Mechanisms of platelet production and release by mammalian megakaryocytes are poorly understood. We used thrombocytopenic knockout mice to better understand these processes. Proplatelets are filamentous extensions of terminally differentiated megakaryocytes that are thought to represent one mechanism of platelet release; however, these structures have largely been recognized in cultured cells and there has been no correlation between thrombocytopenia in vivo and proplatelet formation. Mice lacking transcription factor NF-E2 have a late arrest in megakaryocyte maturation, resulting in profound thrombocytopenia. In contrast to normal megakaryocytes, which generate abundant proplatelets, cells from these mice never produce proplatelets, even after prolonged stimulation with c-Mpl ligand. Similarly, thrombocytopenic knockout mice with lineage-selective loss of transcription factor GATA-1 produce proplatelets very rarely. These findings establish a significant correlation between thrombocytopenia and proplatelet formation and suggest that the latter represents a physiologic mechanism of platelet release. We further show that proplatelet formation by normal megakaryocytes and its absence in cells lacking NF-E2 are independent of interactions with adherent (stromal) cells. Similarly, thrombocytopenia in NF-E2−/− mice reflects intrinsic defects in the megakaryocyte lineage. These observations improve our understanding of platelet production and validate the study of proplatelets in probing the underlying mechanisms.

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thrombocytopenia are as obscure as the mechanisms of platelet formation and release by normal megakaryocytes.

We report here on our studies on three aspects of thrombocytopenia by normal and mutant megakaryocytes. First, megakaryocytes cultured from murine fetal livers in the presence of the c-Mpl ligand develop large numbers of proplatelets. In contrast, megakaryocytes lacking NF-E2 never generate proplatelets and GATA-1-deficient megakaryocytes do so very rarely. These findings provide a strong correlation between proplatelet formation in vitro and thrombocytopenia in vivo and lend support to the view that proplatelet formation represents a physiologic mechanism of platelet release. Second, we provide more detailed characterization of normal murine proplatelets. Finally, we demonstrate that proplatelet formation by normal megakaryocytes in vitro and thrombocytopenia resulting from absence of NF-E2 in vivo occur independently of interactions with other cell types. The sum of these observations furthers our understanding of thrombocytopoiesis and enhances the utility of genetic models of thrombocytopenia to analyze the mechanism of platelet production.

**MATERIALS AND METHODS**

**Megakaryocyte culture.** p45 NF-E2 and GATA-1 heterozygous mice were maintained on an inbred 129/Sv genetic background. Livers were recovered from mouse fetuses between embryonic day (E) 13 and 15. Single-cell suspensions, prepared by successive passage through 22- and 25-gauge needles, were cultured in Dulbecco’s modified Eagle’s medium (GIBCO BRL, Bethesda, MD) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 0.1 µg/mL polyethylene glycol-conjugated recombinant human c-Mpl ligand (Amgen, Thousand Oaks, CA). Between the days 4 and 6, these fetal liver cultures contained 50% to 60% acetylcholinesterase-positive cells. c-Mpl ligand was added only at the initiation of liquid cultures.

Conditioned medium was obtained from megakaryocyte liquid cultures on day 5 and contaminating cells were removed by passage through 0.45-µm filters. Undiluted conditioned medium, without determination of cytokine content, was used to replace the original medium in experiments testing its role in influencing proplatelet formation.

To establish stromal cell layers, fetal liver or bone marrow cells were cultured on the stromal cell layers, as described above. Proplatelet formation was studied after 7 days in culture at 37°C.

**Detection and characterization of proplatelets.** Proplatelets were detected and scored by phase contrast microscopy of cells growing in suspension in liquid culture. A total of 10^5 to 10^6 cells were cyt centrifuged onto coated glass slides and acetylcholinesterase activity was detected as previously reported. For indirect immunofluorescence, cyto centrifuged cell preparations were fixed in methanol for 1 minute, washed, blocked with 2% goat serum in tris-buffered saline, and successively incubated with 1:100 dilutions of rabbit antiplatelet antiserum (gift of C.W. Jackson, St Jude Children’s Research Hospital, Memphis, TN) and fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG (Pharmingen, Los Angeles, CA).

**Electron microscopy.** To preserve proplatelet integrity, cells were concentrated by gentle centrifugation (200g for 4 minutes) and then adhered by gravity to poly-L-lysine (Sigma, St Louis, MO)-coated glass cover slips resting in a Petri dish. After 15 minutes, fixative (1.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4) was added slowly into the Petri dish and the cells were fixed for 4 hours. After embedding in Epoxy resin in an inverted Beem capsule, the single layer of megakaryocytes was detached from the cover slips by immersion in liquid nitrogen, and ultrathin sections were cut with a Dupont MT6000 microtome (Dupont, Newton, CT), stained with uranyl acetate and lead citrate, and examined with a JEOL 100CX-II transmission electron microscope (JEOL, Peabody, MA) at an accelerating voltage of 60 kV.

For scanning electron microscopy (SEM), cover slips with cells were taken through a series of alcohols and dried using a Ladd Model 28000 Critical Point Dryer (Ladd Research Industries, Inc, Hatfield, PA). The cover slips were then mounted onto SEM specimen tabs, sputter-coated with Polaron SEM coating system (Polaron Instruments, Inc, Burlington, VT), and examined with a JEOL JSM-35CF Scanning Electron Microscope at an accelerating voltage of 20 kV.

**Fetal liver transplantation.** Livers were recovered from the fetuses of p45 NF-E2 heterozygote intercrosses on postcoital day 14 and single-cell suspensions were prepared by passage through a syringe and 22-gauge needle. Pending determination of the p45 NF-E2 genotype of each fetus, these cells were cultured overnight in Dulbecco’s modified Eagle’s medium (GIBCO BRL, Bethesda, MD) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 0.1 µg/mL polyethylene glycol-conjugated recombinant human c-Mpl ligand (Amgen, Thousand Oaks, CA). Between the days 4 and 6, these fetal liver cultures contained 50% to 60% acetylcholinesterase-positive cells. c-Mpl ligand was added only at the initiation of liquid cultures.
Eagle’s medium supplemented with 20% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% spleen cell conditioned medium. The next day, 14 adult 129/SvJ adult females were treated with whole-body irradiation at two doses of 500 cGy each 3 hours apart. Six mice were then injected intravenously with 6 to 8 $3 \times 10^6$ fetal liver cells derived from p45 NF-E2$^+/+$ or p45 NF-E2$^-/-$ donors and 6 mice with the same number of cells cultured from p45 NF-E2$^-/-$ donor fetuses. One recipient from the test group and 2 mice from the control group showed either endogenous or chimeric reconstitution at 3 to 5 weeks, indicating sublethal irradiation; the analysis presented here is limited to the majority of mice with complete hematopoietic reconstitution by the donor cells. Histologic analysis of reconstituted spleen and bone marrow and Southern analysis, using a flanking genomic DNA probe, were performed as described previously. At various times, 50 to 100 µL of blood was removed from the retro-orbital sinus and diluted in Unopette buffer (Becton Dickinson, Franklin, NJ), and leukocyte and platelet counts were determined by manual counting under light microscopy.

RESULTS

Abundant proplatelet formation by cultured murine megakaryocytes. We cultured murine fetal liver cells in liquid or semisolid medium in the presence of fetal bovine serum and the c-Mpl ligand and observed the cultures over several days. Under these conditions, mature megakaryocytes develop in large numbers and produce an impressive array of cell projections (Fig 1a) that appear identical to structures previously observed.
described in human,\textsuperscript{17,22} rat,\textsuperscript{19} guinea pig,\textsuperscript{11} and bovine\textsuperscript{12} megakaryocytes cultured in vitro. These proplatelets, as they have been termed,\textsuperscript{6} are seen very rarely when c-Mpl ligand is excluded from the cultures. The formation of proplatelets correlates well with the degree of megakaryocyte maturity, being undetectable over the first 2 days and then increasing in frequency as the culture becomes dominated by large, polyploid, acetylcholinesterase-positive cells; in wild-type or p45 NF-E2\textsuperscript{1–/–} megakaryocyte cultures, 40\% to 50\% of the acetylcholinesterase-positive cells display proplatelets between culture days 4 and 6. In contrast, megakaryocytes lacking NF-E2 grow appreciably larger than control cells and acquire morphologic and molecular markers of maturity but are never observed to generate proplatelets (Fig 1b), even after prolonged culture. This finding provides the first correlation between proplatelet formation in vitro and thrombopoiesis in vivo.

Proplatelet formation has not previously been reported in cells isolated from the laboratory mouse, a species in which genetic models of thrombocytopenia are available. In large part, this reflects the relatively recent isolation of the c-Mpl ligand, which is required to propagate and drive complete maturation of sufficient numbers of megakaryocytes. A tendency to study cells derived from adult mice may also have been a factor; in our hands, culture of bone marrow cells under the same conditions as fetal liver cells yields significantly fewer megakaryocytes. However, mature megakaryocytes cultured from wild-type or p45 NF-E2\textsuperscript{2–/–} adult bone marrows also produce large numbers of proplatelets over the same culture period as do fetal liver cells, whereas cells derived from p45 NF-E2\textsuperscript{2–/–} adults fail to do so (data not shown).

Biochemical characterization of proplatelets. Although proplatelets are believed to form by eversion of the megakaryocyte cytoplasm through the system of demarcation membranes, their specific composition is not fully characterized. Wild-type murine proplatelets show strong acetylcholinesterase activity (Fig 2a), a specific marker of the rodent megakaryocyte and platelet cytoplasm,\textsuperscript{21} throughout their length. This finding indicates the incorporation of cytoplasmic contents into these structures. Furthermore, a rabbit antimouse platelet antiserum\textsuperscript{23} readily stains the surfaces of the largest cells (megakaryocytes) in both control and p45 NF-E2\textsuperscript{2–/–} cultures, including the full length of proplatelets in the former (Fig 2b and c). Taken together, these data suggest that proplatelet formation represents cytoplasmic and membrane reorganization of mature megakaryocytes. This dramatic aspect of terminal megakaryocyte differentiation is notably missing in cells cultured from the profoundly thrombocytopenic mice lacking p45 NF-E2.

Electron microscopy of proplatelets. To better appreciate the structural basis of proplatelet formation in wild-type megakaryocytes and its absence in cells lacking NF-E2, we performed electron microscopy. Scanning electron micrographs of megakaryocytes cultured from normal fetal livers provide high-resolution images of the proplatelet surface and show these to be structures that emanate from the cell (Fig 3a and b). The long filamentous connections between individual platelet-size structures are also readily shown by this technique.

![Fig 3](image-url)
In contrast, the much larger megakaryocytes from NF-E2-deficient mice (Fig 3c) show numerous small, villous projections but do not display the proplatelets characteristic of normal mature megakaryocytes. Transmission electron microscopy confirms that the proplatelet cytoplasm is contiguous with that of the cell (Fig 4). Although the static images obtained by this technique are insufficient to draw conclusions about the dynamic process by which proplatelets are generated by mature megakaryocytes, these data are consistent with internal fragmentation of the megakaryocyte cytoplasm, as suggested by earlier studies. Indeed, ultrastructural features of cytoplasmic reorganization that appear to precede proplatelet formation, including dilatation of demarcation membranes, are also notably absent from megakaryocytes lacking NF-E2 (data not shown). Nevertheless, cultured p45 NF-E2−/− megakaryocytes have many of the same properties seen in vivo, including dearth of platelet-specific granules and abundance of demarcation membranes, indicating that maturation of the cells in culture parallels megakaryocyte differentiation in vivo.

**Impaired proplatelet formation by GATA-1-deficient megakaryocytes.** We have recently established a distinct murine model of thrombocytopenia. Mice with a targeted deletion within the 5′-flanking region of the GATA-1 gene display megakaryocyte-selective loss of GATA-1 expression, dysregulated growth of megakaryocyte progenitors, and platelet counts that are reduced to approximately 15% of normal. The ultrastructure of GATA-1-deficient megakaryocytes shows a striking and early block in cytoplasmic maturation, with features distinct from those observed in the absence of p45 NF-E2. If proplatelet formation is a physiologically important aspect of terminal megakaryocyte differentiation, then GATA-1-deficient megakaryocytes also may be expected to be impaired in their ability to generate these structures. Indeed, proplatelet formation by GATA-1-deficient megakaryocytes is observed only rarely in liquid (Fig 5a, see page 1611) or semisolid (data not shown) cultures of the mutant cells or upon staining of the mutant cells with acetylcholinesterase or antiplatelet antiserum (Fig 5b and c, see page 1611). Scanning and transmission
were performed on late megakaryocyte progenitors (human CD34+ bone marrow cells further purified by differential sedimentation or by flow cytometry for the CD38 marker) and thus leave open the possibility that signals delivered at an earlier stage in megakaryocyte differentiation are required for optimal proplatelet production. We therefore depleted the adherent cell fraction from a population of total wild-type fetal liver cells over the first few hours after tissue harvest. Over the following week, the remaining progenitors give rise to megakaryocytes that develop proplatelets in similar numbers and at the same rate as cells from nondepleted cultures (data not shown), indicating that contact with stromal cells is not a requirement for proplatelet formation. Moreover, when p45 NF-E2−/− fetal liver cells are cocultured from the outset with adherent cells derived from normal fetal livers or bone marrows, they remain unable to produce proplatelets; similarly, coculture of wild-type fetal liver cells with adherent cells of p45 NF-E2−/− origin does not inhibit proplatelet formation (Table 1). Hence, the lack of proplatelet formation by the defective megakaryocytes is very likely a cell-autonomous process reflecting a critical requirement for NF-E2 within the megakaryocyte lineage.

Cell-autonomous thrombocytopenia in the absence of NF-E2. The mechanism underlying the inability of NF-E2-null megakaryocytes to produce platelets is unclear. To determine whether this mechanism is also cell-autonomous, we delivered lethal doses of γ-irradiation to wild-type adult mice and then attempted to rescue hematopoesis in these animals by introducing fetal liver cells derived from p45 NF-E2−/− or control fetuses. All animals reconstituted by wild-type donor cells recover normal hematologic profiles, including platelet counts, within 3 weeks; in contrast, mice reconstituted by NF-E2−/− fetal liver cells rapidly recover normal numbers of leukocytes (data not shown) but remain profoundly thrombocytopenic ≥9 weeks after transplantation (Fig 6a); complete reconstitution by cells of donor origin is indicated by Southern analysis of the hematopoietic tissues (Fig 6b). In recipients of p45 NF-E2-null fetal liver cells, megakaryocytes harbor the same morphologic abnormalities seen in p45 NF-E2−/− cells (Fig 6C). These findings establish that the thrombocytopenia resulting from absence of NF-E2 function reflects deficiencies within the daughters of a radiation-sensitive cell population, most likely the megakaryocyte progenitors.

**Discussion**

The cellular mechanisms by which megakaryocytes produce and release millions of platelets daily are uncertain and difficult to study. In large part, this is because megakaryocytes are among the rarest of hematopoietic cells and because platelet release is a dynamic process that occurs over an unspecified time period in an unknown location. Nevertheless, ultrastructural analysis of megakaryocytes in vivo has provided valuable insights into potential mechanisms of platelet release; images of parasinusoidal megakaryocytes extending processes that include nascent platelets (proplatelets) into the vascular space led to the hypothesis that formation, and subsequent fragmentation, of these structures contributes in some manner to the pool of circulating platelets. Before the identification of the c-Mpl ligand as the major regulator of megakaryocyte growth and differentiation (reviewed in Kaushansky96), proplatelet forma-
tion could consistently be demonstrated only in cells derived from a limited number of species and under specific culture conditions (reviewed in Choi). Although wider use of the c-Mpl ligand to culture megakaryocytes in vitro has led to increasing recognition of the proplatelet as a genuine subcellular entity, it has remained difficult to establish a link between proplatelet formation in vitro and thrombopoiesis in vivo.

We show here that wild-type murine megakaryocytes develop a dramatic array of proplatelets during culture in the c-Mpl ligand, thus providing evidence that proplatelet formation is an integral aspect of late megakaryocyte maturation in all species studied to date. Furthermore, the kinetics of proplatelet formation closely parallels megakaryocyte differentiation, i.e., both the frequency of proplatelet-bearing megakaryocytes and the average number of proplatelets per cell (data not shown) increase between 2 and 7 days of culture, before cell viability decreases. These proplatelets express both surface and cytoplasmic markers of the megakaryocyte/platelet lineage (Fig 2) and are shed into the culture medium in large numbers, where they either appear as platelet-size particles or remain in the beads on a string form that emanates from the mature megakaryocytes (Figs 1 and 3).

Proplatelets are observed infrequently when either human...
or murine (our observations) megakaryocytes are cultured in the absence of the c-Mpl ligand. This may explain why these structures proved difficult to demonstrate in early studies of thrombocytopoiesis in mice and other species. Our demonstration of abundant proplatelets in normal murine megakaryocytes may also be traced in part to the choice of cultured tissue. Culture of adult bone marrow or spleen cells under identical conditions yields many fewer megakaryocytes than that of mouse fetal liver cells (data not shown), perhaps reflecting the more limited proliferation potential of adult progenitors. However, we do not observe substantial differences in the acetylcholinesterase staining or ultrastructure of proplatelets obtained from fetal and adult sources. Moreover, known features of murine fetal liver-derived cells, such as the ability to produce circulating platelets in the fetus and to reconstitute hematopoiesis in lethally irradiated recipients, indicate that the fetal liver is a physiologically relevant source of megakaryocytes for study.

Our most important observation is that proplatelets are absent from cultures of p45 NF-E2−/− megakaryocytes and greatly reduced in cultures of GATA-1-deficient cells; the respective knockout mice display either profound or moderately severe thrombocytopenia. In each case, the development of megakaryocytes in culture shows other features that are also seen in vivo, such as unusually large megakaryocytes in the case of NF-E2 loss and smaller megakaryocytes admixed with an excess of immature cells in the case of GATA-1 deficiency (Vyas et al, manuscript in preparation). The striking differences between the wild-type and mutant cells with respect to generation of proplatelets thus establish a strong correlation between proplatelet formation in vitro and platelet production in vivo and support the view that proplatelets contribute to the mechanism by which terminally differentiated megakaryocytes release platelets.

Besides addressing the mechanism of platelet production in a species that is amenable to genetic manipulation, our studies underscore the value of examining this mechanism in culture, thus circumventing the low likelihood of identifying platelet-producing megakaryocytes in vivo. At the same time, it is worth emphasizing that the absence or decrease of proplatelet formation by megakaryocytes lacking NF-E2 or GATA-1, respectively, does not by itself point to the complete mechanism of thrombocytopenia in these animal models. Although our findings establish a critical link between proplatelet formation in vitro and thrombocytopoiesis in vivo, the arrest in megakaryocyte cytoplasmic maturation that characterizes these knockout mice19,20 might well precede the maturational stage at which generation of proplatelets is feasible. Although a requirement for NF-E2 or GATA-1 in aspects of proplatelet production cannot be ruled out, it is also possible that other cytoplasmic abnormalities of the defective megakaryocytes, such as reduced numbers of granules and disorganized demarcation membranes, simply preclude progression of the cell through terminal differentiation, including proplatelet formation.

Early observations of proplatelet formation in vivo by megakaryocytes in close contact with marrow stromal cells generated the notion that platelet production might depend on such cell-cell interactions. We have extended here previous studies7,18 to demonstrate that proplatelet formation can occur independently of interactions between megakaryocytes and stromal cells. The results summarized in Table 1 further indicate that the lack of proplatelet formation in the absence of NF-E2 function is not rescued by culture supernatant or adherent (stromal) cells from wild-type cultures, suggesting that this defect is intrinsic to the megakaryocyte. Additional proof for a cell-autonomous defect in NF-E2-null megakaryocytes is provided by our observation that the phenotype of NF-E2 knockout mice is reproduced in its entirety in lethally irradiated wild-type mice that receive a transplant of fetal liver cells derived from the knockout mice. Thus, it is unnecessary to invoke the existence of a secreted platelet-shedding factor solely on the basis of the phenotype of mice lacking NF-E2. Although it is likely that megakaryocytes receive, and depend on, signals delivered by other cells early in their differentiation, our results with the normal and mutant cells argue that terminal aspects of megakaryocyte differentiation, especially proplatelet formation, are largely independent of such signals. One might therefore speculate that platelet production and release are programmed aspects of the maturing megakaryocyte once the cell has committed to polyploidization and a megakaryocyte-specific pattern of gene expression. Of course, this does not exclude the possibility that extraneous signals serve to modify the number or qualitative characteristics of the proplatelets produced by mature megakaryocytes, as suggested previously.3,11,32

In conclusion, we report that proplatelet formation is absent or severely reduced in two distinct knockout mouse models of thrombocytopenia that result from primary disturbances in platelet production. These findings strongly suggest a physiologic role for proplatelet formation in the normal mechanisms of platelet production and release in vivo.

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REFERENCES

1. Trowbridge EA, Martin JF, Slater DN: Evidence for a theory of physical fragmentation of megakaryocytes implying that all platelets are produced in the pulmonary circulation. Thromb Res 28:461, 1982


23. McDonald TP, Jackson CW: Thrombopoietin derived from human embryonic kidney cells stimulates an increase in DNA content of murine megakaryocytes in vivo. Exp Hematol 18:758, 1990


Mice Lacking Transcription Factor NF-E2 Provide In Vivo Validation of the Proplatelet Model of Thrombocytopoiesis and Show a Platelet Production Defect That Is Intrinsic to Megakaryocytes

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