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Tie2-Cre–Induced Inactivation of a Conditional Mutant *Nf1* Allele in Mouse Results in a Myeloproliferative Disorder that Models Juvenile Myelomonocytic Leukemia

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ABSTRACT

Neurofibromatosis type one (NF1) is a common genetic disorder affecting 1:4000 births and is characterized by benign and malignant tumors. Children with NF1 are predisposed to juvenile myelomonocytic leukemia. The *Nf1* gene encodes neurofibromin, which can function as a Ras GTPase-activating protein. Neurofibromin deficiency in mice leads to mid-gestation lethality due to cardiovascular defects. We have previously shown that conditional inactivation of *Nf1* using Tie2-Cre recapitulates the heart defects seen in $Nf1^{-/-}$ embryos. Tie2-Cre transgenic mice express Cre recombinase in all endothelial cells. Here, we show that Tie2-Cre–mediated deletion of *Nf1* also leads to excision of *Nf1* in the hematopoietic lineage. Surviving mice exhibit a myeloproliferative disorder similar to juvenile myelomonocytic leukemia seen in NF1 patients. These mice provide a useful model to study neurofibromin deficiency in hematopoiesis. Furthermore, defects in Tie2-Cre–expressing progenitors that result in heart and blood defects suggest that related heart and blood disorders in NF1 and other syndromes represent disorders of the hemangioblast. **(*Pediatr Res* 55: 581–584, 2004)**

Abbreviations

CBC, complete blood counts
FACS, fluorescence-activated cell sorting
H&E, hematoxylin and eosin
JMML, juvenile myelomonocytic leukemia
LOH, loss of heterozygosity
MPD, myeloproliferative disorder
NF1, neurofibromatosis type 1
GAP, ras GTPase-activating protein
WBC, white blood cell

NF1 is a common genetic disorder in which affected individuals are predisposed to tumors of both neural crest and non-neural crest origin. Cardiovascular defects and a predisposition to leukemia contribute to NF1. The incidence of JMML is increased 200- to 500-fold in children with NF1 (1). The *Nf1* gene encodes neurofibromin, a large protein that can function as a GAP. Neurofibromin functions as a tumor suppressor and tumor cells from NF1 patients exhibit LOH at the *Nf1* locus. Attempts to generate mouse models of NF1 have been hampered by the fact that inactivation of *Nf1* in mice leads to mid-gestation lethality due to developmental cardiovascular defects (2–4). However, mice heterozygous for a targeted disruption of *Nf1* develop tumors and are predisposed to myeloid leukemia. This occurs at low frequency after 1.5–2 y and is accompanied by loss of the normal *Nf1* allele in somatic cells (3). A "floxed" allele of *Nf1* has been generated, allowing for tissue-specific inactivation (5). Conditional inactivation of *Nf1* in the Schwann cell lineage bypasses the early embryonic lethality and leads to neural crest–derived tumors similar to those seen in humans with NF1 (6). $Nf1^{-/-}$ fetal liver cells exhibit hypersensitivity to granulocyte-macrophage colo-

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ny-stimulating factor (GM-CSF) *in vitro*, similar to cells from JMML patients. Furthermore, lethally irradiated wild-type mice reconstituted with *Nf1*$^{-/-}$ fetal liver cells develop myeloid leukemia characterized by over-proliferation of mature myeloid cells that infiltrate the liver and spleen (7, 8). This model faithfully models JMML seen in NF1 patients, though the complexity of *Nf1*$^{-/-}$ fetal cell isolation and subsequent bone marrow transplantation limit utility.

We have recently used a Cre/lox approach to show that endothelial, but not neural crest restricted inactivation of *Nf1* leads to embryonic cardiovascular defects (9). To inactivate *Nf1* in endothelium, we used Tie2-Cre transgenic mice crossed to a conditional allele of *Nf1*. Tie2-Cre mice express Cre recombinase in all endothelial cells as well as in the hematopoietic lineage (10, 11). The conditional allele of *Nf1* (Nf1^{flox}) contains exons 31 and 32 that are *f*lanked by *lox* P sequences (producing a "floxed" allele), enabling deletion by Cre-mediated recombination (5). We crossed Tie2-Cre mice with *Nf1*$^{+/-}$ mice to obtain *Nf1*$^{+/-}$;Tie2-Cre offspring, and then we crossed these mice with Nf1$^{flox/flox}$ mice to obtain Nf1$^{flox/-}$;Tie2-Cre mice. A subset of these Nf1$^{flox/-}$;Tie2-Cre mice died at mid-gestation of heart defects resembling homozygous null embryos. These defects include thickening of the cardiac valves and outflow tract abnormalities. We demonstrated that neurofibromin is required in endothelial cells to regulate ras signaling. Increased ras signaling in neurofibromin-deficient endothelial cells leads to enhanced epithelial-mesenchymal transformation and over-proliferation in the endocardial cushions both *in vitro* and *in vivo* (4, 9). During our analysis of the embryonic cardiac phenotype, we observed animals that survived embryogenesis and were viable. Further analysis of Tie2-Cre transgenic mice revealed Cre recombinase activity in the hematopoietic as well as endothelial lineages, consistent with recent reports (11). Here, we show that the floxed *Nf1* allele is completely recombined by Tie2-Cre in the blood. Consistent with a requirement for neurofibromin function in normal hematopoiesis, these mice exhibit a MPD at 3 mo and exhibit defects that closely resemble JMML. The development of this and other Cre/lox mouse models of leukemia in NF1 will be useful for testing potential therapeutics as well as screening for genetic modifiers.

METHODS

Mouse breeding and genotyping. We isolated genomic DNA from peripheral blood samples using the Gentra Systems DNA isolation kit (Minneapolis, MN, U.S.A.). We genotyped *Nf1*$^{+/-}$, Nf1$^{flox/-}$, and Tie2-Cre, as described previously (9). The institutional animal care and use committee of the University of Pennsylvania approved all animal protocols.

Analysis of blood parameters. We obtained approximately 250 μL of blood from each mouse through retroorbital venipuncture and analyzed samples using a Hemavet@850 machine (CDC Technologies Inc., Oxford, CT, U.S.A.).

FACS analysis. We harvested and stained peripheral blood, spleen, and bone marrow cells from wild-type and Nf1$^{flox/-}$;Tie2-Cre mice with FITC-conjugated anti-Gr1 and phycoerythrin (PE)-conjugated anti-Mac1 antibodies (gift from Diana Ramirez-Bergeron). We analyzed cells with a Becton-Dickinson FACScalibur and CellQuest software (BD Biosciences, San Jose, CA, U.S.A.).

Histology. Fixation and immunohistochemistry protocols are available at http://www.uphs.upenn.edu/mcrc. We fixed spleens in 10% neutral buffered formalin. Ten micormolar sections were stained with H&E. We fixed bone marrow from the femur as above, decalcified, and stained with H&E.

RESULTS

Tie2-Cre efficiently recombines the floxed* Nf1 *allele in most hematopoietic cells. It has been shown previously that enhancer elements from the *Tie2* gene are capable of directing tissue specific expression in both endothelium and hematopoietic cells (11). We sought to determine whether Tie2-Cre had recombined the floxed *Nf1* allele in blood from Nf1$^{flox/-}$;Tie2-Cre animals. We used two PCR assays to analyze the status of the floxed *Nf1* allele. The first reaction was used to amplify the recombined allele. The presence of a PCR product in this reaction indicates that the floxed *Nf1* allele has been recombined by Cre, and thus the *Nf1* gene product inactivated. A second reaction was used to amplify the nonrecombined allele. We used this reaction to assess the efficiency of Cre-mediated recombination. The absence of a PCR product in this reaction indicates that the floxed *Nf1* allele has been completely recombined. As shown in Figure 1*A*, PCR failed to detect the presence of a nonrecombined floxed *Nf1* allele in genomic DNA isolated from peripheral blood from 3-mo-old Nf1$^{flox/-}$;Tie2-Cre mice, whereas this allele was readily detected in Nf1$^{flox/-}$ mice that did not carry the Tie2-Cre transgene. The recombined Nf1$^{flox/-}$ allele was easily detected in Nf1$^{flox/-}$;Tie2-Cre samples. Hence, Nf1$^{flox/-}$;Tie2-Cre mice that survive embryogenesis lack a functional *Nf1* gene in blood.

Leukocytosis and splenomegaly in Nf1$^{flox/-}$;Tie2-Cre mice. Before 3 mo of age, Nf1$^{flox/-}$;;Tie2-Cre mice appeared healthy and active. As shown in Figure 1*B*, CBC revealed elevated WBC counts compared with wild-type littermates. Hb and platelet counts were similar to wild type (data not shown). The WBC counts we observe are similar to those observed when bone marrow from lethally irradiated wild-type mice are reconstituted with *Nf1*-deficient fetal liver cells (8). In four mice examined serially, WBC numbers continued to increase in Nf1$^{flox/-}$;Tie2-Cre but not wild-type mice at 6 mo and 9 mo. We observed four Nf1$^{flox/-}$;Tie2-Cre mice with WBC counts $>$20,000 cells/μL at 6 mo and three Nf1$^{flox/-}$;Tie2-Cre mice with WBC counts $>$60,000 cells/μL at 9 mo. Peripheral blood smears performed on these mice also revealed the presence of an increased number of monocytes (Fig. 1*C*) and immature granulocytes (Fig. 1*D*), consistent with clinical manifestations of JMML present in NF1 patients (Fig. 1, *C* and *D*). Quantitative analysis with FACS demonstrated a 15-fold increase in Gr1$^+$/Mac1$^+$ cells (0.02% in wild type *versus* 0.30% in Nf1$^{flox/}$;Tie2-Cre) (Fig. 1, *E* and *F*). Gr1 is expressed on granulocytes. Mac1 is expressed on both granulocytes and macrophages. Increases in the numbers of Mac1$^+$/Gr1$^-$ and Mac1$^+$/Gr1$^+$ cells confirm the expansions of macrophages and granulocytes. By 9 mo, animals with the most severe elevation of WBC

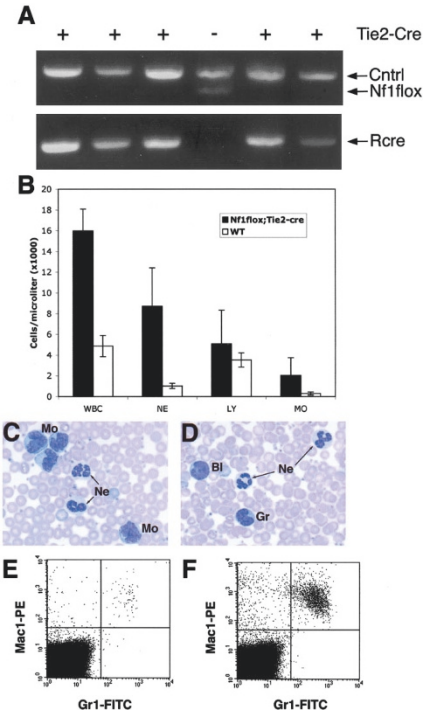

Figure 1. Leukocytosis in $Nf1^{flox/-}$;Tie2-Cre mice. (*A*) PCR was used to detect the presence of a recombined $Nf1^{flox}$ allele (*Rcre*) in genomic DNA isolated from either $Nf1^{flox/+}$;Tie2-Cre or wild-type peripheral blood. The completion of Cre-mediated recombination was demonstrated by the absence of the $Nf1^{flox}$ (*Nf1flox*) band. This band was absent from all Cre-positive (+) lanes and present in the Cre-negative (−) lane, indicating complete excision of the floxed *Nf1* allele in blood from all Cre-expressing animals and presence of the nonrecombined allele in blood from animals lacking Cre expression. PCR amplification of the nonfloxed (*Cntrl*) allele from all samples is shown as a control. (*B*) CBC performed on peripheral blood from 3-mo- ld $Nf1^{flox/-}$;Tie2-Cre or wild-type (*WT*). The number of WBC and neutrophils (*NE*) were significantly elevated in $Nf1^{flox/-}$;Tie2-Cre mice. Values represent the mean of five counts ± SD. Peripheral blood smears from $Nf1^{flox/-}$;Tie2-Cre (*C, D*) showed increased numbers of monocytes (*Mo*) and neutrophils (*Ne*); occasional granulocytic precursors such as myelocytes/metamyelocytes (*Gr*) and myeloblasts (*Bl*) were also seen. (*E, F*) Increased percentage of $Mac1^{+}/Gr1^{-}$ and $Mac1^{+}/Gr1^{+}$ cells in peripheral blood from a 9-mo-old $Nf1^{flox/-}$;Tie2-Cre mouse (*F*) compared with a wild-type littermate (*E*).

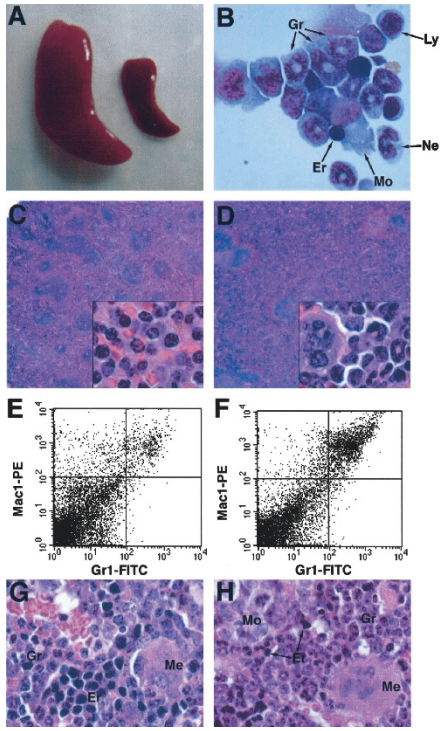

Figure 2. Splenomegaly and myeloproliferative disorder in $Nf1^{flox/-}$;Tie2-Cre mice. (*A*) Massively enlarged spleen (eight times normal weight) from $Nf1^{flox/-}$;Tie2-Cre mouse (*left*) compared with spleen from wild-type mouse (*right*). (*B*) Wright-stained cytospin preparation of cells from enlarged $Nf1^{flox/-}$;Tie2-Cre spleen shows the presence of neutrophils (*Ne*) and metamyelocytes/myelocytes (*Gr*) with occasional monocytes (*Mo*), nucleated red blood cells (*Er*), and lymphocytes (*Ly*). (*C, D*) H&E-stained sections of wild-type (*C*) and $Nf1^{flox/-}$;Tie2-Cre (*D*) spleens (250× magnification) with higher magnification insets of the red pulp (1000×). The $Nf1^{flox/-}$;Tie2-Cre spleen has an expansion of the red pulp by a cellular infiltrate composed of myeloid cells and megakaryocytes. (*E, F*) Increased percentage of $Gr1^{+}/Mac1^{+}$ cells in spleen cells from a 9-mo-old $Nf1^{flox/-}$;Tie2-Cre mouse (*F*) compared with a wild-type littermate (*E*). (*G, H*) H&E-stained sections of wild-type (*G*) and $Nf1^{flox/-}$;Tie2-Cre (*H*) bone marrows, (1000× magnification). The $Nf1^{flox/-}$;Tie2-Cre marrow is hypercellular with decreased number of erythroid precursors (*Er*), increased numbers of maturing granulocytic cells (*Gr*) and megakaryocytes (*Me*), and numerous small aggregates of monocytes (*Mo*).

counts appeared lethargic and one animal died, presumably due to severe leukemia. The rest were killed for autopsy. Consistent with a MPD, the spleens were massively enlarged compared with wild-type littermates (Fig. 2*A*). Cytospin preparations of cells from $Nf1^{flox/-}$;Tie2-Cre spleens showed increased numbers of maturing monocytic/granulocytic cells (Fig. 2*B*). These cells infiltrate and expand the splenic red pulp in H&E-stained sections of the $Nf1^{flox/-}$;Tie2-Cre spleens (Fig. 2, *C* and *D*). Similar to the peripheral blood, FACS analysis revealed an increased percentage of $Gr1^{+}/Mac1^{+}$ cells in spleen cells (10% in wild type *versus* 25% in $Nf1^{flox/-}$;Tie2-Cre) (Fig. 2, *E* and *F*). We also observed a similar, though milder, increase in the bone marrow (46% in wild type *versus* 60% in $Nf1^{flox/-}$;Tie2-Cre). H&E sections of the $Nf1^{flox/-}$;Tie2-Cre bone marrow showed hypercellularity with myeloid and megakaryocytic hyperplasia (Fig. 2, *G* and *H*). Hence, inactivation of *Nf1* in blood, using Tie2-Cre, is sufficient to produce a MPD similar to JMML seen in some NF1 patients.

DISCUSSION

We have developed a NF1 mouse model of JMML using Tie2-Cre to conditionally inactivate the *Nf1* gene. A previous mouse model of JMML relied on transplanting $Nf1^{-/-}$ fetal liver cells into lethally irradiated wild-type hosts. The $Nf1^{flox/-}$;Tie2-Cre mice we describe here represent a significant advance and provide a tractable mouse model for the study of JMML, for the testing of experimental therapeutics, and screening for genetic modifiers.

Loss of *Nf1* in hematopoietic cells has been shown to lead to growth factor independent growth, GM-CSF hypersensitivity, and p21-ras activation (7, 8). These features are also seen in hematopoietic cells from JMML patients (1, 12). We did not directly test these properties in cells from $Nf1^{flox/-}$;Tie2-Cre mice. Future work will be needed to examine the similarities and differences between $Nf1^{flox/-}$;Tie2-Cre mice and JMML. Nevertheless, we have shown that $Nf1^{flox/-}$;Tie2-Cre mice lack a functional *Nf1* gene in hematopoietic cells. Therefore, it is

likely that cells from these mice will behave similarly to other *Nf1*-deficient hematopoietic cells.

An additional limitation to our JMML model should be pointed out. Some offspring from crosses to generate $Nf1^{flox/-}$;Tie2-Cre mice succumb to cardiovascular defects during midgestation, due to *Nf1* inactivation in endothelium. Therefore, to obtain sufficient numbers of $Nf1^{flox/-}$;Tie2-Cre adult mice for future studies including testing of therapeutics may require an alternative breeding strategy. One possibility would be to harvest bone marrow and spleen from surviving $Nf1^{flox/-}$;Tie2-Cre mice and transplant these cells into irradiated wild-type hosts. This procedure is straightforward and does not require performing timed matings and fetal liver cell isolation, which would be required if $Nf1^{-/-}$ embryos were used as the donor. Alternatively, the floxed *Nf1* allele could be crossed onto different genetic backgrounds, which might alter the penetrance of the cardiovascular phenotype upon endothelial-specific inactivation.

Our results presented here and our previous work demonstrate that inactivation of *Nf1* in Tie2-Cre–expressing precursors leads to cardiovascular defects and to leukemia. These abnormalities are remarkably similar to those seen in a subset of patients with NF1. In addition to being predisposed to JMML, NF1 patients have an increased incidence of pulmonic stenosis (13). Interestingly, cardiac valvular hyperplasia and JMML are also associated with Noonan syndrome caused by mutation in *PTPN11*, the gene encoding the protein-tyrosine-phosphatase Shp2 (14, 15). Furthermore, inactivation of the mouse *Shp2* gene leads to cardiovascular defects similar to *Nf1* mutants, and this phenotype is enhanced by loss-of-function mutations in the epidermal growth factor receptor (16). The *Nf1* gene product, neurofibromin, has been implicated as a regulator of ras activation by epidermal growth factor signaling. Future work will be aimed at elucidating the molecular details involving how neurofibromin, EGF receptor, and Shp2 function together to coordinate growth factor signaling involved in cardiovascular and hematologic development.

There is emerging evidence suggesting common origins of cardiovascular and blood components. Mouse knockout studies have identified genes required for both vascular and blood development (17–20). Recent studies indicate that endothelial cells and blood derive from a common precursor, the hemangioblast (21). It is likely that defects of the hemangioblast account for the coincidence of heart and blood defects in $Nf1^{flox/-}$;Tie2-Cre mice, in human NF1 patients, and in other human syndromes. Future work will be necessary to determine whether cells from $Nf1^{flox/-}$;Tie2-Cre mice are defective in hemangioblast colony-forming assays. Also, *Nf1* is likely to be inactivated in blood vessels of surviving $Nf1^{flox/-}$;Tie2-Cre mice. Although we do not observe gross vascular phenotypes in these mice, a more careful examination is warranted.

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