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Geminin deletion from hematopoietic cells causes anemia and thrombocytosis in mice

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HSCs maintain the circulating blood cell population. Defects in the orderly pattern of hematopoietic cell division and differentiation can lead to leukemia, myeloproliferative disorders, or marrow failure; however, the factors that control this pattern are incompletely understood. Geminin is an unstable regulatory protein that regulates the extent of DNA replication and is thought to coordinate cell division with cell differentiation. Here, we set out to determine the function of Geminin in hematopoiesis by deleting the Geminin gene (*Gmnn*) from mouse bone marrow cells. This severely perturbed the pattern of blood cell production in all 3 hematopoietic lineages (erythrocyte, megakaryocyte, and leukocyte). Red cell production was virtually abolished, while megakaryocyte production was greatly enhanced. Leukocyte production transiently decreased and then recovered. Stem and progenitor cell numbers were preserved, and *Gmnn*−/− HSCs successfully reconstituted hematopoiesis in irradiated mice. CD34+ *Gmnn*−/− leukocyte precursors displayed DNA overreplication and formed extremely small granulocyte and monocyte colonies in methylcellulose. While cultured *Gmnn*−/− megakaryocyte-erythrocyte precursors did not form erythroid colonies, they did form greater than normal numbers of megakaryocyte colonies. *Gmnn*−/− megakaryocytes and erythroblasts had normal DNA content. These data led us to postulate that Geminin regulates the relative production of erythrocytes and megakaryocytes from megakaryocyte-erythrocyte precursors by a replication-independent mechanism.

Introduction

Stem cells maintain adult tissues by replacing cells that are lost through normal attrition, damage, or disease. Stem cell division patterns are unusual in that they produce 2 different types of daughter cells. Some daughters maintain their identity as stem cells, while others enter a pathway of terminal differentiation and ultimately become mature somatic cells. Stem cell division and differentiation must be carefully balanced in order to supply the proper numbers and proportions of mature cells. The factors that control this balance are incompletely understood. In most cases, it is not even known whether stem cell division is symmetric, producing either 2 stem cells or 2 differentiating cells, or if it is asymmetric, generating 1 stem cell and 1 differentiating cell. One model proposes that the choice between self renewal and terminal differentiation is stochastic (i.e., random), while another proposes that the choice is driven by cytokines in response to environmental stimuli (1, 2).

The unstable regulatory protein Geminin (Gmnn) is thought to control patterns of cell division and differentiation (3, 4). Two different molecular functions have been described for Geminin. One function is to limit the extent of DNA replication to 1 round per cell cycle by binding and inhibiting the essential replication factor Cdt1 (5–7). Geminin is destroyed by ubiquitin-dependent proteolysis during mitosis, allowing for a new round of replication in the succeeding cell cycle. Overreplication is also suppressed by a redundant Geminin-independent mechanism: Cdt1 itself is destroyed by ubiquitin-dependent proteolysis when replication origins fire (8–11). Because of this redundancy, it is not known whether Geminin is absolutely required to prevent overreplication in all types of adult somatic cells.

In addition to regulating DNA replication, Geminin also affects cell differentiation in the central nervous system, the axial skeleton, and the eye. Using 2-hybrid assays, Geminin has been found to bind several different transcription factors in the Homeobox (*Hox*) and sine oculis families (12, 13). Geminin also binds 2 chromatin-remodeling proteins: Brg1, the ATPase subunit of a SWI/SNF remodeling complex; and Scmh1, a component of polycomb repressive complex 1 (13, 14). Overexpression and knockdown of Geminin in cultured cells and in various embryonic systems has suggested that Geminin influences cell fate by inhibiting the function of these proteins. Because it regulates both the cell cycle and tissue-specific transcription factors, it has been postulated that Geminin somehow coordinates cell division with cell differentiation.

This hypothesis has been tested by deleting Geminin from model organisms. *Caenorhabditis elegans* embryos that have been treated with Geminin RNAi grow to adulthood, but approximately 20% of them display cytological abnormalities in their germ cells and are sterile (15). A similar proportion of geminin (*RNAi*) worms show anaphase chromosome bridges in intestinal epithelial cells, suggesting a defect in DNA replication. *Gmnn*−/− *Drosophila* embryos die at larval stages (16). They also show anaphase chromosome bridges, and an extended period of DNA replication has been detected in ovarian follicle cells. Geminin-deficient *Xenopus* embryos stop dividing after the 13th cleavage division and disintegrate during gastrulation (17). Their primary defect is overreplication of their DNA, which activates the DNA replication checkpoint and arrests the cells in G_2 phase. *Gmnn*−/− mouse embryos also stop dividing at the early blastula stage, as soon as the maternal stockpile of Geminin is exhausted (18, 19). At the time of the arrest, their cells have a greater DNA content than normal. Intriguingly, all the blastomeres prematurely differentiate as trophoblast cells and none show markers of embryonic stem (ES) cells. Heterozygous *Gmnn*+/− mice are phenotypically

normal. Taken together, these studies provide good evidence that Geminin deficiency disrupts DNA replication and causes cell-cycle abnormalities. Geminin's effects on cell differentiation have been difficult to assess in these systems because the population of differentiating cells is small and nonuniform.

To more rigorously examine the role of Geminin in regulating cell division and differentiation, we have developed a mouse model with a conditional floxed Geminin allele and deleted the protein from hematopoietic cells using an interferon-inducible *Cre* driver. The hematopoietic system is ideal for these studies because the stem cells have been well defined, their pattern of differentiation has been mapped out, and the numbers of different types of cells in the differentiation tree can be quantified by staining with cell-type–specific antibodies. We found that Geminin deletion caused only subtle abnormalities in DNA replication but had profound effects on the pattern of cell differentiation.

Results

Geminin is expressed in stem cells, erythroid precursors, and megakaryocytes. First, we measured the abundance of Geminin mRNA in different FACS-sorted hematopoietic cell populations by quantitative real-time PCR (RT-PCR). Geminin is expressed in the erythrocyte/megakaryocyte lineage, including megakaryocyte-erythrocyte progenitors (MEPs), $CD71^+Ter119^+$ erythroblasts, and mature $CD41^+$ megakaryocytes (Figure 1A). Geminin is also broadly expressed in most progenitor cells, including common myeloid progenitors (CMPs), granulocyte monocyte progenitors (GMPs), and Lin⁻Sca1⁺c-Kit⁺ (LSK) stem cells. LSK cells represent the most undifferentiated population and include the long-term HSCs that are able to reconstitute hematopoiesis in an irradiated animal (20). In contrast, mature marrow $Gr1^+Mac1^+$ wbc and peripheral blood leukocytes had very low levels of Geminin expression.

Construction of mice with a conditional targeted deletion of Geminin. The mouse genome contains a single Geminin gene that is composed of 7 exons (Figure 1B). We constructed a conditional Geminin floxed allele (*Gmnn*ᶠˡ) by flanking exons 5, 6, and 7 with *loxP* sites. These exons encode Geminin's dimerization domain and its binding site for Cdt1, both of which are essential for the protein's biological function (21). We inserted the *Gmnn*ᶠˡ allele into the mouse genome through homologous recombination in ES cells (Figure 1C), then generated a strain of *Gmnn*ᶠˡ/⁺ mice by injecting the targeted ES cells into mouse blastocysts. *Gmnn*ᶠˡ/⁺ and *Gmnn*ᶠˡ/ᶠˡ mice are completely viable and fertile (not shown).

To specifically delete Geminin from hematopoietic cells, *Gmnn*ᶠˡ/ᶠˡ mice were crossed to transgenic mice that express *Cre* recombinase from the interferon-responsive Mx1 promoter (22). To induce *Cre* expression, *Mx1-Cre/Gmnn*ᶠˡ/ᶠˡ mice and littermate controls were injected with polyinosine-polycytosine (pIpC), which mimics a viral infection. With this protocol, *Cre*-mediated recombination through the *loxP* sites efficiently excises exons 5, 6, and 7 from the Geminin gene and generates a Geminin-null allele (*Gmnn*ᐃ) (Figure 1D). RT-PCR of total marrow RNA showed that *Cre* induction brings about a 95%–99% reduction in the amount of *Gmnn* mRNA in marrow cells (Figure 1E). A time course demonstrated that Geminin RNA has largely disappeared by 48 hours after the first dose of pIpC (not shown). To document loss of the Geminin protein, we raised an antibody against mouse Geminin in rabbits. The antibody recognizes approximately 20 kDa protein on immunoblots that matches the predicted molecular weight of Geminin (23.3 kDa) and comigrates with Geminin that has been translated in vitro (Figure 1F). This protein was not detected in *Mx1-Cre/Gmnn*ᶠˡ/ᶠˡ bone marrow cells after pIpC injection. This result attests to the specificity of the antibody and also documents disappearance of the Geminin protein after Cre induction. Finally, we crossed our *Mx1-Cre/Gmnn*ᶠˡ/ᶠˡ mice to *R26R-GFP* mice, which carry a loxP-flanked transcription/translation STOP cassette inserted between the ROSA promoter and the GFP coding sequence (23). *Cre*-mediated recombination deletes the STOP cassette and brings about GFP expression. We found that after pIpC injection, *Mx1-Cre(–) Gmnn*ᶠˡ/ᶠˡ marrow cells remained GFP negative, while 80–90% of *Mx1-Cre/Gmnn*ᶠˡ/ᶠˡ cells became GFP positive (Figure 1G). Control experiments demonstrate that the GFP-negative cells consist of both nonhematopoietic (CD45⁻) cells and hematopoietic (CD45⁺) cells that express low levels of Geminin mRNA by RT-PCR (Supplemental Figure 3).

Geminin deletion causes anemia and thrombocytosis. After pIpC injection, *Mx1-Cre/Gmnn*ᶠˡ/ᶠˡ mice showed poor growth compared with littermate controls and appeared sickly. Most of them (~90%) died within 3 weeks, while few of the pIpC-injected control *Mx1-Cre/Gmnn*ᶠˡ/⁺ mice died (Figure 2A). We have not determined the cause of death in pIpC-injected *Mx1-Cre/Gmnn*ᶠˡ/ᶠˡ mice (hereafter referred to as *Gmnn*ᐃ/ᐃ mice). At the time of sacrifice, 2 of them were found to have multiple bacterial abscesses in their livers with little surrounding inflammatory reaction, suggesting that they may have died of overwhelming infection (not shown). All *Gmnn*ᐃ/ᐃ mice exhibited pathological abnormalities in the peripheral blood, bone marrow, and spleen. *Mx1-Cre/Gmnn*ᶠˡ/ᶠˡ mice had about one-third as many marrow cells as controls (Figure 2B). The loss of Geminin strikingly affected the production of mature blood cells in all 3 hematopoietic lineages.

The erythrocyte and megakaryocyte lineages were the most severely affected. *Gmnn*ᐃ/ᐃ mice developed a profound and progressive anemia; their average red cell count was 40%–60% less than littermate controls (Figure 2C). Their red cell indices (MCV, MCH, and MCHC) were normal (not shown). Immunocytochemistry revealed a virtual absence of Ter119⁺ red cell precursors in *Gmnn*ᐃ/ᐃ bone marrow (Figure 2G). By flow cytometry, the numbers of all stages of erythroid precursors were greatly reduced compared with controls, including R1 $CD71^{hi}Ter119^{lo}$ proerythroblasts, R2 $CD71^{hi}Ter119^{hi}$ basophilic erythroblasts, R3 $CD71^{med}Ter119^{hi}$ polychromatophilic erythroblasts, and R4 $CD71^{lo}Ter119^{hi}$ orthochromatophilic erythroblasts and reticulocytes (Figure 2K). The loss of Ter119⁺ progenitors was also apparent in red pulp of the spleen, a common site of erythropoiesis in younger mice. The red pulp was paucicellular compared with controls, while the white pulp appeared normal (Figure 2J). *Gmnn*ᐃ/ᐃ mice had markedly elevated serum erythropoietin (EPO) levels compared with controls, consistent with their severe anemia (Figure 2N).

In striking contrast to the anemia, the number of platelets was vastly increased. *Gmnn*ᐃ/ᐃ mice had platelet counts that were 5–10 times those of control mice, up to $6 \times 10^6/\mu l$ (Figure 2D). The platelets appeared morphologically normal (Figure 2I) and had a normal volume by flow cytometry (not shown). They also activated normally in response to thrombin (not shown). Reflecting the high peripheral platelet count, both the marrow and spleen were densely infiltrated with large numbers of megakaryocytes (Figure 2H). Flow cytometry showed that the number of $CD41^+$ megakaryocytes in the marrow was increased by about 10-fold (Figure 2L). The number of Lin⁻Sca1⁻c-Kit⁺CD9⁺CD41⁺ megakaryocyte precursors was also increased (Supplemental
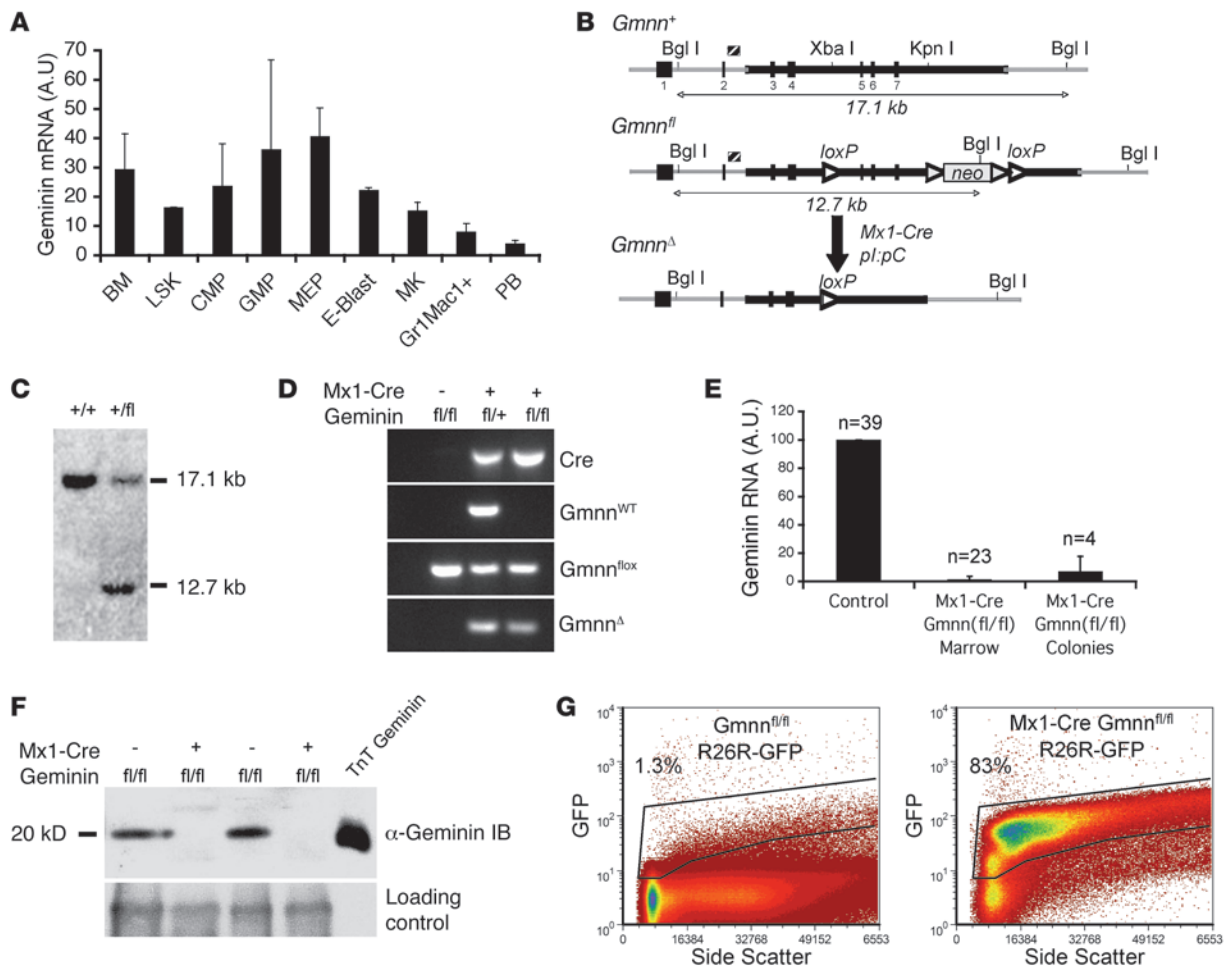
Figure 1
Targeted deletion of geminin from hematopoietic cells. (**A**) Geminin mRNA expression in sorted hematopoietic cell populations. CMP, common myeloid progenitor; GMP, granulocyte monocyte progenitor; MEP, megakaryocyte-erythrocyte progenitor; E-blast, erythroblast; MK, mega-karyocyte; Gr1+Mac1+, Gr1+Mac1+mononuclear cells; PB, peripheral blood. (**B**) Targeting strategy. The thick black line represents sequences encompassed by the targeting construct. (**C**) Southern blot of ES cell DNA showing homologous recombination of the targeting construct into the Geminin locus. The hatched box in **B** indicates the location of the hybridization probe. (**D**–**G**) Mice with the indicated genotypes were injected with pIpC, then analyzed. (**D**) PCR reaction showing deletion of exons 5–7 after pIpC injection. The first lane is a control with no DNA. (**E**) RT-PCR showing reduced Geminin expression pIpC-treated *Mx1-Cre/Gem*$^{fl/fl}$ marrow and colonies. (**F**) Immunoblot showing undetectable Geminin expression in *Mx1-Cre/Gmnn*$^{fl/fl}$ bone marrow cells after pIpC injection. TnT, in vitro transcription and translation. (**G**) Flow cytometry showing Mx1-Cre induction in the majority of marrow cells.

Figure 2; supplemental material available online with this article; doi:10.1172/JCI43556DS1). Serum thrombopoietin (TPO) levels were not significantly different in *Gmnn*$^{\Delta/\Delta}$ mice and littermate controls, indicating that the thrombocytosis was not being driven by TPO (Figure 2O).

The wbc were also affected by Geminin deletion, but not to the same extent as the erythroid cells and megakaryocytes. Control mice showed a sharp rise in peripheral wbc after pIpC injection, but *Gmnn*$^{\Delta/\Delta}$ mice showed a decrease (Figure 2, E and F). The number of neutrophils was more severely affected than the number of lymphocytes or monocytes. Near the nadir, flow cytometry showed that the number of mature Gr1+Mac1+ leukocytes in *Gmnn*$^{\Delta/\Delta}$ marrow was greatly decreased (Figure 2M). These differences were transient and normalized within 2–3 weeks (Figure 2, E and F). Furthermore, when *Gmnn*$^{\Delta/\Delta}$ marrow cells were transplanted to an

irradiated host to bypass the mortality, the number of peripheral Gr1+Mac1+ leukocytes normalized with time (see below).

Gmnn$^{\Delta/\Delta}$ *stem and progenitor cells show reduced proliferation in vitro.* Next we tested how Geminin deletion affects HSCs, multipotent progenitor cells, and committed precursor cells. By flow cytometry, the absolute number of LSK (Lin–Sca1+c-Kit+LSK) stem cells was increased in *Gmnn*$^{\Delta/\Delta}$ mice (Figures 3, A and B). The number of SLAM+ LSK cells, which represent a more definitive HSC population, was also preserved (Supplemental Figure 1). There was also a nonsignificant trend toward greater numbers of CMPs and MEPs but not GMPs (Figure 3B). Stem and progenitor cells constituted a greater fraction of the marrow in *Gmnn*$^{\Delta/\Delta}$ mice because of the loss of more mature types of cells (not shown). Because stem and progenitor cell populations are preserved, Geminin must affect hematopoiesis at later stages.
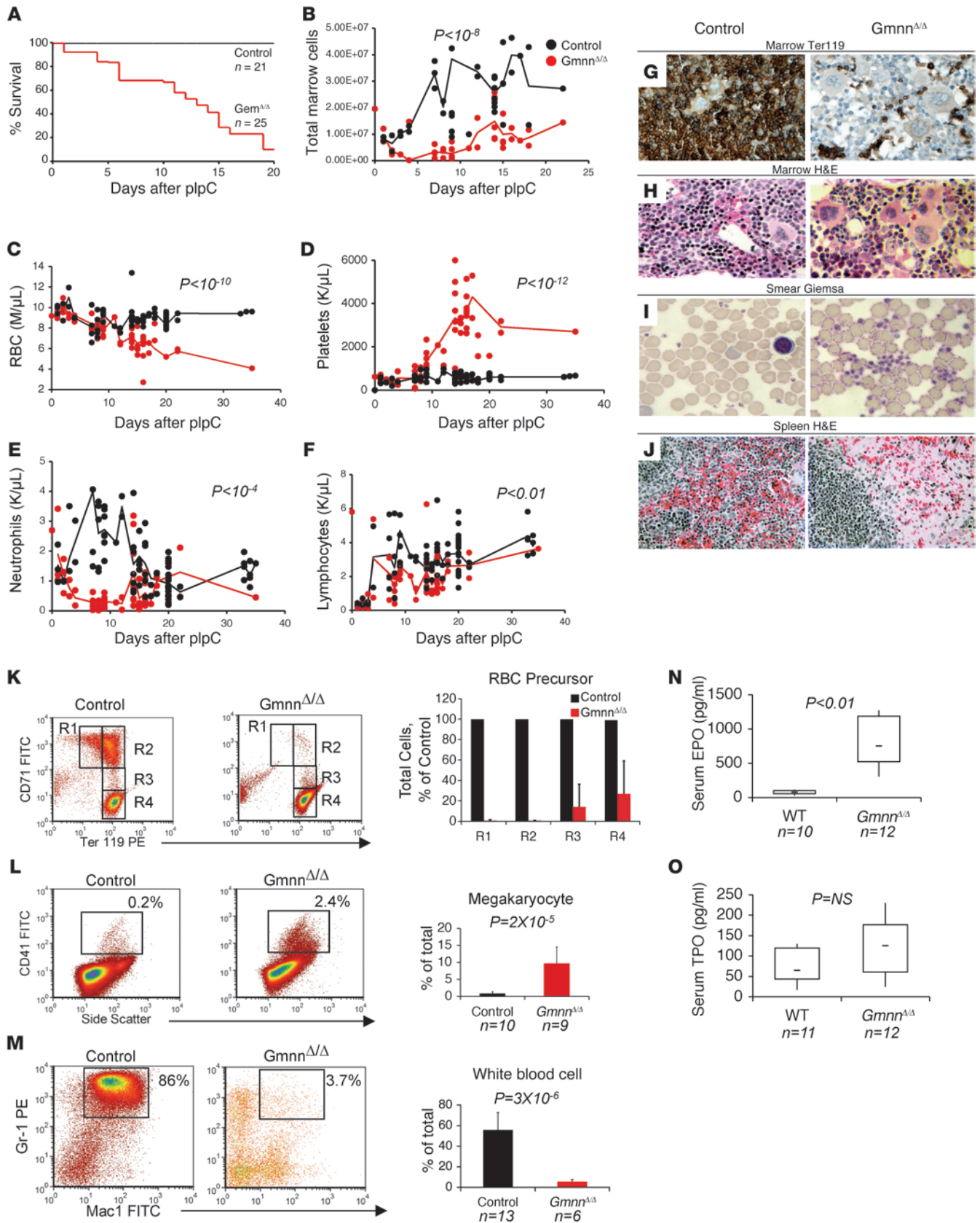
Figure 2
Geminin deletion affects all 3 hematopoietic lineages. (**A**) Kaplan-Meier survival curve of pIpC-treated *Mx1-Cre/Gem^fl/fl* mice (red) and control littermates (black). (**B**–**F**) Total marrow cells and complete blood counts after pIpC induction. Red circles, pIpC-treated *Mx1-Cre/Gem^fl/fl* mice; black circles, pIpC-treated control littermates. Each circle represents a single mouse, and the line graph represents the average value at each time point. (**G**–**J**) Bone Marrow sections (**G** and **H**), blood smears (**I**), and spleen sections (**J**) from pIpC-treated *Mx1-Cre/Gmnn^fl/fl* mice (*Gmnn^Δ/Δ*) and pIpC-treated control littermates (control). Original magnification, ×400 (**G**, **H**, and **J**); ×1000 (**I**). (**K**–**M**) Typical flow cytometry profiles of marrow cells from pIpC-treated *Mx1-Cre/Gem^fl/fl* mice (*Gem^Δ/Δ*) and pIpC-treated control littermates (control). Quantification of data from several mice is shown in the right panels. The rectangles indicate the mean value and the error bars indicate the standard deviation of the mean. (**N** and **O**) EPO levels (**N**) and TPO levels (**O**) for control and pIpC-treated *Mx1-Cre/Gem^fl/fl* mice. The horizontal bar indicates the median, box indicates 25th to 75th percentiles, and whiskers indicate extremes. *P* values calculated from Student's *t* test.

Although *Gmnn^Δ/Δ* stem and progenitor cells were maintained, they had a dramatically reduced ability to form colonies in methylcellulose (Figure 3, C and D). *Gmnn^Δ/Δ* cells did not form erythroid colonies at all, reflecting the loss of marrow Ter119⁺ erythroid precursor cells seen in vivo. They formed granulocyte and monocyte colonies, but both the total number of colonies and the size of individual colonies were severely reduced (Figure 3, C and D). The distribution of different types of myeloid colonies was normal. When the *Gmnn^Δ/Δ* myeloid colony cells were recovered from the plate, we found that the Geminin message was still undetectable by RT-PCR, indicating that the Geminin gene had been deleted from the committed precursors (Figure 1F).

In contrast, *Gmnn^Δ/Δ* marrow cells formed about 3 times as many megakaryocyte colonies as control cells (Figure 3E). To see if this was caused by an increased number of MEPs or by a change in their differentiation pattern, we plated a fixed number of purified MEPs from *Gmnn^Δ/Δ* and littermate control mice in methylcellulose. When plated in erythrocyte growth medium, we found that control MEPs produced 25 times as many erythroid colonies as *Gmnn^Δ/Δ* MEPs (Figure 3F). When plated in megakaryocyte growth medium, the ratio was reversed: *Gmnn^Δ/Δ* MEPs produced 3–4 times as many megakaryocyte colonies as control MEPs (Figure 3G). These data indicate that the thrombocytosis in *Gmnn^Δ/Δ* mice is due to an increased propensity of the MEPs to differentiate as megakaryocytes.

In summary, the ability of marrow cells to form colonies in culture paralleled the hematological abnormalities seen in intact mice. These results indicate that the abnormalities are intrinsic to the marrow cells themselves and not driven by changes in the hematopoietic microenvironment. They also indicate that, except for the megakaryocyte lineage, there is a defect in the ability of individual *Gmnn^Δ/Δ* stem and precursor cells to proliferate in vitro.

Geminin is not required for maintenance of HSCs. We next performed transplantation experiments to determine whether Geminin is required for self renewal and long-term maintenance of HSCs. Marrow cells from *Mx1-Cre/Gmnn^fl/fl/R26R-GFP/CD45.2* mice were injected into lethally irradiated WT *CD45.1* mice along with a small rescue dose of WT *CD45.1* marrow cells (Figure 4A). We also transplanted cells from *Mx1-Cre/Gmnn^fl/+/R26R-GFP/CD45.2* littermates as a control. We monitored engraftment by flow cytometry of peripheral wbc, using the CD41 polymorphism to

distinguish the transplanted CD45.2⁺ cells from the recipient's endogenous CD45.1⁺ cells (Figure 4B). Five weeks after transplantation, approximately 65% of the peripheral white cells were CD45.2⁺, indicating successful engraftment in all cases (Table 1). We then deleted the *Gmnn* gene by injecting the mice with pIpC following the same regimen as before. Some mice in each group were left uninjected as a control. After pIpC treatment, 70%–90% of the peripheral CD45.2⁺ cells became GFP positive, indicating successful Cre induction (Figure 4C). Immediately after induction, the mice transplanted with *Mx1-Cre/Gmnn^fl/fl* marrow showed a precipitous drop in the peripheral wbc count and the fraction of CD45.2⁺ cells decreased to 30%–40% of the total (Figure 4, D and E). After approximately 5 weeks, however, the white cell count normalized and the fraction of CD45.2⁺ cells returned to approximately 65%. Mice transplanted with the 3 different types of control marrow showed only a slight drop in the total white count and no significant change in the fraction of CD45.2 cells after pIpC injection. These results mirror what we observed before: that the white count drops transiently in response to Geminin deletion, then recovers.

The mice transplanted with *Mx1-Cre/Gmnn^fl/fl/R26R-GFP/CD45.2* marrow developed anemia and thrombocytosis after pIpC injection, while the control mice did not (Figure 4, F and G). This confirms that the hematological abnormalities associated with Geminin deletion are caused by a primary defect within the marrow cells themselves and not from a change in the hematopoietic microenvironment. The anemia and thrombocytosis were not as severe as in *Mx1-Cre/Gmnn^fl/fl* mice; they were probably ameliorated by the presence of normal CD45.1⁺ marrow cells. None of the mice transplanted with *Mx1-Cre/Gmnn^fl/fl* marrow and then injected with pIpC died during the course of the experiment.

The *Gmnn^Δ/Δ* CD45.2⁺ cells were maintained in the peripheral blood for 22 weeks after transplantation, indicating long-term survival of the Geminin-deficient stem cells and precursor cells (Figure 4D). After 22–24 weeks, the mice were sacrificed and the marrow was examined. In the mice that were transplanted with *Mx1-Cre/Gmnn^fl/fl* cells and injected with pIpC, CD45.2⁺ cells constituted 90%–100% of the marrow, confirming that the *Gmnn^Δ/Δ* cells were able to persist (Table 1). The CD45.2⁺ cells were purified by flow cytometry, and RT-PCR confirmed that these cells lacked Geminin RNA (Figure 4H). In contrast, the small number of remaining CD45.1⁺ cells and both the CD45.1⁺ and the CD45.2⁺ cells from controls had normal amounts of Geminin message. This confirms that the Geminin gene had been deleted from the stem and precursor cells and that the marrow had not become repopulated by rare CD45.2⁺ cells that did not delete Geminin. The absolute number of CD45.2⁺ LSK stem cells and CD45.2⁺ Lin⁻Kit⁺Sca1⁻ progenitors was the same in all 4 groups of mice (Figure 4I). These results indicate that *Gmnn^Δ/Δ* CD45.2⁺ stem and progenitor cells can persist in the marrow for at least 22 weeks. Geminin does not seem to be required for long-term marrow reconstitution or for self renewal of HSCs.

In other respects, the marrow of the mice transplanted with *Mx1-Cre/Gmnn^fl/fl* cells resembled the marrow from pIpC-injected *Mx1-Cre/Gmnn^fl/fl* mice. It was densely infiltrated with megakaryocytes (not shown), and the number of CD45.2⁺Ter119⁺CD71⁺ erythrocyte precursors was reduced compared with controls (Figure 4I). One difference was that the number of mature Gr1⁺Mac1⁺ leukocytes had partially recovered, in accordance with the transient nature of the reduction in wbc numbers (Figure 4I). When
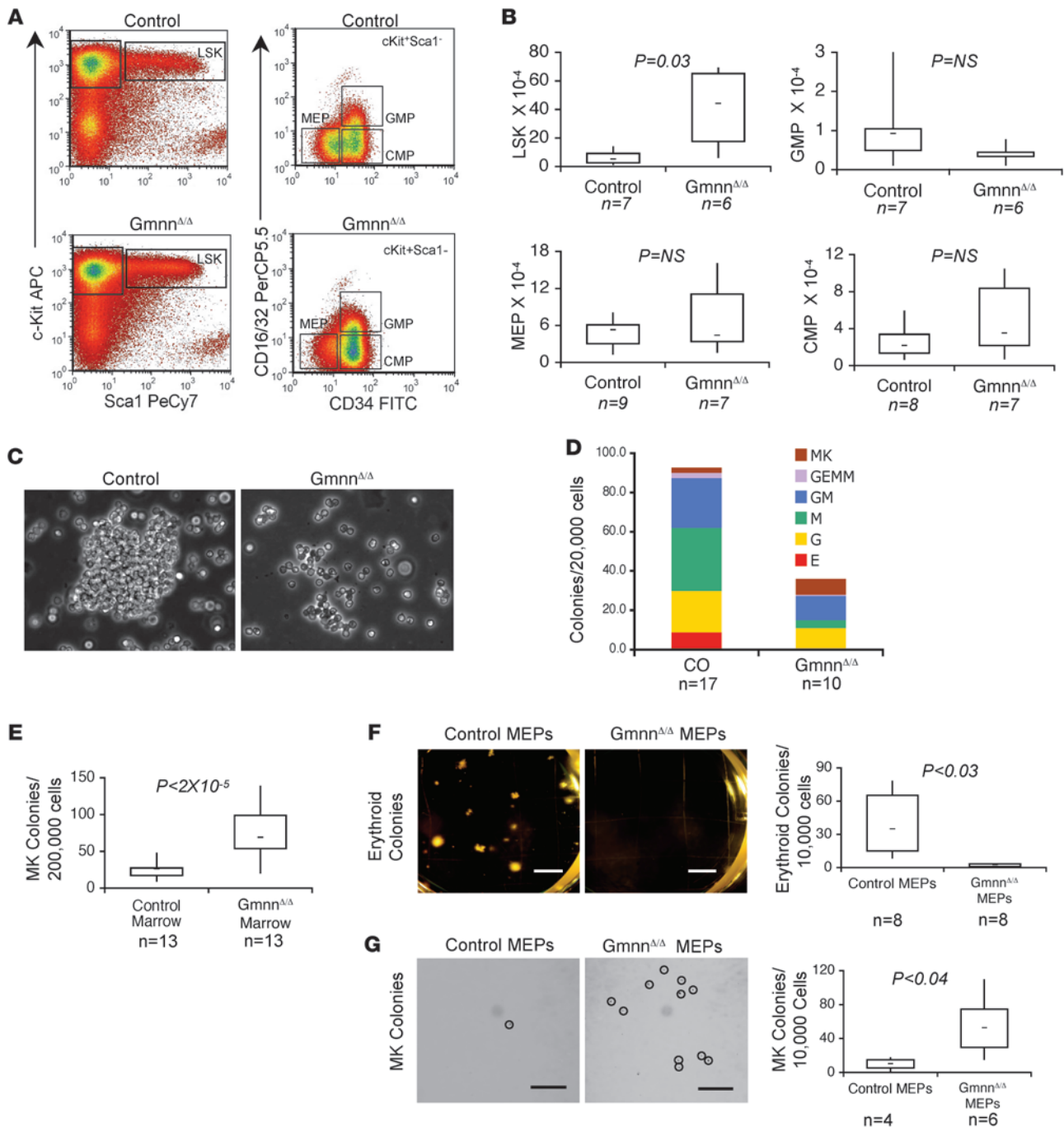
Figure 3
Defective stem and progenitor cell proliferation in $Gmnn^{\Delta/\Delta}$ mice. (**A**) Typical stem cell flow cytometry profiles from pIpC-treated $Mx1$-Cre/$Gem^{fl/fl}$ mice ($Gmnn\Delta/\Delta$) and pIpC-treated control littermates (control). (**B**) Quantification of stem cell populations. (**C**) Typical appearance of myeloid colonies grown from control and $Gmnn^{\Delta/\Delta}$ marrow cells. Original magnification, ×200. (**D** and **E**) Number of erythroid and myeloid colonies (**D**) or megakaryocyte colonies (**E**) grown from control or $Gmnn^{\Delta/\Delta}$ marrow cells. (**F**) Control or $Gmnn^{\Delta/\Delta}$ MEPs were plated for erythroid colonies. Left, photographs of colony plates; right, number of colonies produced. Scale bar is 5 mm. (**G**) Control or $Gem^{\Delta/\Delta}$ MEPs were plated for megakaryocyte colonies. Left, photographs of colony plates (megakaryocyte colonies are circled); right, number of colonies produced. Scale bar is 0.5 mm. For box-and-whisker plots, horizontal bar indicates the median, box indicates 25th to 75th percentiles, and whiskers indicate extremes. P values calculated from Student's t test.

the recovered CD45.2$^+$ $Gmnn^{\Delta/\Delta}$ cells were plated in methylcellulose, they readily produced megakaryocyte colonies but not erythroid colonies (Figure 4J). The number of leukocyte colonies was severely reduced, and the few that formed were small and contained few cells. The reduction in colony number was more pronounced than in pIpC-injected $Mx1$-Cre/$Gmnn^{fl/fl}$ mice (compare Figure 3D and Figure 4J). In contrast, CD45.1$^+$ cells from the same mice formed near normal numbers of colonies, as did

both CD45.2$^+$ and CD45.1$^+$ cells from all 3 groups of control mice. These results confirm that the hematopoietic defects are intrinsic to the $Gmnn^{\Delta/\Delta}$ cells.

$Gmnn^{\Delta/\Delta}$ cells engraft poorly. To see if the $Gmnn^{\Delta/\Delta}$ cells could persist indefinitely, marrow from each of the transplanted mice was infused into a second lethally irradiated CD45.1 recipient. In this experiment, we noted that $Gmnn^{\Delta/\Delta}$ cells displayed a severe engraftment defect. Three of the 6 irradiated mice infused with $Gmnn^{\Delta/\Delta}$ marrow died within 3 weeks of the procedure (Figure 4K). One mouse that survived had a very low percentage (2%–5%) of CD45.2$^+$ cells in his peripheral blood, indicating that the marrow had been reconstituted largely by $Gmnn^{+/+}$ CD45.1 cells. Engraftment was successful in only 2 of the 6 cases. In contrast, all 14 recipient mice that were infused with $Mx1-Cre/Gmnn^{fl/+}$ marrow or $Mx1-Cre/Gmnn^{fl/fl}$ marrow without pIpC treatment had normal engraftment and survival. In these mice, the peripheral blood showed about the same proportion of CD45.2$^+$ cells as was present in the donor (Figure 4K). These results indicate that $Gmnn^{\Delta/\Delta}$ stem and progenitor cells engraft poorly when transferred to a new host.

In the 2 cases where the $Gmnn^{\Delta/\Delta}$ cells successfully engrafted, they came to constitute 30%–70% of the recipient's marrow. The proportion of CD45.2$^+$ cells generally increased with time in these mice, confirming that $Gmnn^{\Delta/\Delta}$ stem cells can persist after transplantation. Both mice showed severe thrombocytosis, anemia, and transient leukopenia (Table 2), again confirming that these abnormalities are intrinsic to the $Gmnn^{\Delta/\Delta}$ cells. These 2 mice died at 45 and 120 days after the procedure. These results suggest that pIpC-injected $Mx1-Cre/Gmnn^{fl/fl}$ mice die primarily from their hematological abnormalities and not because of deletion of Geminin from some other cell type.

To confirm that $Gmnn^{\Delta/\Delta}$ cells engraft poorly, we performed a direct transplantation experiment. We injected $CD45.2/Mx1-Cre/Gmnn^{fl/fl}$ mice and $CD45.2/Mx1-Cre/Gmnn^{fl/+}$ controls with pIpC to delete the Geminin gene, and 4 weeks later, we transplanted the cells into irradiated $CD45.1$ recipients along with a rescue dose of WT CD45.1 cells (Figure 4L). After 2 weeks, we found that the mice transplanted with pIpC-treated $CD45.2/Mx1-Cre/Gmnn^{fl/fl}$ cells had only CD45.1$^+$ cells in their bloodstream, while the mice transplanted with control $CD45.2/Mx1-Cre/Gmnn^{fl/+}$ cells contained an equal mixture of CD45.1$^+$ and CD45.2$^+$ cells. This confirms that $Gmnn^{\Delta/\Delta}$ cells engraft poorly.

$Gmnn^{\Delta/\Delta}$ wbc precursor cells overreplicate their DNA. One well-established function of Geminin is to limit the extent of DNA replication to 1 round per cell cycle (24). We next sought to determine whether abnormalities in $Gmnn^{\Delta/\Delta}$ marrow could be caused by overreplication of the DNA. Two weeks after pIpC injection, $Mx1-Cre/Gmnn^{fl/fl}$ mice and littermate controls were given a single injection of ethinyl-deoxy uridine (EdU) to label cells in S-phase and sacrificed 90 minutes later. We then determined the fraction of the marrow cells in each phase of the cell cycle by measuring the amount of EdU incorporation and the total DNA content by flow cytometry (Figure 5, A and B). Both WT and $Gmnn^{\Delta/\Delta}$ cells vigorously incorporated EdU. Slightly fewer $Gmnn^{\Delta/\Delta}$ cells had entered S phase, but otherwise the cell-cycle distribution was normal. We could not detect any evidence of overreplication in $Gmnn^{\Delta/\Delta}$ cells, which would be manifest as an increased number of cells with DNA contents greater than 4n. We also analyzed the DNA content of $Gmnn^{\Delta/\Delta}$ cells in the first few days after pIpC injection, since this is the time when the cell populations are changing the most. Under these conditions, we again could not detect an increased number of cells with DNA content greater than 4n (Supplemental Figure 4A).

We also measured the extent of replication in subpopulations of hematopoietic cells that were identified by staining with specific antibodies. CD41$^+$ megakaryocytes can become polyploid with DNA contents up to 32n (Figure 5C). We found that there was no significant difference in the distribution of DNA content between control and $Gmnn^{\Delta/\Delta}$ megakaryocytes (Figure 5C). The small number of remaining $Gmnn^{\Delta/\Delta}$ CD71$^+$Ter119$^+$ red cell precursors vigorously incorporated EdU but did not detectably overreplicate their DNA (not shown), but so few cells could be analyzed that it was difficult to form a firm conclusion. To circumvent this problem, we measured the DNA content of $Gmnn^{\Delta/\Delta}$ CD71$^+$Ter119$^+$ cells in the first few days after pIpC injection and again found no evidence of overreplication (Supplemental Figure 4, B and C).

Because $Gmnn^{\Delta/\Delta}$ cells formed smaller and fewer granulocyte and monocyte colonies in vitro, we also tested whether cultured $Gmnn^{\Delta/\Delta}$ wbc precursors overreplicated their DNA. We purified CD34$^+$ leukocyte precursors from pIpC-injected $Mx1-Cre/Gmnn^{fl/fl}$ and control mice, cultured them in liquid medium for 48 hours, and then measured their DNA content by flow cytometry (Figure 5, E and F). In this case, we found a significant population of $Gmnn^{\Delta/\Delta}$ cells with DNA content greater than 4n, indicating overreplication, while control cells showed no such population ($P = 0.003$). The distribution of cells in different phases of the cell cycle was otherwise normal. We also detected overreplication in CD34$^+$ cells isolated directly from the marrow without culture and in granulocytes and monocytes recovered from colonies in methylcellulose (Supplemental Figure 5). These results indicate that the poor growth of $Gmnn^{\Delta/\Delta}$ wbc in culture is caused by a replication defect.

To see whether $Gmnn^{\Delta/\Delta}$ cells undergo apoptosis, we measured the number of annexin V$^+$ cells at different times after Mx1-Cre induction. We could detect no consistent difference in the total number of apoptotic cells between $Mx1-Cre/Gmnn^{fl/fl}$ and control mice at any time (Figure 5, G and H). These results indicate that the changes in marrow cell populations after Geminin deletion are not caused by widespread cell death.

Discussion

Geminin is an unstable regulatory protein that is thought to coordinate cell division and cell differentiation (3, 4). Geminin limits the extent of DNA replication to 1 round per cell cycle by binding and inhibiting the replication factor Cdt1 (5–7). In several systems, Geminin has also been found to inhibit cell differentiation by binding and inhibiting various transcription factors and chromatin remodeling proteins. In this study, we examined the effect of Geminin deletion on the proliferation and differentiation of HSCs. Surprisingly, Geminin is not required for accurate DNA replication in most marrow cells; deletion of the protein does not cause overreplication, apoptosis, or a cell-cycle arrest. Geminin is probably dispensable because of redundant mechanisms that inhibit a second round of DNA synthesis during S and G$_2$ phase, such as the ubiquitin-dependent proteolysis of Cdt1 (8–11). We did find, however, that Geminin profoundly affects the production of blood cells in all 3 hematopoietic lineages.

Geminin's effects on the wbc lineage are the least extreme. The neutrophil count drops precipitously when $Mx1-Cre/Gmnn^{fl/fl}$ mice are given pIpC, then recovers within a few weeks. The drop in leukocyte production seems to be caused by a replication defect. $Gmnn^{\Delta/\Delta}$ CD34$^+$ myeloid precursor cells overreplicate their DNA in vivo and in vitro, and $Gmnn^{\Delta/\Delta}$ marrow cells show a markedly
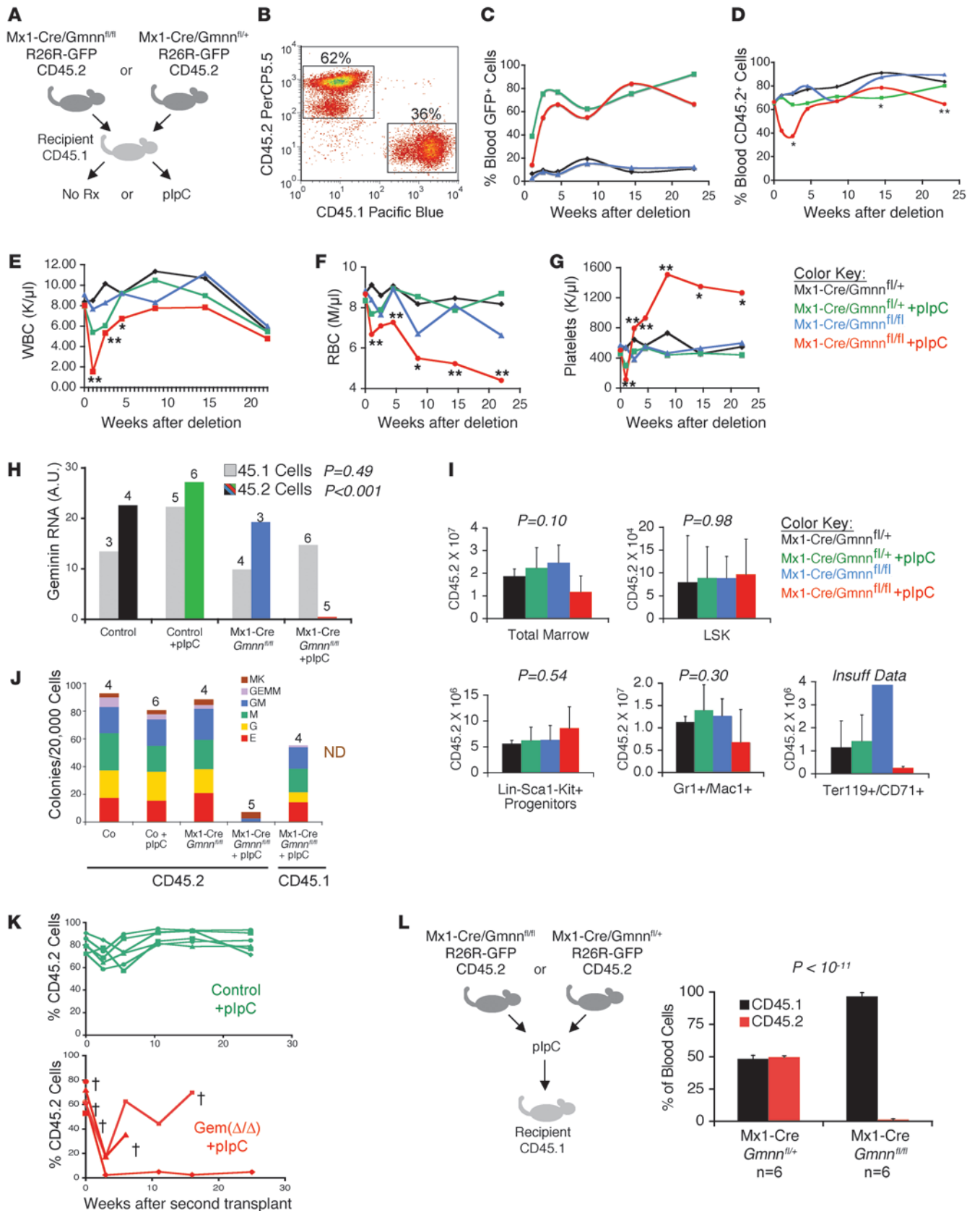
Figure 4

Geminin is not required for HSC self renewal. (**A**) Experimental protocol. Recipient CD45.1 mice were irradiated and transplanted with CD45.2 marrow from *R26R-GFP/Mx1-Cre/Gem^fl/fl* or *R26R-GFP/Mx1-Cre/Gem^fl/+* donors. After engraftment, the mice were either left untreated or treated with pIpC. (**B**) Typical flow profile quantifying CD45.1+ and CD45.2+ populations. (**C–G**) Time-dependent changes in peripheral blood GFP+ cells (**C**), CD45.2+cells (**D**), wbc count (**E**), rbc count (**F**), and platelet count (**G**) in transplanted mice after pIpC injection. Black, uninjected control mice; green, pIpC-injected control mice; blue, uninjected *Mx1-Cre/Gem^fl/f* mice; red, pIpC-injected *Mx1-Cre/Gem^fl/f* mice. Average of 6 measurements (pIpC-treated mice) or 4 measurements (untreated mice). *P < 0.05; **P < 0.01. (**H**) Geminin mRNA levels in CD45.1 and CD45.2 cells recovered from transplanted mice. Number of measurements is shown above each column. Black bar, uninjected control mice; blue bar, uninjected *Mx1-Cre/Gem^fl/f* mice; red bar, pIpC-injected *Mx1-Cre/Gem^fl/f* mice. (**I**) Absolute numbers of recovered CD45.2+ marrow cells by flow cytometry. Color code is the same as in (**H**). (**J**) Colony types grown from CD45.1 or CD45.2 cells recovered from transplanted mice. Number of mice is shown above each column. Red, erythroid colonies (E); yellow, granulcoyte colonies (G); green, monocyte colonies (M); blue, granulocyte-monocyte colonies (GM); lavender, granulocyte-erythrocyte-megakaryocyte colonies (MK). (**K**) Recovered marrow cells (CD45.1+CD45.2) were retransplanted into irradiated CD45.1 recipients. Time-dependent changes in CD45.2 peripheral blood cells in individual mice transplanted with cells from pIpC-treated control mice (green curves) or with cells from pIpC-treated *Mx1-Cre/Gem^fl/fl* mice (red curves). Cross symbol indicates death. Green lines, donor cells from pIpC-injected control mice; red lines, donor cells from pIpC-injected *Mx1-Cre/Gem^fl/fl* mice. (**L**) Right, experimental protocol. Mice were treated with pIpC and 4 weeks later marrow cells were transplanted into irradiated recipients. Left, number of CD45.1+ and CD45.2+ cells in the peripheral blood 2 weeks after transplantation. Black, percentage of CD45.1+ cells; red, percentage of CD45.2+ cells. *n* = 6 for both groups. *P* values calculated by ANOVA.

reduced ability to form granulocyte and monocyte colonies when plated in methylcellulose. These results indicate that Geminin is required to suppress overreplication in leukocytes. Although *Gmnn^Δ/Δ* white cells grow poorly in culture, overreplication of the DNA appears to be well tolerated in vivo. *Gmnn^Δ/Δ* stem and progenitor cells are able to produce mature wbc for at least 6 months after transplantation into an irradiated host. The reason for the difference in phenotype between in vivo and in vitro conditions is unclear. We hypothesize that when *Gmnn^Δ/Δ* cells are stressed to divide rapidly, either by growth factors in the culture medium or during a pIpC-inducted interferon response, they may develop more severe replication abnormalities or tolerate them less well. Geminin does not appear to regulate the pattern of cell differentiation in the wbc lineage. Normal proportions of G, M, and GM colonies are produced when *Gmnn^Δ/Δ* cells are plated in methylcel-

lulose, and we have not been able to detect an abnormal population of developmentally arrested white cells in *Gmnn^Δ/Δ* marrow.

Our most striking finding is that Geminin deletion strongly influences the relative production of erythrocytes and megakaryocytes, corresponding with the high level of Geminin expression seen in these cells. The 2 lineages are affected in opposite directions — the erythroid cells are virtually eliminated from the marrow while the megakaryocytes are greatly expanded. *Gmnn^Δ/Δ* mice develop both a severe anemia and a massive thrombocytosis. Geminin's effects on erythropoiesis and megakaryopoiesis are intrinsic to the marrow cells themselves since they are reproduced in colony-plating assays and persist through 2 serial transplantations. Red cell production is blocked at a very early stage, since *Gmnn^Δ/Δ* marrow has decreased numbers of both immature CD71+Ter119- and more mature CD71+Ter119+ precursors. In contrast, all stages in the megakaryocyte pathway are increased, including megakaryocyte precursors, mature megakaryocytes, and platelets. These results suggest that Geminin regulates cell fate in the common progenitor cell of both these lineages, the megakaryocyte-erythrocyte progenitor (MEP). In colony-plating assays, *Gmnn^Δ/Δ* MEPs produce far fewer erythroid colonies and far more megakaryocyte colonies than control MEPs, indicating that *Gmnn^Δ/Δ* MEPs are intrinsically more likely to differentiate as megakaryocytes. Our functional studies complement previous reports that Geminin is downregulated when megakaryocytic cell lines are induced to differentiate with TPA (25).

Geminin might influence cell fate in MEPs by a passive replication–based mechanism. Cells in the megakaryocyte lineage, being naturally polyploid, might tolerate overreplication of the genome better than erythroid cells. In contrast to the white cell lineage, however, we could not detect any evidence of overreplication in *Gmnn^Δ/Δ* megakaryocytes or erythroid precursors. Furthermore, a passive mechanism cannot easily explain why the megakaryocyte number is so vastly increased in *Gmnn^Δ/Δ* mice. The thrombocytosis does not seem to be driven by hematopoietic growth factors because the serum TPO levels are only slightly increased. The EPO levels are significantly higher (*P* < 0.01), but EPO only mildly stimulates megakaryopoiesis (26, 27) and the effects we observe seem too extreme to be explained by this mechanism. Furthermore, in our cell culture experiments, both *Gmnn^Δ/Δ* and control MEPs were exposed to the same levels of exogenous growth factors, yet *Gmnn^Δ/Δ* MEPs formed more megakaryocyte colonies and fewer erythroid colonies. These results strongly suggest that Geminin influences erythrocyte and megakaryocyte production by a replication-independent mechanism.

We postulate that Geminin actively regulates a transcription factor or a chromatin remodeling protein that determines MEP cell fate. According to this model, the normal function of Geminin is to

Table 1

Transplantation and recovery of control and *Gmnn^Δ/Δ* CD45.2+ cells

| | *Mx1-Cre/Gmnn^fl/+* | | *Mx1-Cre/Gmnn^fl/fl* | | |
Time	Control (*n* = 4)	+pIpC (*n* = 6)	Control (*n* = 4)	+pIpC (*n* = 6)	*P* value
Engraftment	64.6 ± 7.6	65.5 ± 6.9	68.2 ± 9.8	66.3 ± 5.7	0.91
22–24 weeks	83.6 ± 8.3	80.1 ± 7.3	89.5 ± 5.5	64.6 ± 9.2	0.001
Marrow at harvest	96.5 ± 1.6	92.3 ± 4.8	97.9 ± 0.9	87.5 ± 10.0	0.16

Numbers represent percentages of experimental (CD45.2+) cells. *P* values calculated by ANOVA.

Table 2
Complete blood counts after retransplantation

Weeks	Gmnn^Δ/Δ recipient no. 1			Gmnn^Δ/Δ recipient no. 2			Average Gmnn+ recipient		
	rbc	wbc	Plt	rbc	wbc	Plt	rbc	wbc	Plt
3	6.13	0.52	754	7.04	0.60	2079	8.12	4.57	526
6	6.98	3.92	894	7.19	0.19	3365	8.74	7.05	540
11	5.59	3.54	2215	–	–	–	8.42	10.11	579
16	3.80	3.76	2119	–	–	–	8.02	9.58	569

rbc, red blood cell count (M/μl); wbc, white blood cell count (K/μl); Plt, platelet count (K/μl)

promote erythrocyte differentiation at the expense of megakaryocyte differentiation. A phenotype of anemia and thrombocytosis has previously been observed in mice that carry hypomorphic mutations in the transcription factor c-Myb or a mutation in the KIX domain of the transcriptional coactivator p300 (28–32). We have not been able to demonstrate a physical interaction between Geminin and either c-Myb or p300 by coprecipitation in several different lines of hematopoietic cells, nor have we been able to demonstrate an effect of Geminin on either c-Myb or p300 transcriptional activity in reporter assays (data not shown). This suggests that Geminin controls MEP cell fate by a Myb-independent mechanism.

Geminin is not strictly required for the self renewal of long-term HSCs. Flow cytometry demonstrates that stem and progenitor cell numbers are preserved after Geminin deletion and Gmnn^Δ/Δ HSCs that are generated in a host animal continue to supply the blood with mature white cells and platelets for at least 6 months. Nevertheless, Gmnn^Δ/Δ HSCs may have a subtle stem cell defect. After 6 months, the number of wbc derived from the transplant was significantly less in animals that harbored Gmnn^Δ/Δ marrow cells compared with controls (Table 1). Furthermore, the Gmnn^Δ/Δ cells recovered from the marrow after 6 months are less efficient at forming colonies than the Gmnn^Δ/Δ cells isolated shortly after Mx1-Cre induction. In their unperturbed state, HSCs divide on average once every 57 days, so that it might take many months to exhaust the pool if there were a slight cell-cycle defect. We are now observing transplanted mice for longer periods of time to see whether Gmnn^Δ/Δ HSCs undergo senescence. A subtle deficiency in the HSCs may account for the observed engraftment defect in Gmnn^Δ/Δ cells, although we cannot rule out a homing defect.

Our model of how Geminin regulates hematopoiesis is summarized in Figure 6. One function of Geminin is to prevent overreplication of the DNA, but different types of hematopoietic cells might have differing requirements for Geminin depending on how fast they cycle, the efficiency of DNA repair pathways, and the effectiveness of redundant Geminin-independent mechanisms that prevent rereplication. The wbc precursors rely on Geminin to suppress overreplication, but under baseline conditions, they seem to tolerate the excess DNA. Stem and progenitor cells may also depend on Geminin to suppress overreplication. When stressed to divide rapidly by growth factors or after transplantation, cells with overreplicated DNA may fail to divide further or undergo apoptosis. Although we could not detect an overall increase in apoptotic cells in Gmnn^Δ/Δ mice, if most apoptosis occurs within the small population of stem and progenitor cells, it would have little effect on the overall number.

A replication-based mechanism, however, cannot readily explain why the megakaryocyte population is so vastly expanded when Geminin is deleted. We hypothesize that Geminin has a second replication-independent function in controlling the relative production of erythroid cells and megakaryocytes from MEPs. Geminin has previously been shown to physically bind and regulate several different Homeobox (Hox) transcription factors, the SWI/SNF chromatin remodeling ATPase Brg1, and the Polycomb protein Scmh1 (13, 14). More recently, Geminin has been shown to inhibit the histone acetylase HBO1 (33). Many of these proteins are known to regulate hematopoiesis (34–37), and abnormal regulation of one or more of these factors may be responsible for the anemia and thrombocytosis seen in Gmnn^Δ/Δ mice. We are now conducting experiments to evaluate these possibilities.

Methods

Construction of conditional Gmnn^Δ/Δ mice. A BAC clone of C57BL/6 DNA that included the Geminin locus (RP23-92G13) was obtained from the Children's Hospital of Oakland Research Institute (Oakland, California, USA). A 14.5-kb fragment containing Geminin exons 3 through 7 (thick line in Figure 1B) was subcloned in to pBluescript SK using the recombineering technique (38). In this fragment, exons 5 through 7 are flanked by unique XbaI and KpnI sites. The FRT-NEO-FRT-LOXP fragment of plasmid PL451 was amplified by PCR and inserted into the KpnI site, and a LOXP sequence was inserted into the XbaI site using synthetic DNA fragments. The orientation and sequence of both inserts was confirmed by DNA sequencing. The targeting construct (pGem.lox.neo) was electroporated into ES cells from strain Sv129. G418-resistant colonies were selected and screened long range by PCR to see if they had undergone a homologous recombination event. Of the 297 neomycin-resistant colonies screened, we found 23 that gave a PCR product indicating homologous recombination (~8%). Genomic Southern blots confirmed homologous insertion in 21 of the 23 ES cell clones (Figure 1C). Gmnn^fl/+ ES cells were injected into mouse blastocysts to generate 26 chimeric mice. Seven of the chimeric mice transmitted the Gmnn^fl allele when mated to wild-type C57BL/6 mice (stock 664; Jackson Laboratory). Gmnn^fl/+ mice were mated among themselves and to Mx1-Cre mice (stock 3556; Jackson Laboratory) to generate Mx1-Cre/Gmnn^fl/fl and Mx1-Cre/Gmnn^fl/+ mice. Genotypes were determined by PCR of tail DNA. The following primers were used for amplification: Mx1-Cre, forward (5′-GCCTGCATTACCGGTCGATGCAACGA-3′), Mx1-Cre reverse (5′-GTGGCAGATGGCGCGGCAACACCATT-3′); Geminin^WT/fl, forward (5′-GCTCAGAGGTTTCAGGG-3′), Geminin^WT, reverse (5′-CATCAGGTGTTCTCTCAAGTGTCTG-3′); Geminin^fl reverse (5′-GCTACTTCCATTTGTCACGTCC-3′); Geminin^Δ, forward (5′-CTAGCCACAGATGTTGAGCTTG-3′), and Geminin^Δ, reverse (5′-CTAGATGGGATGTATTGTATGAGAG-3′). To induce the Mx1-Cre gene, mice were injected intraperitoneally with 3 doses of polyinosine:polycytosine (10 μg/g body weight; InvivoGen) dissolved in PBS on alternate days between the ages of 11 and 35 days. Mice were analyzed 14–18 days after the first pIpC injection unless otherwise noted. In the Figures, day 1 is the first day of pIpC injection. Unless otherwise indicated, controls were either Gmnn^fl/fl or Mx1-Cre/Gmnn^fl/+ littermates. These 2 genotypes were phenotypically indistinguishable (not shown). All animal studies were approved by the Northwestern University Animal Care and Use Committee (Chicago, Illinois, USA).

Anti-Geminin antibody. The mouse Geminin coding sequence was amplified by PCR and subcloned into pET Duet 1 between the BamHI and EcoRI sites. The protein was expressed in bacteria, purified by nickel-NTA chromatography, and used to immunize rabbits (Covance). Anti-Geminin antibodies were affinity purified by passing the crude immune serum
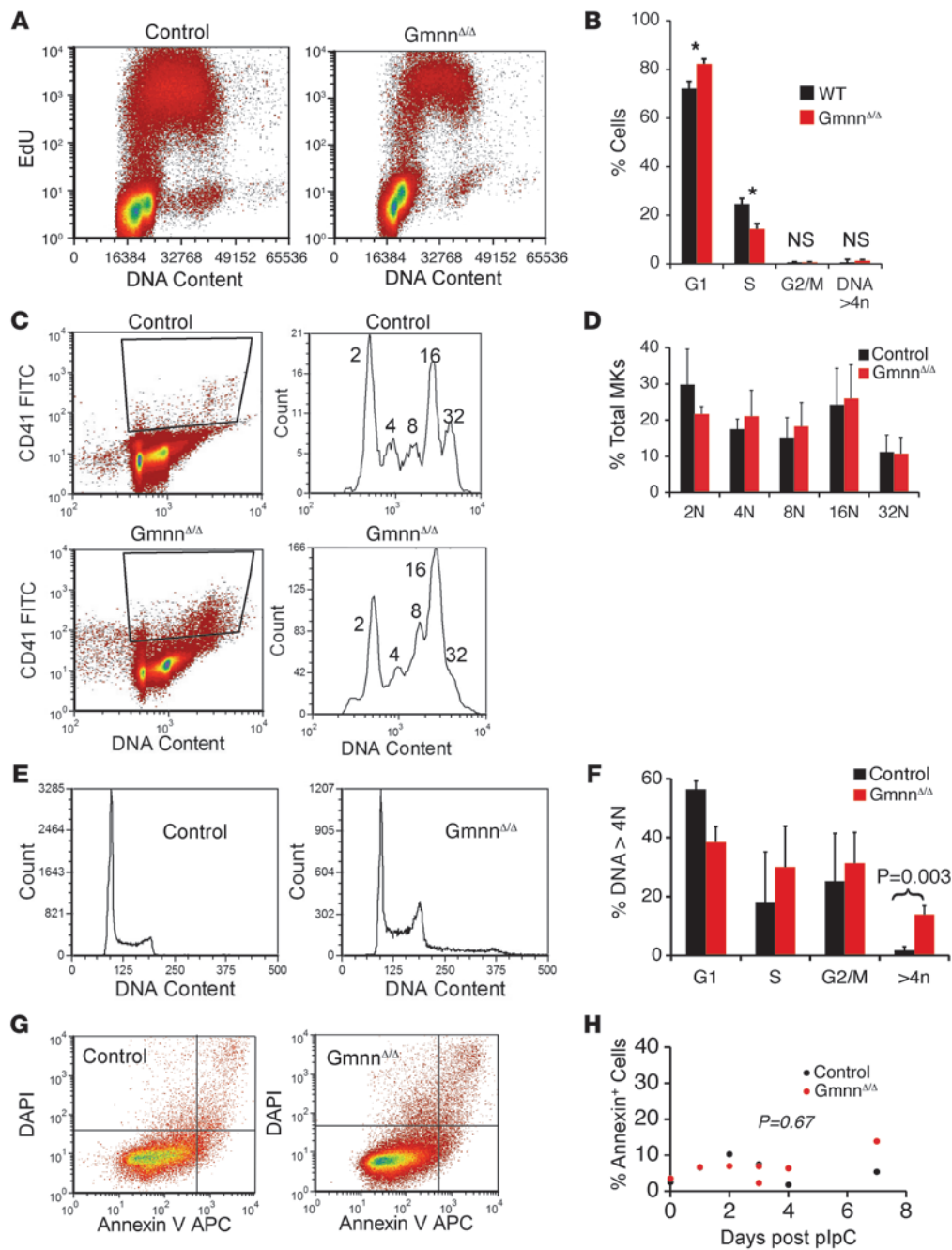
Figure 5

Replication defects in Geminin-deficient cells. (**A**) pIpC-treated *Mx1-Cre/Gem^fl/+* (Control) or *Mx1-Cre/Gem^fl/fl* (*Gem^Δ/Δ*) mice were injected with EdU, then sacrificed. Flow cytometry profile of EdU incorporation versus DNA content. (**B**) The percentage of cells in each phase of the cell cycle is graphed. $n = 3$ for each genotype. *$P < 0.05$ (Student's *t* test). (**C**) Bone marrow cells from pIpC-treated *Mx1-Cre/Gem^fl/+* (control) or *Mx1-Cre/Gem^fl/fl* (*Gem^Δ/Δ*) mice were stained with antibodies to CD41 and for DNA content. The histogram of DNA contents for CD41+ megakaryocytes is shown at the right. (**D**) Ploidy analysis of megakaryocytes from control or *Gmnn^Δ/Δ* mice. $P > 0.30$ for all DNA contents (Student's *t* test). (**E**) DNA content of CD34+ cells from pIpC-treated *Mx1-Cre/Gem^fl/+* (control) or *Mx1-Cre/Gem^fl/fl* (*Gmnn^Δ/Δ*) mice after culture in vitro for 2 days. (**F**) Cell-cycle distribution of control or *Gem^Δ/Δ* CD34+ cells after culture. $n = 3$ for each genotype. (**G** and **H**) Control and *Mx1-Cre/Gmnn^fl/fl* mice were treated with pIpC, and at various times afterwards the percentage of apoptotic cells was measured by annexin V or FLICA staining. The right shows a graph of the percentage of apoptotic cells versus time. Black dots represent individual control mice, and red dots represent individual *Mx1-Cre/Gem^fl/fl* mice. *P* values calculated by Student's *t* test.

over a column of recombinant mouse Geminin attached to CNBr-sepharose beads (Sigma-Aldrich) and eluting bound antibody with 100 mM glycine, pH 2.5. Geminin protein was translated in vitro from a pCS2⁺ mouse Geminin plasmid using the TnT system (Promega).

Flow cytometry. Marrow cells were flushed from dissected femurs with 3 ml PBS. Mature rbc were removed by lysis in ACK buffer (150 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, pH 7.2-7.4) (39). Cells were stained with antibodies diluted in PBS/1% BSA. Antibodies were purchased from either
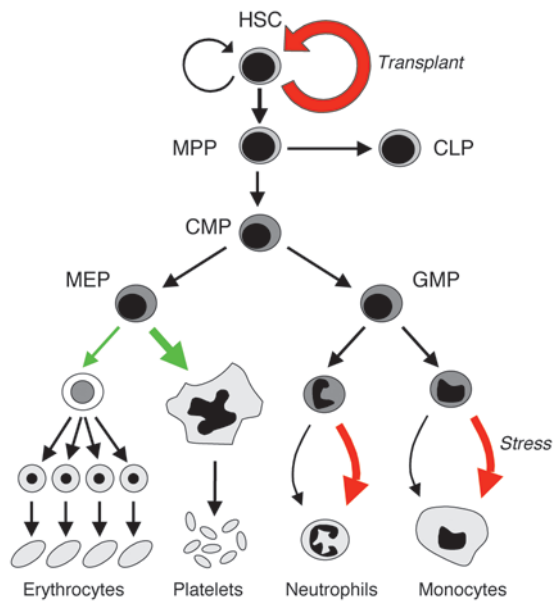
Figure 6
Model. Geminin is required for stress production of granulocytes and monocytes and amplification of HSCs after engraftment (thick red arrows). Geminin may also be required for proliferation of erythroblasts (small red arrows). Geminin is not required for self renewal of HSCs or baseline production of granulocytes or monocytes (black arrows). Geminin also influences the relative production of megakaryocytes and erythroid cells from MEPs (green arrows). MPP, multipotent progenitor; CLP, common lymphocyte progenitor.

eBioscience or BD Biosciences. The lineage cocktail contained PE-conjugated antibodies to Ter119, Gr1 (RB6-8C5), Mac1 (M1/70), CD4 (RM4-4), CD5 (50-7.3), CD8 (53-6.7), B220 (RA3-6B), and CD3 (500A2) (BD Biosciences). Depending upon the experiment, cells were also stained with fluorescently labeled antibodies to CD71 (R17217), CD41 (MWReg30), Sca1 (D7), c-Kit (2B8), CD34 (RAM34), or CD16/32 (clone 93). To avoid nonspecific antibody binding to Fc receptors, unlabeled antibody to CD16/32 (2.4G2; BD Biosciences) was included in all staining reactions except those in which CD16/32 fluorescent staining was performed. To pulse label cells in S phase, mice were injected intraperitoneally with EdU (ref. 40; 0.5 mg/ml in PBS/5% DMSO; Invitrogen) 90 minutes before sacrifice. EdU incorporation was visualized using the manufacturer's protocol. To measure DNA content, cells were fixed with paraformaldehyde to preserve the antibody staining and then stained with propidium iodide (41). Apoptotic cells were labeled with either APC-annexin, biotin-annexin (BD Biosciences), or FLICA reagent (42) (Immunocytochemistry Technologies) and DAPI. Cells were counted using a CyAn Flow cytometer and FlowJo software.

Peripheral blood analysis. Blood was obtained by retro-orbital puncture using heparinized capillaries and collected in tubes containing EDTA. Complete blood counts were determined using a Hemavet 950 cell counter. EPO and TPO levels were measured using Quantikine kits (R&D Systems).

Histology. The sternum and spleen were fixed in formalin, embedded in paraffin, and stained with H&E using standard procedures. Sections were 5 μM thick. Immunocytochemistry was performed with anti-Ter119 antibody (BD Biosciences).

RT-PCR. RNA was isolated using Trizol reagent (Invitrogen). cDNA synthesis was carried using a standard kit (Ambion), and RT-PCR was performed using an Applied Biosystems 7500 Fast Real Time PCR

System. Primers and fluorescently labeled probes for RT-PCR were designed using Primer Design software (Applied Biosystems). All RNA levels were normalized to the amount of 18S ribosomal RNA in each sample. Geminin RNA primer sequences were 5′-ACGGATGCTAGGCC-GTGTAC-3′ (forward), 5′-GCACCGTGTAGTTAGTTTACCAAGAG-3′ (reverse), and 5′-ACGCACTGCCAGCGTTGCCC-3′ (probe). 18S RNA primer sequences were 5′-AACGAGACTCTGGCATGCTAACT-3′ (forward), 5′-CGCCACTTGTCCCTCTAAGAA-3′ (reverse), and 5′-TTAC-GCGACCCCCGAGCGG-3′ (probe).

Colony assays and cell culture. ACK-treated marrow cells were plated in methylcellulose using the MethoCult m3434 system (Stem Cell Technologies) containing stem cell factor, IL-3, IL-6, and EPO. Colonies were scored after 12 days using an inverted phase contrast microscope. For megakaryocyte colonies, cells were plated in a collagen-based gel containing 50 ng/ml TPO, 20 ng/ml IL-6, 50 ng/ml IL-11, and 10 ng/ml IL-3 using the MegaCult system (Stem Cell Technologies). After 12 days, colonies were stained for acetylcholinesterase using the manufacturer's protocol, except that the gel was not dried and the staining solution was added directly to the wells. MEPs (Lin⁻Kit⁺Sca1⁻CD34⁻CD16/32⁻) were purified using the protocol illustrated in Figure 3A. We plated 10,000–40,000 MEPs per well for megakaryocyte colony assays and 9,000 cells per well for erythrocyte colony assays. Sorted CD34⁺ cells were cultured for 48 hours in DMEM with 10% FBS and 100 ng/ml stem cell factor, 10 ng/ml GM-CSF, and 10 ng/ml IL-3.

Bone marrow transplantation. For donor CD45.2⁺ mice, *Mx1-Cre/Gmnn^{fl/fl}* mice were crossed to *Gt(ROSA)26^{Sor}-GFP* mice (strain 4077; Jackson Laboratories) to generate *Mx1-Cre/Gmnn^{fl/fl}/Gt(ROSA)26^{Sor}-GFP* mice (experimental) and *Mx1-Cre/Gmnn^{+/fl}/Gt(ROSA)26^{Sor}-GFP* mice (control). Recipient CD45.1⁺ C57BL/6 mice were purchased from the Jackson Laboratory (stock 2014). Recipients were lethally irradiated with 1200 Gy in split doses (800 Gy, then 400 Gy 4 hours later) using a cesium source. Recipients were anesthetized with isoflurane and injected in the tail vein with a mixture of 1 × 10⁶ donor marrow cells along with a rescue dose of 2 × 10⁵ CD45.1⁺ marrow cells. After 5 weeks, 6 mice in each group were injected with pIpC to induce deletion of the Geminin gene and 4 were left uninjected as controls. Peripheral blood was obtained by retro-orbital puncture, and after ACK lysis the percentage of CD45.1⁺ and CD45.2⁺ wbc was determined by flow cytometry using fluorophore-conjugated antibodies against CD45.1 and CD45.2 (BD Biosciences). At 22–24 weeks after induction, the mice were sacrificed and marrow cells were isolated, analyzed by flow cytometry, and retransplanted (1 × 10⁶ cells/mouse) into fresh irradiated CD45.1⁺ recipients without rescue marrow cells.

For direct transplantation after pIpC induction (Figure 4L), *Mx1-Cre/Gmnn^{fl/fl}/Gt(ROSA)26^{Sor}-GFP* (experimental) and *Mx1-Cre/Gmnn^{+/fl}/Gt(ROSA)26^{Sor}-GFP* (control) mice were given 3 doses of pIpC as described above. After 4 weeks, 5.5 × 10⁵ marrow cells were transplanted into irradiated CD45.1⁺ recipient mice along with a rescue dose of 4.5 × 10⁵ CD41⁺ cells. We noted poor engraftment of both *Gmnn^{Δ/Δ}* and control cells when transplantation was performed immediately after pIpC injection (data not shown).

Statistics. For comparison between 2 groups, *P* values were calculated using either paired or unpaired 2-tailed Student's *t* tests. For comparison among 3 or more groups, *P* values were calculated by analysis of variance. Unless otherwise indicated, significance was defined as *P* < 0.05. Numerical values are reported as mean ± SD.

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