under adherent conditions, more than 85% of the macrophages derived from either the wild-type or mafB mutants differentiated into F4/80-expressing cells (Fig. 7A, red lines in the left and right panels). Real-time PCR analysis of nonadherent macrophages showed a 50% reduction in F4/80 mRNA levels in the homozygous mutant macrophages (Fig. 7C). Following the time course on nonadherent dishes, the MafB deficiency suppressed F4/80 expression from day 2 onward, although Mac-1 expression was unaffected (Fig. 7B).

To further confirm F4/80 suppression in the mafB homozygous mutant macrophages under other nonadherent conditions, we analyzed e14.5 fetal liver-derived macrophages in methylcellulose supplemented with M-CSF. A total of 81.1% of mafB homozygous mutant fetal liver cells gave rise to GFP⁺ macrophages, although most of them failed to express F4/80 (Fig. 8A to D), while under identical conditions, 92% of wild-type cells gave rise to F4/80⁺ macrophages (Fig. 8C). These combined results demonstrate that MafB is required for F4/80 expression under nonadherent conditions.

To test the hypothesis that MafB directly regulates the F4/80 gene, we performed reporter assays using a 668-bp F4/80 promoter/luciferase construct that was cotransfected with a MafB expression vector (36, 37). A well-conserved MARE half-site (CTTTCTGTGCTAGTTGCCA [underlined letters are the consensus MARE half-site]) from −130 to −113 bp (37) was identified within the F4/80 promoter. The F4/80-directed reporter was activated (ca. 10-fold) by MafB cotransfection in a dose-dependent manner after transfection into the murine macrophage cell line RAW264.7, suggesting that MafB directly regulates the F4/80 promoter region (Fig. 8E).

For characterization of their differentiated phenotype, fetal liver-derived macrophages (cultured on nonadherent dishes) were assessed for their ability to engulf Nile red fluorescent carbosylate-modified microspheres; the mean overall phagocytic activities of macrophages derived from the wild type and heterozygous and homozygous mafB mutants were indistinguishable (data not shown). The production of nitric oxide and inflammatory/anti-inflammatory cytokines such as IL-6, IL-12, and IL-10 treated with lipopolysaccharide was also quantified. No significant difference in production of those cytokines was observed (data not shown), and differences in nitric oxide production were minimal (our unpublished observations), suggesting that germ line inactivation of mafB does not significantly affect conventional macrophage functions.

**DISCUSSION**

In this study, we report that the kidneys of mice in which both mafB alleles have been disrupted display renal dysgenesis and non-cell-autonomous apoptotic cell death in tubular epithelial cells. The substantial reduction of tubular epithelial cell populations in the renal medulla and the accompanying increase of renal mesenchymal cells caused a visible dystrophic appearance of the mafB⁻/⁻ kidney. In addition, the expression of kidney disease-related genes nephrin, podocin, and CD2AP were significantly reduced in mafB⁻/⁻ mice. Recently, Kr⁺/⁻ mutant mice were reported to display abnormal foot process formation, although differences in the expression levels of these same kidney disease-related genes were minimal (39). These observations are consistent with the hypothesis that the Kr⁺/⁻ mutation is a hypomorphic allele of the mafB gene. The Kr⁺/⁻ mutation, a missense mutation at a conserved asparagine residue in the MaB DNA binding domain, is therefore likely to retain partial function in the absence of DNA binding, possibly through interactions with other bZip transcription factors or cofactors. One recent report indeed
FIG. 6. F4/80 expression is suppressed in MafB-deficient macrophages. (A and C) Reduction of the F4/80-positive population and the increase in the GFP⁺ F4/80⁺ population in neonatal peripheral blood and spleen cells. The Mac-1-positive macrophage population was unaffected (C). (B) F4/80 and Mac-1 expression profiles in GFP-positive (MafB-expressing) macrophages. A total of 0.6% of GFP-positive cells (red gates in B) from each genotype were analyzed. In the mafB⁻/⁻ cells, an increase of F4/80⁻ Mac-1⁻ premature macrophages and a reduction of F4/80⁺ Mac-1⁺ mature macrophages were observed. Seven independent animals of each genotype were analyzed. The data were recorded as means ± SD. The statistical significance of differences was determined by Student’s t test. WT, wild type.
showed that \( kr^{ENV} \) was able to form dimers with wild-type MafB and trans-activate a reporter gene (40). The \( mafB^{-/-} \) mice reported here, representing a complete loss of MafB function, poorly activate the nephrin, podocin, and CD2AP genes, demonstrating that they are clearly under the (direct or indirect) influence of MafB in developing podocytes of the kidney.

We also observed increased NEPH1 expression in \( mafB^{-/-} \) neonatal kidneys. NEPH1 is a molecule related to nephrin and belongs to a family of three closely related proteins that interact with the C-terminal domain of podocin (11, 43). NEPH1 forms a heterooligomeric complex with nephrin in the plane of the membrane that appears to interact across the foot process intercellular junction (3). These data suggest that the inhibition of podocin and nephrin might induce compensatory NEPH1 expression for the maintenance of the slit diaphragm structure in \( mafB^{-/-} \) neonatal kidneys.

Deficiencies in nephrin, podocin, or CD2AP commonly lead...
to nephrosis and massive proteinuria, so the observed perinatal anuria might not simply represent a reduction in the expression of these genes. Podocalyxin is a podocyte membrane protein with a highly charged cytoplasmic tail. On mature podocytes, podocalyxin is essential for foot process formation and for maintaining the spacing between the interdigitating foot processes by charge repulsion (41). In podocalyxin mutant mice, the reduced permeability of the glomerular filter due to increased tight junctions between adjacent podocytes leads to the observed perinatal anuria phenotype (12). Despite a similar anuric phenotype detected in mafB mutant newborns, podocalyxin expression was barely affected in the mafB mutant kidney (Fig. 3A), suggesting that the phenotypes observed in the mafB and podocalyxin mutants lie in different biochemical and genetic pathways. It is nonetheless possible that MafB regulates other (undefined) genes that are required to preserve glomerular permeability.

To our surprise, we also observed severe proximal and distal tubular dysgenesis, which are equally likely to be the direct cause of the observed anuric phenotype in the mafB−/− animals. MafB is not expressed in renal tubuli as reflected by GFP immunohistochemistry, in accord with previously reported in situ hybridization studies (18, 39). Despite this lack of mafB expression, TUNEL staining clearly demonstrated significantly increased apoptotic cell death of renal tubular epithelial cells in mafB−/− kidneys, leading to the conclusion that tubular apoptosis is a non-cell-autonomous effect of the MafB deficiency.

In early morphogenesis of the kidney and urinary tract, the elongation and branching of the nephric duct are promoted by a number of humoral factors secreted from the metanephric mesenchyme, including primitive glomerular epithelial cells (6, 32). Although MafB expression is first detected at a relatively late stage of kidney development, it is intriguing to hypothesize that the mature podocyte may secrete a humoral factor that is required for the maintenance of tubular epithelial cell growth and differentiation. It may thus be particularly germane that we also observed significantly increased expression of c-Myc, a well known apoptosis inducer, in mafB−/− kidneys (Fig. 3A). Autosomal dominant polycystic kidney disease, one of the most frequent human genetic disorders that constitutes a major cause of end-stage renal failure worldwide, is associated with increased levels of c-Myc (9). Indeed, a model for the human disease, the autosomal dominant polycystic kidney disease transgenic mouse, was generated by targeted overexpression of c-Myc to the renal tubular epithelium, and this mouse displays severe renal anomalies characteristic of polycystic kidney disease (47). Thus, we suggest that the tubular dysgenesis and cyst formation observed in the mafB−/− kidney may be mediated by c-Myc-induced apoptosis. Since their GFP fluorescence will enable us to collect podocytes from mafB−/+ or mafB−/− kidneys by flow cytometry, we may be able to identify candidate target genes of MafB, including this hypothetical tubular growth factor.

Although it has been shown that MafB is expressed selectively in myelomonocytic cells and plays key roles in differentiating macrophages, a similar demonstration has never been confirmed in vivo. In this report, we show that MafB was expressed in a subpopulation of differentiated macrophages. Flow cytometric analysis of fetal liver cells revealed that MafB was expressed in distinct subsets of differentiated macrophages that expressed Mac-1 and F4/80. In mafB−/− mice, a substantial proportion of fetal liver, neonatal spleen, and peripheral macrophages failed to express F4/80, indicating that MafB is required for the full differentiation of this distinct subset of macrophages.

F4/80, a member of the epidermal growth factor-TM7 (transmembrane-domain) family, has been established as a specific cell surface marker for murine macrophages (2, 31). The precursor of tissue macrophages, the blood monocyte, is known to express less F4/80 than its mature counterparts (14). Although the function of F4/80 in macrophages remains unknown, one recent report demonstrated that F4/80 expression in APCs was essential for the induction of immunological tolerance by generating antigen-specific efferent regulatory T (Treg) cells that suppress antigen-specific immunity (28). During peripheral immunotolerance induction, F4/80+ APCs first appear in the bloodstream and then circulate to the splenic marginal zone, where they interact with NK-T cells, which in turn generate Treg cells. Moreover, F4/80 expression in blood APCs is essential for Treg induction, presumably through the F4/80-mediated direct interaction between the two different cell types (28). In the present study, we showed that MafB is essential for F4/80 expression, especially in nonadherent macrophages. Given the importance of F4/80 expression in circulating APCs for the induction of peripheral immunotolerance, it is intriguing to hypothesize an essential role for MafB as a regulator of immunological tolerance by maintaining F4/80 expression in circulating tolerogenic APCs.

Assuming a direct regulatory interaction between MafB and F4/80, we examined the sequence of the mouse F4/80 proximal promoter region. One site identified is a well-conserved half-site MARE (actual sequence, CTTTCTGCTGAGTTGCCA, where the underlined letters are the consensus MARE half-site) from −130 to −113 bp of the F4/80 promoter. A recent report showed that 5′ AT-rich MARE half-sites retain Maf binding site potency as strong as the classical palindromic MARE (49). Moreover, it was reported recently that a serial deletion study of that promoter revealed strong enhancer activity between −187 and −117 bp, which includes the predicted MARE element (37). It is therefore quite plausible that MafB binds to the F4/80 promoter at that site to stimulate F4/80 transcription in mature macrophages.

In summary, the results presented here demonstrate an essential role for MafB in the development of the mature glomerulus, in renal tubules (unexpectedly), and in F4/80-expressing macrophages. Further analysis to determine the biological consequences of diminished F4/80 expression in the MafB-deficient macrophages, especially in adolescent animals, is ongoing.

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