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E2F-1 Blocks Terminal Differentiation and Causes Proliferation in Transgenic Megakaryocytes

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The transcription factor E2F-1 plays a central role in the cell cycle through its ability to activate genes involved in cell division. E2F-1 activity is regulated by a number of proteins, including the retinoblastoma susceptibility gene product, cyclin-dependent kinases, and their inhibitors, proteins that have been implicated in the control of certain developmental processes. To investigate a potential role of E2F-1 in differentiation, we assayed the ability of megakaryocytes to form platelets in an in vivo transgenic model. E2F-1 expression in megakaryocytes blocked differentiation during maturation, resulting in severe thrombocytopenia. Ultrastructural analysis of megakaryocytes revealed abnormal development characterized by hyperdemarcation of cytoplasmic membranes and reduced numbers of alpha granules. Administration of megakaryocyte growth and development factor or interleukin 6 could not overcome the differentiation block. Additionally, E2F-1 caused massive megakaryocyte accumulation in both normal and ectopic sites, first evident in E15 embryonic liver. Furthermore, significant apoptosis was observed in transgenic megakaryocytes. These data indicate that E2F-1 can prevent terminal differentiation, probably through its cell cycle-stimulatory activity.

E2F-1 is a transcription factor that is thought to play a central role in the control of cell division (30, 37). It is one member of a family of at least four E2F proteins whose functions appear to be similar (4, 11, 16, 22, 27, 32, 49). The DNA binding and transcriptional activity of E2F-1 is maximally activated by heterodimerization with the DP-1 family of proteins (2, 12, 17, 28), suggesting that the active complex is an E2F-DP heterodimer. E2F binds the consensus sequence TTTCGCGC and activates transcription of a number of genes involved in the G_1 and S phases of cell division. Such genes include *myc* (35), *myb* (29), and the dihydrofolate reductase (53), thymidine kinase (7), thymidylate synthetase (34), DNA polymerase α and cyclin D1 (45), and E2F-1 (20, 25, 36) genes. These genes contain E2F consensus sites in their promoters and/or are transcriptionally responsive to E2F protein.

The expression and effects of E2F-1 in cultured cells have been well characterized. E2F-1 mRNA is cell cycle regulated, appearing during G_0/G_1 through S (27, 49, 53). Overexpression of E2F-1 in 3T3 cells causes reentry into the cell cycle (26, 58) and, in primary fibroblasts, causes transformation (24, 51). E2F-1 is positively correlated with cell proliferation, as E2F-1 is expressed in all tumor cell lines assayed (16, 32) and is undetectable in senescent cells in culture (6).

E2F function is thought to be regulated by a cascade of cell cycle-regulatory proteins. E2F-1 is a target of the tumor suppressor Rb, the product of the retinoblastoma susceptibility gene and a protein with an inhibitory effect on proliferation. E2F-1 interacts directly with Rb, resulting in suppression of E2F-mediated transactivation (9, 15). Rb, in turn, is negatively regulated through phosphorylation by the cyclin-dependent kinases (CDKs). CDKs are themselves inhibited by the p16 and p21 classes of kinase inhibitors. For instance, E2F-1 can induce DNA synthesis in transforming growth factor β growth-inhibited mink lung epithelial cells (47), suggesting that E2F-1 acts downstream of transforming growth factor β, which has been reported to block the cell cycle through one or more of the CDK inhibitors. Additionally, the p16 CDK inhibitor can block E2F activity in a cyclin D1-stimulated cell (46). Recent data suggest that Rb, cyclin D1, and p21 may play a significant role in differentiation of muscle. Rb appears to be necessary for proper differentiation of cultured myoblasts (13), cyclin D1 can inhibit myogenic differentiation, an effect counteracted by coexpression of p16 or p21 (52), and expression of the p21 CDK inhibitor is positively correlated with muscle differentiation (14, 40). Functional inactivation of the Rb gene in mice results in developmental defects in erythrocytes and some neurons (5, 23, 31). It has been postulated that Rb acts by causing cells to exit the cell cycle (reviewed in reference 56). It is possible that Rb and p21 accomplish this through their ability to directly or indirectly inhibit E2F, suggesting that E2F may act to prevent differentiation. E2F-1 is present in a number of human tissue RNAs (27), including, paradoxically, RNAs of tissues usually associated with very little mitotic activity. The function or regulation of E2F in cells in vivo is not well understood.

To address the role of E2F-1 in differentiation, we examined the consequences of deregulation of E2F-1 expression in vivo by using the well-defined differentiative process of platelet formation as a model (reviewed in reference 10). Megakaryocyte precursors proliferate and then exit the cell cycle as diploid megakaryoblasts. These cells, readily identifiable because of their large size, mature through three morphologically defined stages, finally fragmenting into blood platelets. Experimental manipulation of this process can be easily assayed by monitoring circulating platelet levels in peripheral blood. Furthermore, megakaryocyte proliferation and differentiation can be positively regulated by several factors, including the recently cloned megakaryocyte growth and development factor (MGDF) (3, 33). To target E2F-1 expression to megakaryocytes, we generated transgenic mice expressing the human E2F-1 gene under the control of the megakaryocyte-specific platelet factor 4 (PF4) promoter (42). Expression of this promoter is thought to initiate as the megakaryocytes exit the cell cycle, such that effects of the transgene would be primarily directed to postmitotic megakaryocytes.

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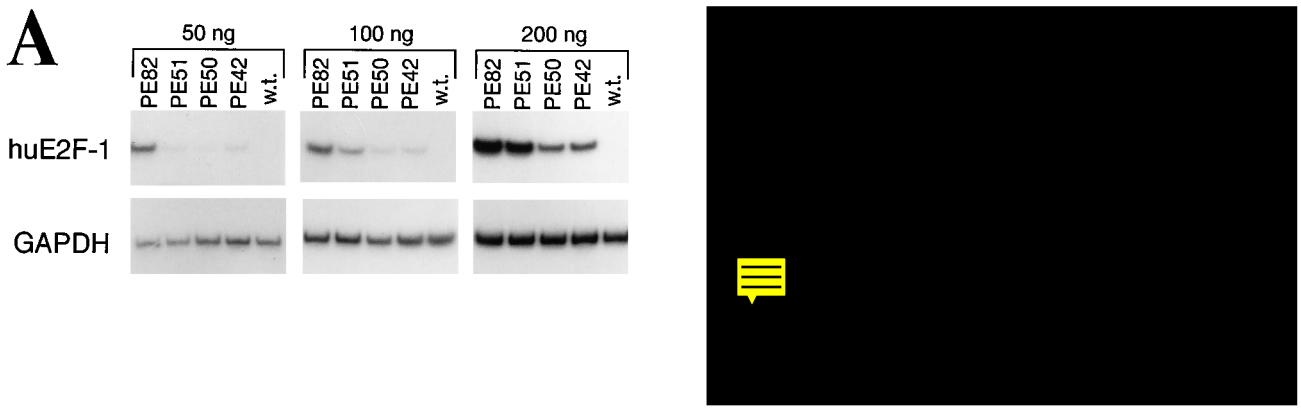

FIG. 1. Expression of human E2F-1 in transgenic megakaryocytes. (A) RT-PCR analysis of huE2F-1 expression in transgenic bone marrow RNA. Fifty, 100, or 200 ng of total RNA from bone marrow of the indicated transgenic (PE series) or wild-type (w.t.) mice was reverse transcribed, amplified for 25 cycles, and analyzed. The amplified products for huE2F-1 and GAPDH were 321 and 352 bp long, respectively. Exposure times were 72 h for E2F-1 and 1 h for GAPDH. (B) Immunostaining of huE2F-1 protein in megakaryocytes. Shown is two-color immunostaining of the indicated bone marrow sections, using antibodies directed against either E2F-1 protein (red) or megakaryocyte marker PF4 (green). The samples were imaged on an ACAS confocal microscope and processed by using Adobe Photoshop 3.0. Magnification, ×62.

MATERIALS AND METHODS

Transgenic mice. A 1.2-kb *Eco*RV-*Bam*HI fragment of the human E2F-1 (huE2F-1) cDNA was filled in with Klenow enzyme and cloned into the *Eco*RV site of an expression cassette containing 1.1 kb of the rat PF4 upstream region 5′ to the *Eco*RV site followed 3′ by the simian virus 40 small-T intron and polyadenylation sequences. The construct was isolated as a *Not*I-*Xho*I fragment, and transgenic mice were generated by pronuclear injection as described previously (19). Transgenic lines were screened by using PCR with oligonucleotide primers used for the reverse transcription (RT)-PCR analysis (see below). Homozygous mice were identified as previously described (44).

Histology. Tissues from adults (4 weeks old) and fetuses (day 15 postcoitus) were harvested from normal and transgenic animals, fixed in 10% zinc-formalin, and embedded in paraffin. Femurs were additionally decalcified (J. T. Baker) for 1 h following fixation. Tissues were cut into 4-μm sections and stained with hematoxylin and eosin (Fischer) for examination. Megakaryocyte numbers were determined by photographing the bone marrow and spleen sections at magnifications of ×400 and ×100, respectively, and then counting megakaryocytes in the photograph and determining the size of the photographic field. Data are presented as cells per 0.5-mm^2 area.

Detection of apoptotic cells. Apoptotic cells were detected by using a modification of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method by amplifying the apoptotic signal with a three-step staining procedure (54). Spleen sections were prepared as described above, and then endogenous peroxidase activity was quenched with a solution of 0.1% of phenylhydrazine in phosphate-buffered saline.

Peripheral blood analysis. One hundred microliters of blood from the lateral tail vein was mixed with 1/10 volume of 3% EDTA and examined on a Technicon H1-E hematology analyzer (Myles, Tarrytown, N.Y.). Blood smears were prepared by spreading peripheral blood onto a glass slide and staining for 1 min with a differential polychrome stain (Fischer). To type megakaryocytes, bone marrow from three animals of each group was extruded by gentle pneumatic pressure from femurs with the ends removed. The marrow was then smeared onto glass slides and stained by using an aerospray hematology slide stainer (model 7120; Wescor, Inc., Logan, Utah). The megakaryocytes were identified and staged according to previously established criteria (39). At least 135 identifiable cells were counted for each mouse.

RT-PCR analysis. For the RT-PCR, 50, 100, or 200 ng of total RNA from bone marrow of the indicated transgenic (PE series) or wild-type mice was reverse transcribed, amplified for 25 cycles by using huE2F-1 primers, and analyzed as described previously (43) except that recombinant *Tth* polymerase (Perkin Elmer, Norwalk, Conn.) was substituted for Moloney murine leukemia virus reverse transcriptase and *Taq* polymerase. Reactions using glyceraldehyde phosphate dehydrogenase (GAPDH) primers were included to demonstrate uniformity of RNA amount and efficiency of amplification among samples. Oligonucleotide primers used for RT-PCR were huE2F-1 (forward, 5′-ACCTTCGTA GCATTGCAGACC-3′; reverse, 5′-TTCTTGCTCCAGGCTGAGTAG-3′) and GAPDH (forward, 5′-GGGTGGAGCCAAACGGGTCATC-3′; reverse, GCCA GTGAGCTTCCCGTTCAGC-3′). Exposure times were 72 h for E2F-1 and 1 h for GAPDH. Quantitation of the amount of radioactivity in each band was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Immunohistochemistry. Four-micrometer sections of paraffin-embedded formalin-fixed bone marrow samples were incubated with 5 μg of a huE2F-1 mouse monoclonal antibody (KH95; Santa Cruz Biotechnology, Santa Cruz, Calif.) per ml and polyclonal rabbit serum specific for PF4 (1:500 dilution; Alexis Corp., Läufelfingen, Switzerland) and then visualized by using species-specific secondary antibodies (anti-mouse antibody conjugated to phycoerythrin and anti-rabbit antibody conjugated to fluorescein isothiocyanate; 1:200 dilution; Dako, Carpinteria, Calif.). The slides were imaged on an ACAS confocal microscope (Meridian Instruments, Okemus, Mich.) and processed by using Adobe Photoshop version 3.0 (Adobe Systems, Inc., Mountain View, Calif.).

Electron microscopy. Femurs from control and transgenic mice were excised, split lengthwise, and immersed in 2.0% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.3. After overnight fixation, the marrow was gently extracted and rinsed in buffer for 24 h. The tissue was postfixed in 1% aqueous osmium tetroxide prior to alcoholic dehydration and infiltration with Medcast epoxy resin (Ted Pella, Redding, Calif.).

Following polymerization, sections were cut from each block and stained with toluidine blue. Selected areas were ultrathin sectioned. The sections were contrast enhanced with uranyl acetate and lead citrate prior to examination on a Philips CM120 transmission electron microscope.

RESULTS

Generation of E2F-1 transgenic mice. Following microinjection, several lines of mice were established, and four of these were assayed for expression of the huE2F-1 mRNA in bone marrow by semiquantitative RT-PCR using huE2F-1 primers (Fig. 1). Primers were chosen such that mouse E2F-1 sequences would not be amplified. All four transgenic lines expressed the transgene, albeit at different levels. Bone marrow RNA from line PE82 appeared to have the highest levels of expression, followed by PE51 > PE50 ≥ PE42 (Fig. 1A). Nontransgenic mice did not show any amplification of huE2F-1 sequences. Quantitation of the DNA in each band with a Molecular Dynamics PhosphorImager showed a 10-fold range of expression between the highest- and lowest-expressing lines.

To determine which cells in the bone marrow were expressing huE2F-1 protein, double immunostaining and confocal microscopic imaging were performed on bone marrow sections from the PE82 line (Fig. 1B). Use of anti-E2F-1 antibodies and a phycoerythrin-labeled secondary antibody revealed nuclear staining in transgenic megakaryocytes. In contrast, no staining was observed in megakaryocytes from nontransgenic bone marrow (Fig. 1B). Although megakaryocytes can be identified by their large size and distinct morphology, immunostaining with antibodies against PF4, a cytoplasmic megakaryocyte-specific marker whose promoter drives E2F-1 expression, was used to confirm the identification of both transgenic and nontransgenic megakaryocytes. Green cytoplasmic fluorescence confirmed the identity of the megakaryocytes (Fig. 1B).

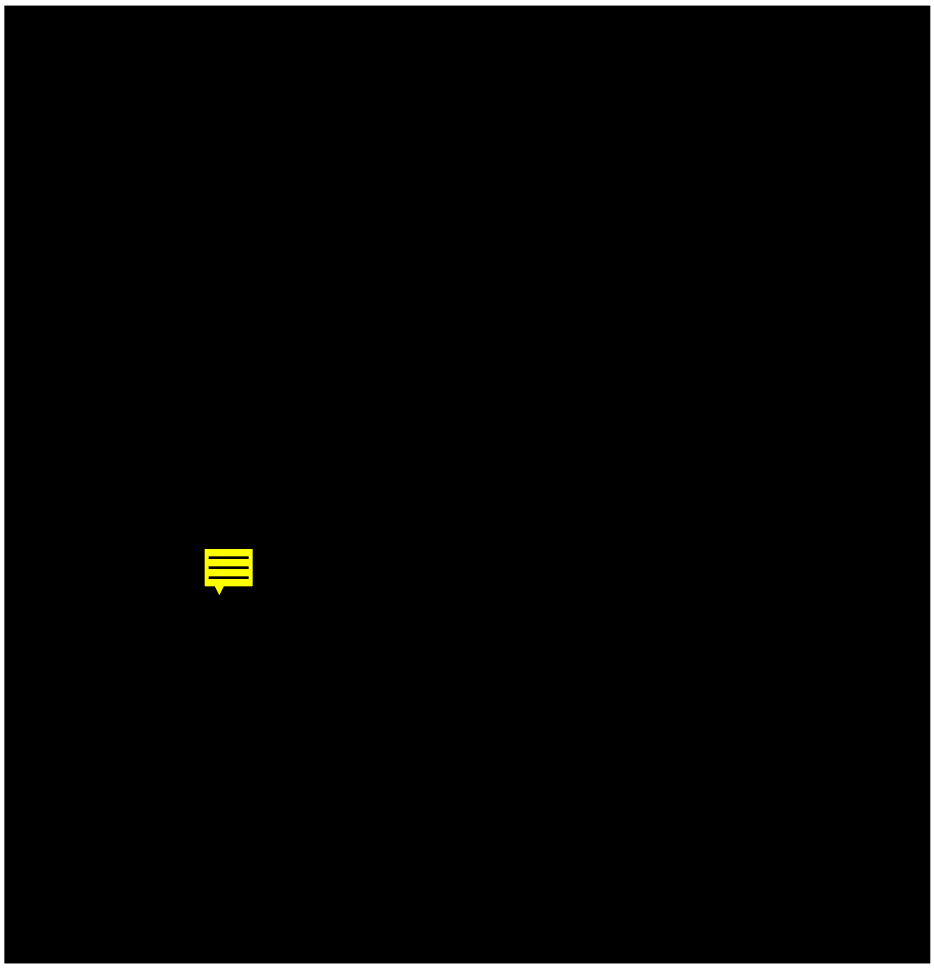

FIG. 2. Differentiation block in E2F-1 transgenic mice. (A) Thrombocytopenia in E2F-1-expressing mice. Platelet counts from several mice ($n \geq 11$) of each indicated transgenic and normal line were determined. The data are presented as means ± SDs of individual determinations. The P value was <0.01 for the difference between the platelet counts of $PE50^{+/+}$ and $PE50^{+/-}$ mice. w.t., wild type. (B) Absence of platelets in the peripheral blood of E2F-1-expressing mice. Representative platelets are indicated by arrowheads. Note the absence of platelets in the blood of the transgenic animal compared with that of a normal littermate. (C) Maturation block in megakaryocytes expressing E2F-1. The data are presented as mean percentages ± SDs.

E2F-1 blocks terminal differentiation of megakaryocytes. To examine the possible effects of E2F-1 expression in megakaryocytes, blood samples from several mice of each line were analyzed by blood smears and by a Technicon H-1E hematology analyzer for alterations in peripheral blood parameters. The highest-expressing line, PE82, exhibited severe thrombocytopenia, with platelet levels measured at $87 \times 10^3/\mu l$ of blood (standard deviation [SD], $21 \times 10^3/\mu l$ of blood), representing only 7% of normal levels (Fig. 2A). This value is likely an overestimate of the actual platelet count as a result of contamination of the platelet window with other cells (data not shown). In support of this assertion, no blood platelets could be detected by cytologic examination (Fig. 2B). The other three lines exhibited lesser degrees of thrombocytopenia (Fig. 2A); platelet numbers given by the H-1E analyzer were consistent with the platelet levels observed in blood smears of each line (data not shown). All other hematopoietic cell values assayed were within the range of the negative littermates (data not shown). Each line maintained a specific level of thrombocytopenia, the severity of which correlated directly with the level of E2F-1 mRNA. Homozygous mice generated from line PE50 confirmed this correlation, as mice carrying two insertions of the transgene had lower platelet counts than their heterozygote littermates (Fig. 2A). This relationship between expression level and degree of thrombocytopenia suggests that the transgene exerts its effects in a dose-dependent manner.

To determine the nature of the defect present in these mice, megakaryocytes in the PE82 bone marrow were further analyzed. The cells appeared larger than normal and displayed unusually large nuclei, some nuclei appearing as ring structures. Despite the unusual appearance of the megakaryocytes, it was possible to determine their maturation stage by using standard criteria (39). The majority (54%) of the megakaryocytes evaluated in the transgenic bone marrow were morphologically judged to be stage 2, characterized by a lobated nucleus, a lightly basophilic cytoplasm, and lack of granulation (Fig. 2C). Normal control mice had equal numbers of the three stages.

Ultrastructural analysis of bone marrow megakaryocytes from the PE82 line demonstrated further evidence of abnormal differentiation. Nontransgenic megakaryocytes exhibited typical networks of cytoplasmic membrane formation that define and demarcate the presumptive nascent platelets (Fig. 3A). In contrast, the cytoplasm of the transgenic megakaryocytes displayed regions of hyperdemarcation, often excluding alpha granules (Fig. 3B and C). The numbers of alpha

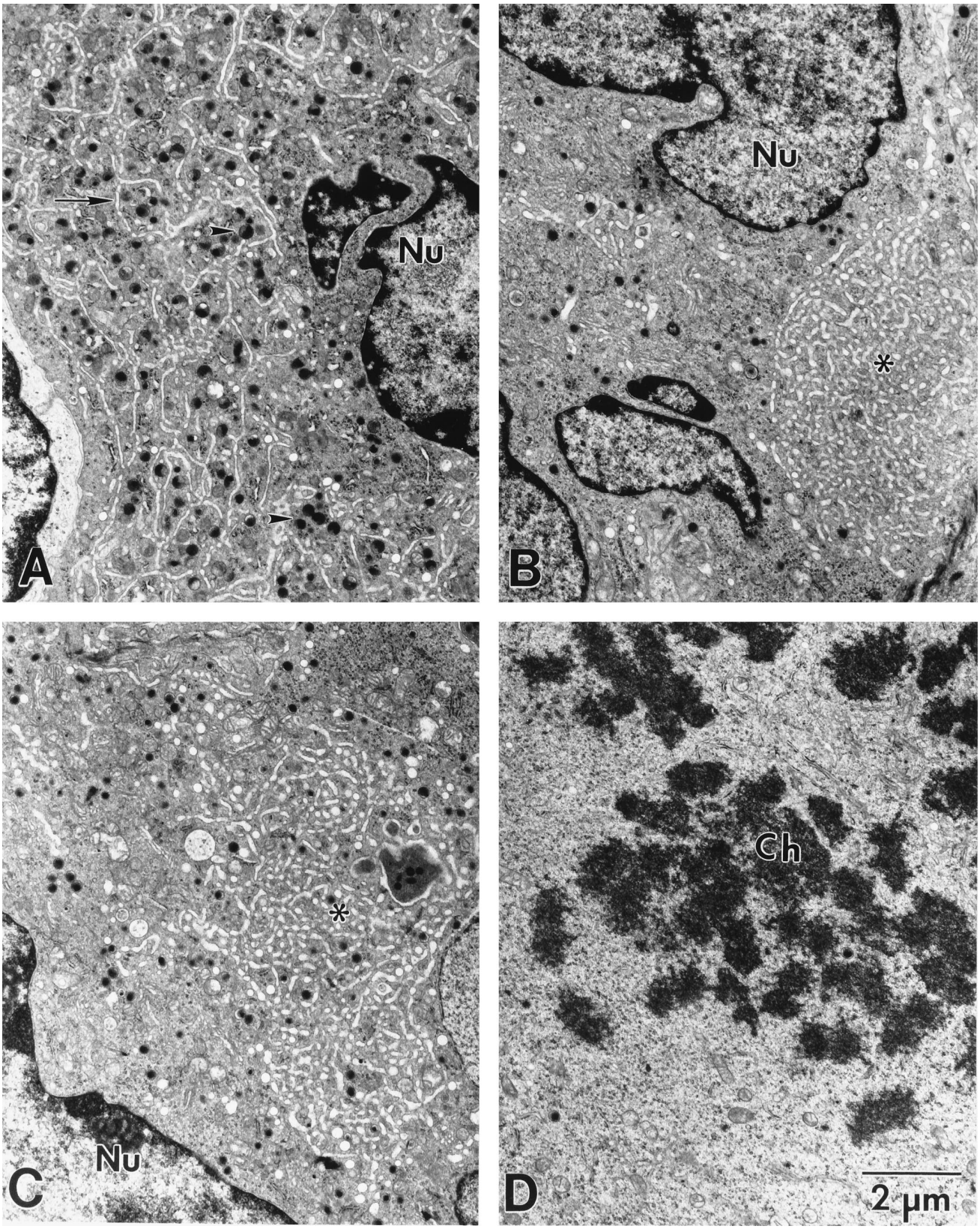

FIG. 3. Ultrastructural defects in PE82 megakaryocytes. (A) Wild-type megakaryocyte showing typical demarcation membrane structure (arrows) and electron-dense alpha granules (arrowheads). This regular pattern defines presumptive regions of nascent platelet formation. Nu, nucleus. (B and C) Typical PE82 megakaryocytes. Note the relative paucity of the alpha granules and the regions of abnormal demarcation membrane formation (asterisks). Disorganized demarcation membranes do not appear to form regions of nascent platelet formation. (D) PE82 endomitosis. Occasional nuclei from PE82 megakaryocytes display chromatin (Ch) condensation and absence of nuclear membrane indicative of a replicating metaphase nucleus.

TABLE 1. E2F-1 transgenic mice do not respond to platelet growth factor administration

Cytokine	Mouse	Mean no. of platelets/μl of blood ± SD		% Change (*P* value)
		Before treatment	After treatment	
MGDF	Nontransgenic	1,360,000 ± 50,000	2,324,000 ± 185,000	+71 (<0.01)
	PE51	219,000 ± 120,000	192,000 ± 111,000	NS[a] (0.70)
	PE82	59,000 ± 22,000	60,000 ± 18,000	NS (0.92)
IL-6	Nontransgenic	1,273,000 ± 320,000	1,679,000 ± 197,000	+32 (0.05)
	PE51	255,000 ± 69,000	167,000 ± 63,000	NS (0.11)
	PE82	175,000 ± 136,000	108,000 ± 30,000	NS (0.36)

[a] NS, not significant.

granules were also reduced in the transgenic mice. This abnormal membrane formation was not uniform, appearing in patches throughout the megakaryocyte cytoplasm. Such structures are not seen in normal megakaryocytes and appear to be inconsistent with nascent platelet formation.

We conclude that E2F-1 expression in megakaryocytes acts to block differentiation during the postmitotic maturation phase. This block in differentiation precludes the formation of platelets, causing extreme thrombocytopenia.

Transgenic megakaryocytes are refractory to treatment with platelet growth factors. In an attempt to overcome the differentiation block observed in the E2F-1-overexpressing mice, five animals each from lines PE82 and PE51 along with five negative littermates were injected daily for 5 days with either 1 μg of recombinant human MGDF or 10 μg of recombinant human interleukin 6 (IL-6). Nontransgenic mice responded predictably to both treatments, exhibiting a 70% increase in peripheral platelet count in response to MGDF and a 30% increase in response to IL-6 (Table 1). In contrast, the transgenic lines were completely refractory to either MGDF or IL-6 administration, exhibiting no significant change in circulating platelet levels. The lack of responsiveness to MGDF was not due to the absence of its cognate receptor, c-mpl, as the MGDF receptor was confirmed on these cells by immunohistochemistry (data not shown). Both MGDF and IL-6 are known to promote differentiation of megakaryocytes (21, 33). Furthermore, IL-6 has been shown to induce terminal differentiation in mature megakaryocytes (1). It appears that E2F-1 overexpression acts to prevent the megakaryocytes from reaching a stage of maturation at which they are able to respond to the differentiative signals initiated by these cytokines.

E2F-1 causes massive megakaryocyte accumulation. To investigate additional changes induced by overexpression of E2F-1 protein, PE82 was chosen for further analysis. Mice from this line exhibited a number of striking characteristics. These mice lived only 9 weeks on average. As mice aged, the erythrocyte count often decreased. Postmortem examination of these animals revealed evidence of internal bleeding, perhaps the cause of death. Additionally, all mice examined displayed splenomegaly, up to approximately 10 times normal spleen weight, as well as grossly enlarged lymph nodes. Hematoxylin-and-eosin-stained sections of decalcified femurs from these animals revealed increased numbers of megakaryocytes in the bone marrow as well as a disruption of the bone marrow architecture (Fig. 4B). The bone marrow sinuses were greatly enlarged, with many containing megakaryocytes. Microscopic examination of the spleen (Fig. 4D) and lymph node (Fig. 4F) revealed a massive accumulation of megakaryocytes. Megakaryocytes in the spleen were primarily found in the red pulp and at the subcapsular region of the tissue. In the lymph node, a tissue normally devoid of megakaryocytes, these cells were observed in clusters throughout the tissue. Megakaryocytes were also found in the blood, lungs, and livers of these animals (data not shown).

To quantitate the number of megakaryocytes in the bone marrow and spleen, cells were enumerated by microscopic examination of tissue sections (Fig. 5A). The bone marrow exhibited an approximately fivefold increase in megakaryocyte number in the two lines examined, PE51 and PE82. Megakaryocyte numbers in the spleen were increased 10-fold in the same lines. In addition to the megakaryocyte numbers, the numbers of megakaryocyte progenitor cells were determined by quantitating the number of megakaryocyte colony-forming units (CFU-Meg) in bone marrow of PE51 and PE82. As shown in Fig. 5B, the number of CFU-Meg was increased between 2.5- and 4-fold, indicating that the number of megakaryocyte progenitors was increased in both transgenic lines assayed.

To determine if the megakaryocytosis was restricted to the adult mice, fetuses from line PE82 were examined at day 15 postcoitus. Examination of embryonic liver, the site of hematopoiesis during fetal development, revealed increased numbers of megakaryocytes, similar to the phenotype seen in adult tissues (Fig. 4H). Every transgenic mouse in the litter exhibited the phenotype. This finding demonstrates that the onset of megakaryocytosis is at least coincident with early fetal hematopoiesis, suggesting that the transgene alone is sufficient to cause the phenotype. Interestingly, large numbers of these cells do not remain in the liver after hematopoiesis moves to adult tissues.

Transgenic megakaryocytes are eliminated by apoptosis. The extent of megakaryocytosis in the transgenic mice suggests that the early megakaryocytes are proliferating. However, it is possible that the megakaryocyte accumulation observed is due to increased survival of cells and not increased proliferation. To address the fate of the transgenic megakaryocytes, further studies were performed. Microscopically, approximately 10% of the megakaryocytes observed appeared to have nuclei with altered staining patterns, suggestive of the appearance of apoptosis. Using a modification of the TUNEL method (54) to detect DNA fragmentation, the presence of apoptosis was confirmed in about 1/10 of the megakaryocytes in the transgenic spleen (Fig. 6A and C). Examination of 60 identifiable megakaryocytes in normal spleen sections (Fig. 6B and D) revealed no evidence of apoptosis, suggesting that apoptosis, if it occurs at all in splenic megakaryocytes, is a rare event. Megakaryocytes are known to undergo endomitosis during the maturation phase, resulting in increases in DNA content up to 128N (18). It is possible that such amplification of DNA leads to a positive signal, using the TUNEL technique. However, because no signal was observed in the nontransgenic megakaryocytes, we consider it unlikely that the signal observed in a subset of the transgenic cells is the result of endomitosis. Preliminary ultrastructure observations using electron microscopy revealed cells

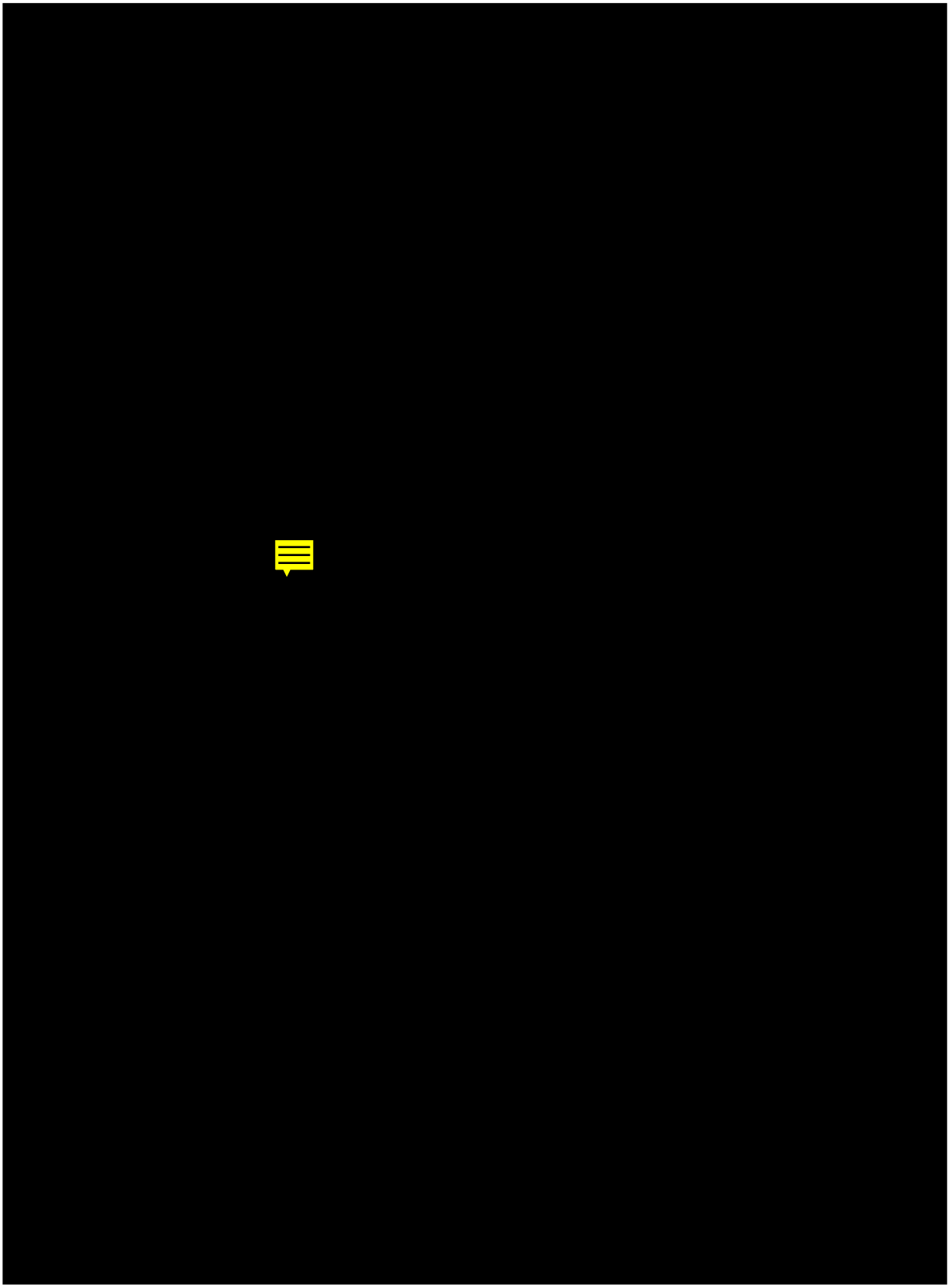

FIG. 4. Proliferation of megakaryocytes in various tissues from E2F-1-expressing mice. (A, C, E, and G) Wild type; (B, D, F, and H) transgenic PE82. (A and B) Bone marrow; (C and D) spleen; (E and F) lymph node; (G and H) embryonic day 15 liver. Representative megakaryocytes are indicated in each panel by arrows. Photomicrographs were taken of hematoxylin-and-eosin-stained 4-μm sections of the indicated tissues. Note the abundance of megakaryocytes in the transgenic tissues compared with normal tissues. Magnification, ×86.

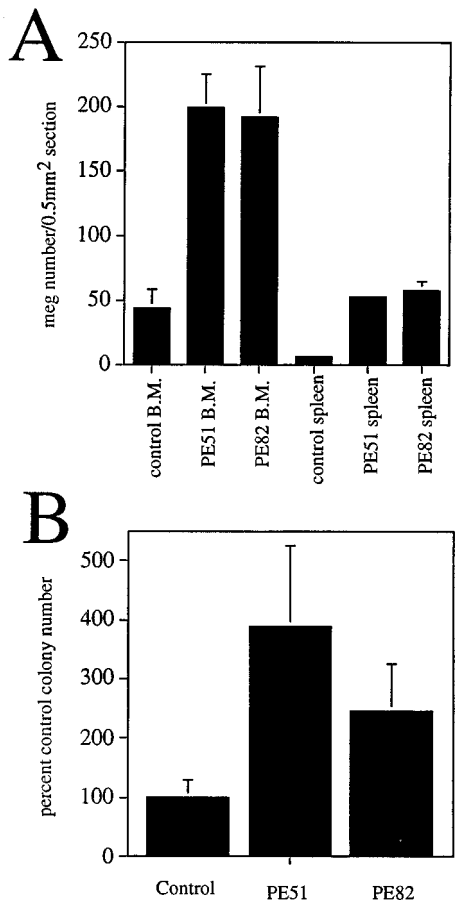

FIG. 5. Megakaryocyte number and CFU-Meg are increased in E2F-1-expressing mice. (A) Megakaryocyte numbers in bone marrow (B.M.) and spleen sections of PE51, PE82, and wild-type mice. The number of megakaryocytes (meg) per field was determined and plotted as cells per 0.5-mm^2 section. At least 50 megakaryocytes were counted per line. The data are presented as means ± SDs of at least three animals per line. (B) Megakaryocyte progenitor numbers in bone marrow of PE51, PE82, and wild-type mice. The number of colonies containing large acetylcholinesterase-positive cells grown in soft agar was determined and plotted as the percentage of wild-type values ± the percent SD. P was <0.01 for both PE51 and PE82 relative to controls.

with apoptotic characteristics, providing support for this conclusion (data not shown). The presence of large numbers of megakaryocytes in normal and ectopic sites, combined with the rapid removal of megakaryocytes through apoptosis, provides strong evidence that the transgene is stimulating a dramatic increase in megakaryocyte production.

DISCUSSION

The decision between proliferation and differentiation requires the concerted activity of genes that regulate the cell cycle. Although E2F-1 is known to have a positive effect on cell division in vitro (24, 26, 51), the data presented here demonstrate that E2F-1 can both stimulate cell division and modulate the differentiative potential of a cell in vivo. E2F-1 expression in the megakaryocyte lineage leads to greatly increased numbers of mature megakaryocytes. However, it appears that the differentiative capacity of these cells is reduced, causing arrest during the maturation phase. Agents known to positively affect terminal differentiation of megakaryocytes cannot function to bypass the differentiation block. Because these megakaryocytes cannot terminally differentiate into platelets, they appear to die through an apoptotic pathway. E2F-1 has been shown previously to cooperate with p53 to induce apoptosis in cultured cells (41, 48, 57). Such a scenario fits with the "clash" hypothesis in which conflicting signals of growth and growth arrest cause entry into the apoptotic pathway (8). The complete penetrance and the early onset of the phenotype suggest that the megakaryocytosis is likely due solely to the effects of E2F-1 expression and not due to secondary events or mutations.

The megakaryocyte accumulation seen in these animals could result from either increased production of early PF4-positive cells (proliferation) or increased survival of the megakaryocytes. The observation that the cells are rapidly being removed through apoptosis suggests that there is increased production of megakaryocytes. However, because these mice are severely thrombocytopenic, endogenous mechanisms should be acting to increase the number and size of the megakaryocytes (10). Such mechanisms do not appear to account for the massive accumulation observed in the E2F-1 transgenic mice. Three other mouse models of severe thrombocytopenia that have been described report two- to fourfold increases in bone marrow megakaryocyte numbers (38, 44, 50), similar to the fivefold increase seen in the E2F-1-expressing mice. However, the E2F-1-expressing mice also have 10-fold increases in the number of splenic megakaryocytes as well as infiltration of megakaryocytes into ectopic sites such as the lymph node and liver. Such numbers and distribution of megakaryocytes have not been observed in the other thrombocytopenia models, suggesting that there is some additional proliferative signal in the E2F-1-expressing mice. We therefore postulate that E2F-1 is able to increase the number of cell divisions in the committed precursor cells.

Although E2F-1 appears to stimulate increased proliferation of megakaryocyte precursors, it is unable to prevent the cells from initiating differentiation. This is likely due to the temporal onset of PF4 promoter function, occurring as megakaryocyte precursors are entering a differentiative phase. Despite expression of E2F-1, these committed precursors can exit the cell cycle and give rise to postmitotic, albeit abnormal megakaryocytes. Targeting expression of E2F-1 to mitotic progenitor cells would address whether the protein could prevent differentiation into megakaryocytes.

Previous experiments suggest that Rb may be important for differentiation of this lineage, as megakaryocytes from mice expressing a megakaryocyte-targeted simian virus 40 temperature-sensitive large T antigen were unable to terminally differentiate (44). A possible mechanism to explain the phenotypes observed in both of these mice stipulates an interaction between Rb and E2F-1 to control differentiation. We propose that Rb, known to be present in megakaryocytes (55), allows the cell to differentiate through inhibition of E2F transcriptional activity. An increase of active E2F-1 (either through overexpression or by blocking Rb) prevents the cell from terminally differentiating. This cell, receiving conflicting signals of differentiation and proliferation, activates an apoptotic pathway leading to cell death. It is formally possible that E2F-1 functions not as a transcriptional activator but only to bind and inactivate Rb. Transgenic expression of E2F-1 mutants deficient for either Rb binding or transcription would be required to rule out this possibility.

These results demonstrate the dramatic effects of deregulation of the E2F-1 protein in vivo and suggest that the cell cycle-regulatory proteins, CDKs, CDK inhibitors, and Rb function to control differentiation through modulation of E2F activity. Furthermore, this mouse may serve as a useful tool to

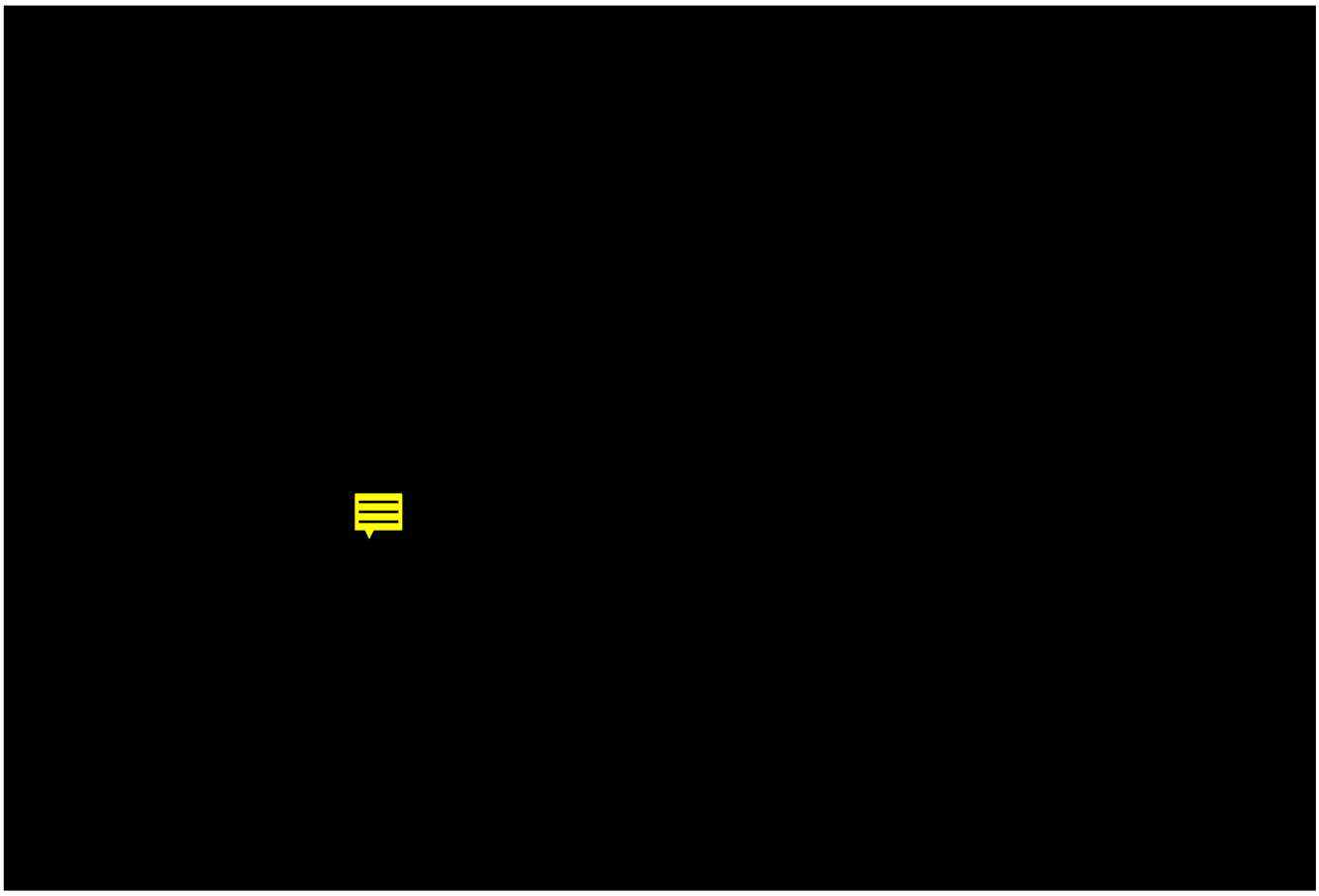

FIG. 6. In situ detection of apoptotic megakaryocytes in the spleens of E2F-1 transgenic animals. Approximately 10% of the megakaryocytes in the spleen sections of E2F-1-expressing mice presented direct evidence of cell death, whereas none were detected in the normal animal. (A and C) PE82 spleen; (B and D) wild-type spleen. Arrowheads indicate apoptotic megakaryocytes. m, nonapoptotic megakaryocyte. Magnifications: (A and B) ca. ×18; (C and D) ×71.

develop reagents that impair E2F-1 function, reagents that may have a therapeutic use in cancer treatment.

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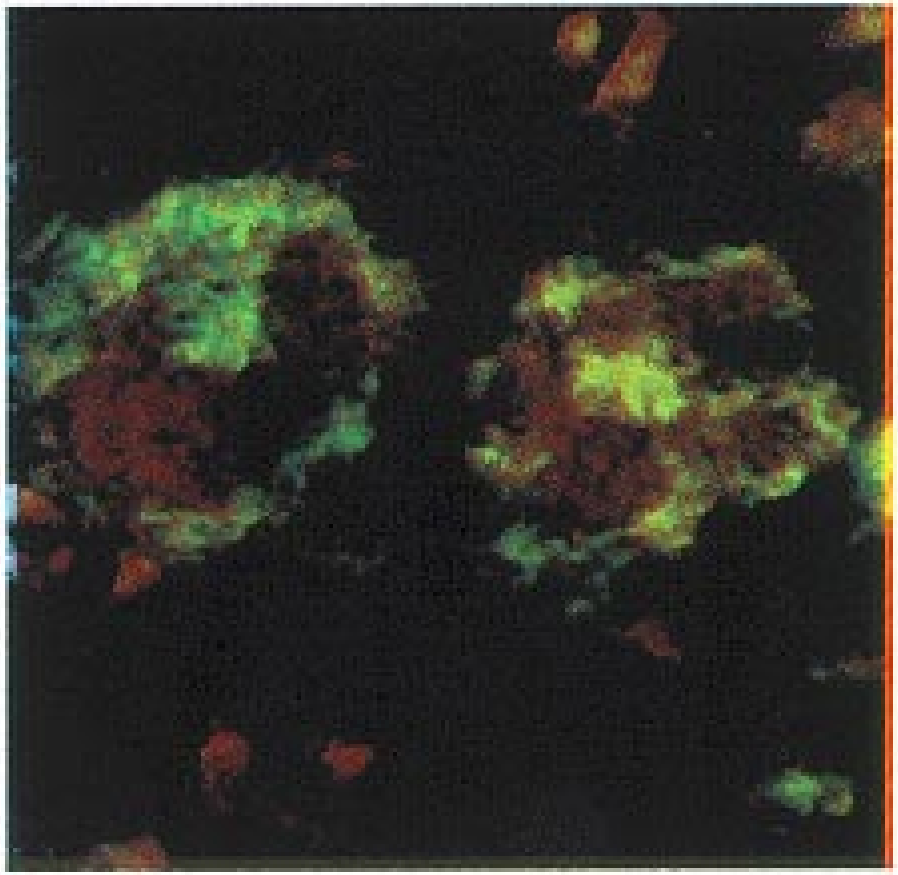

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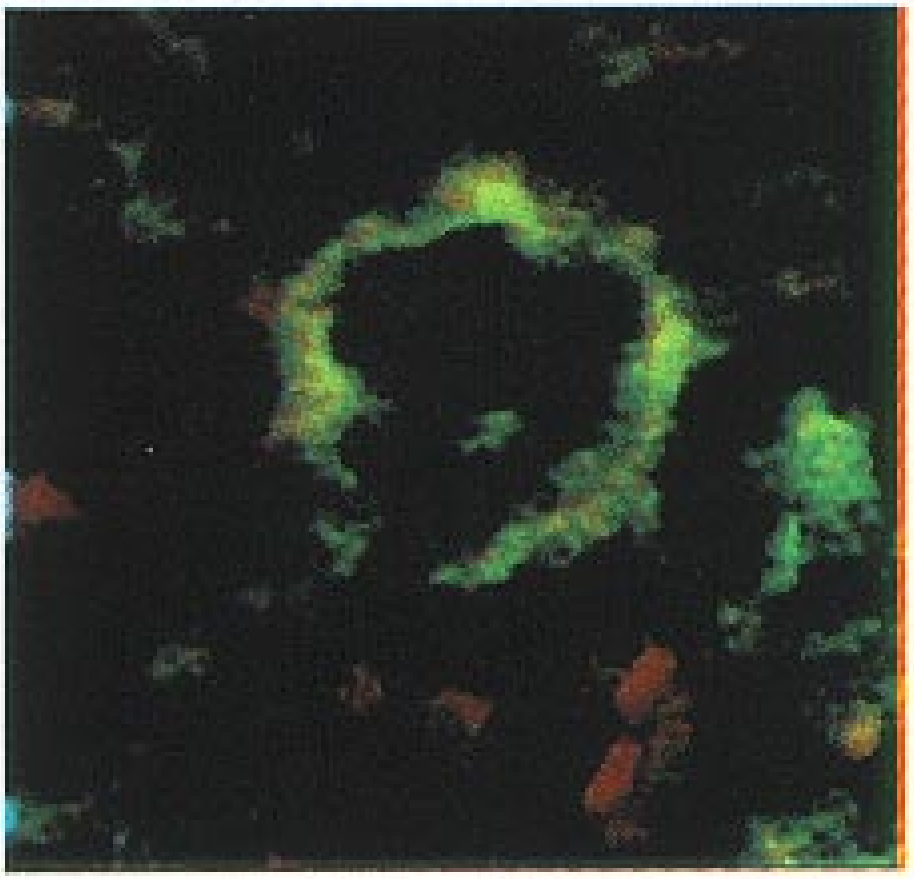

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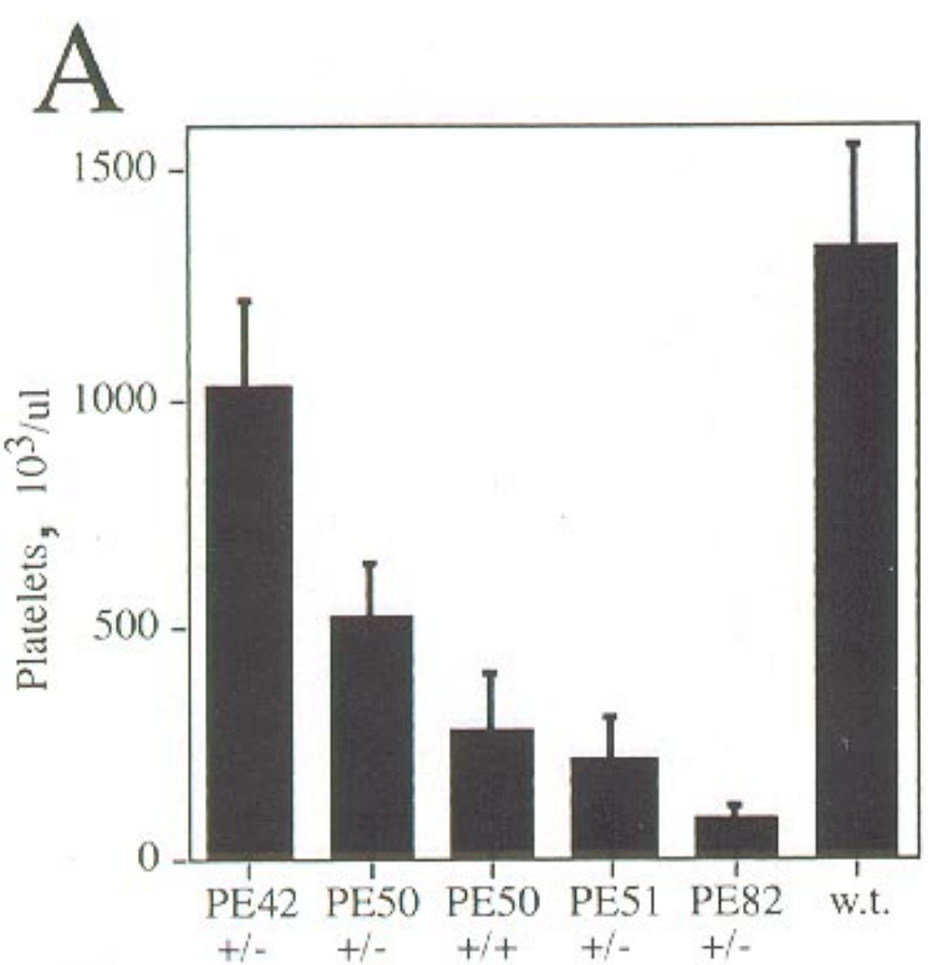
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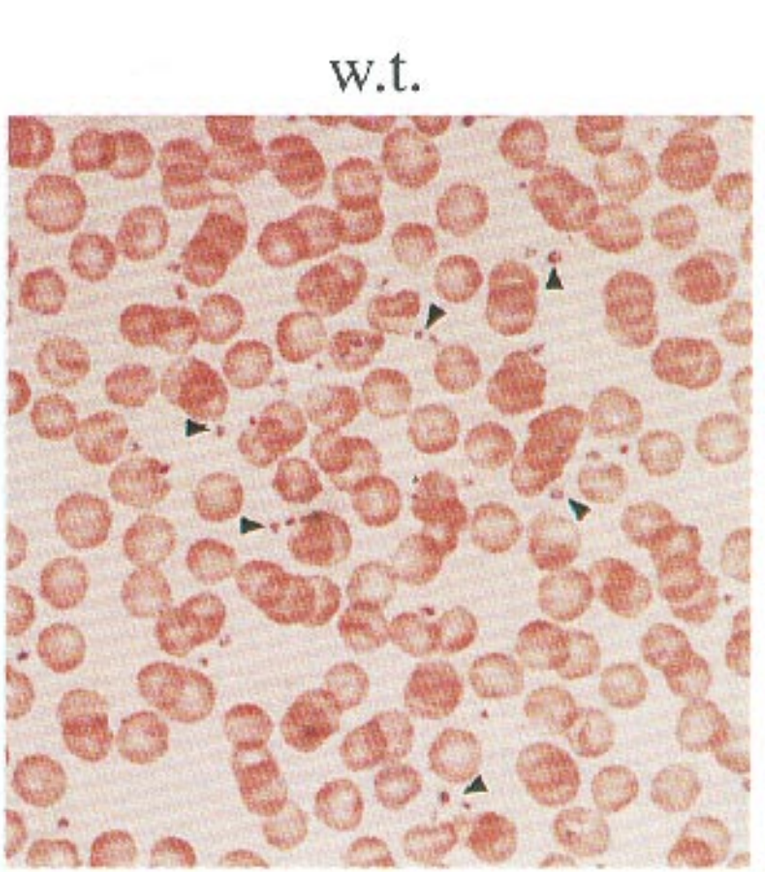
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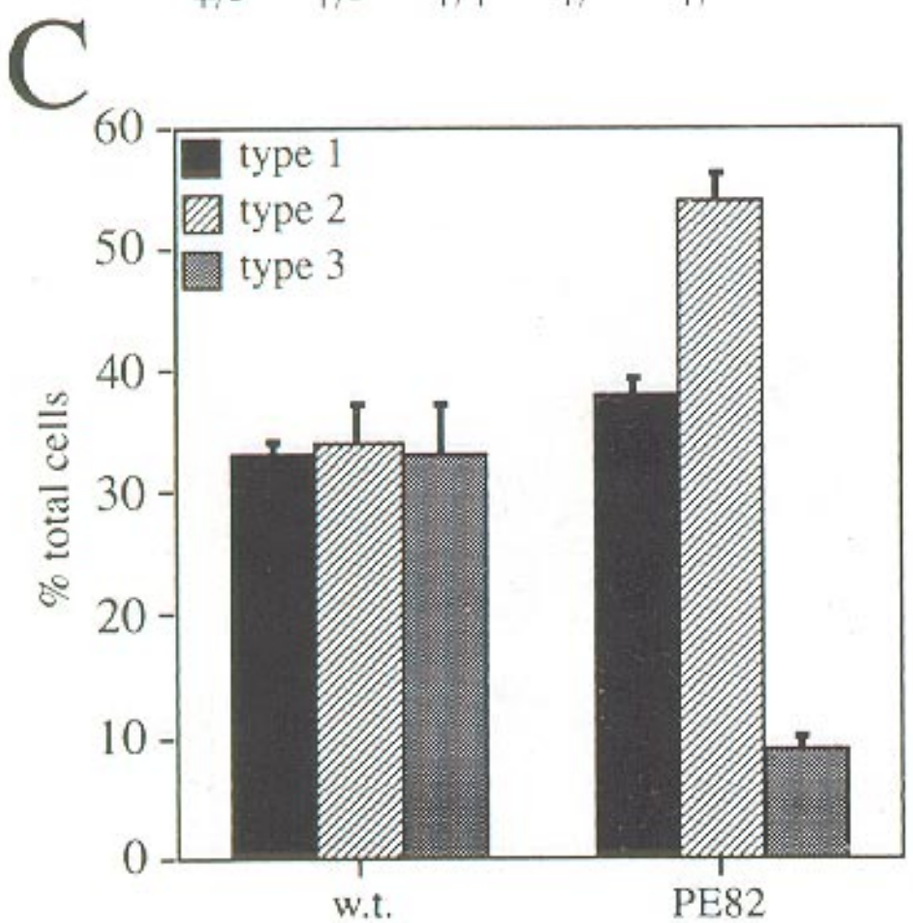
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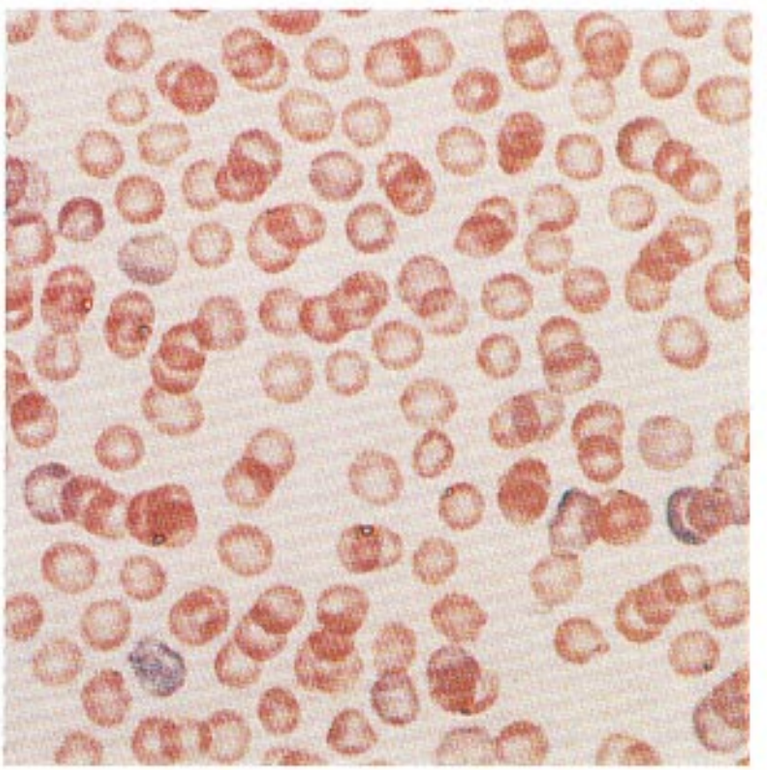
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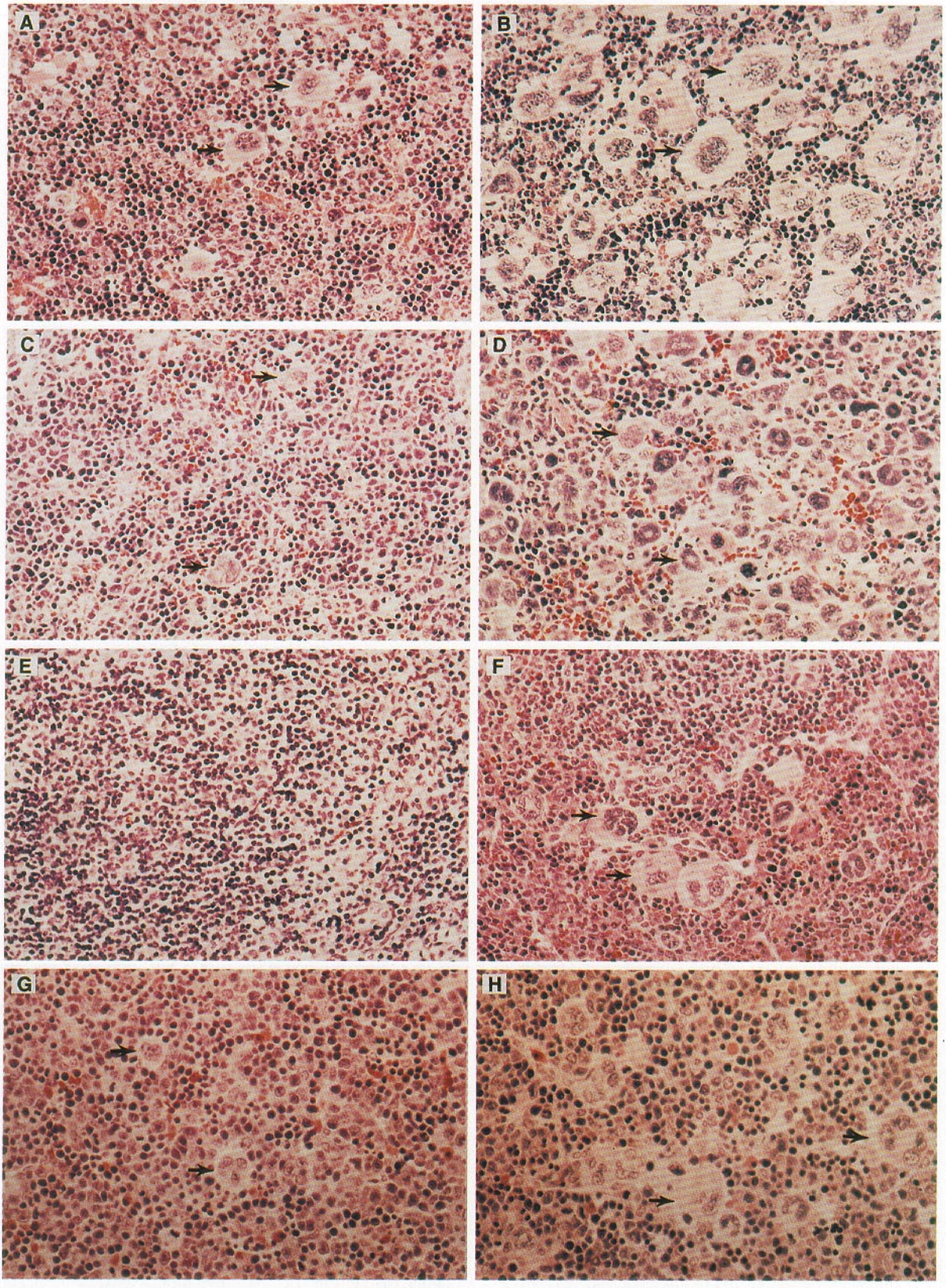
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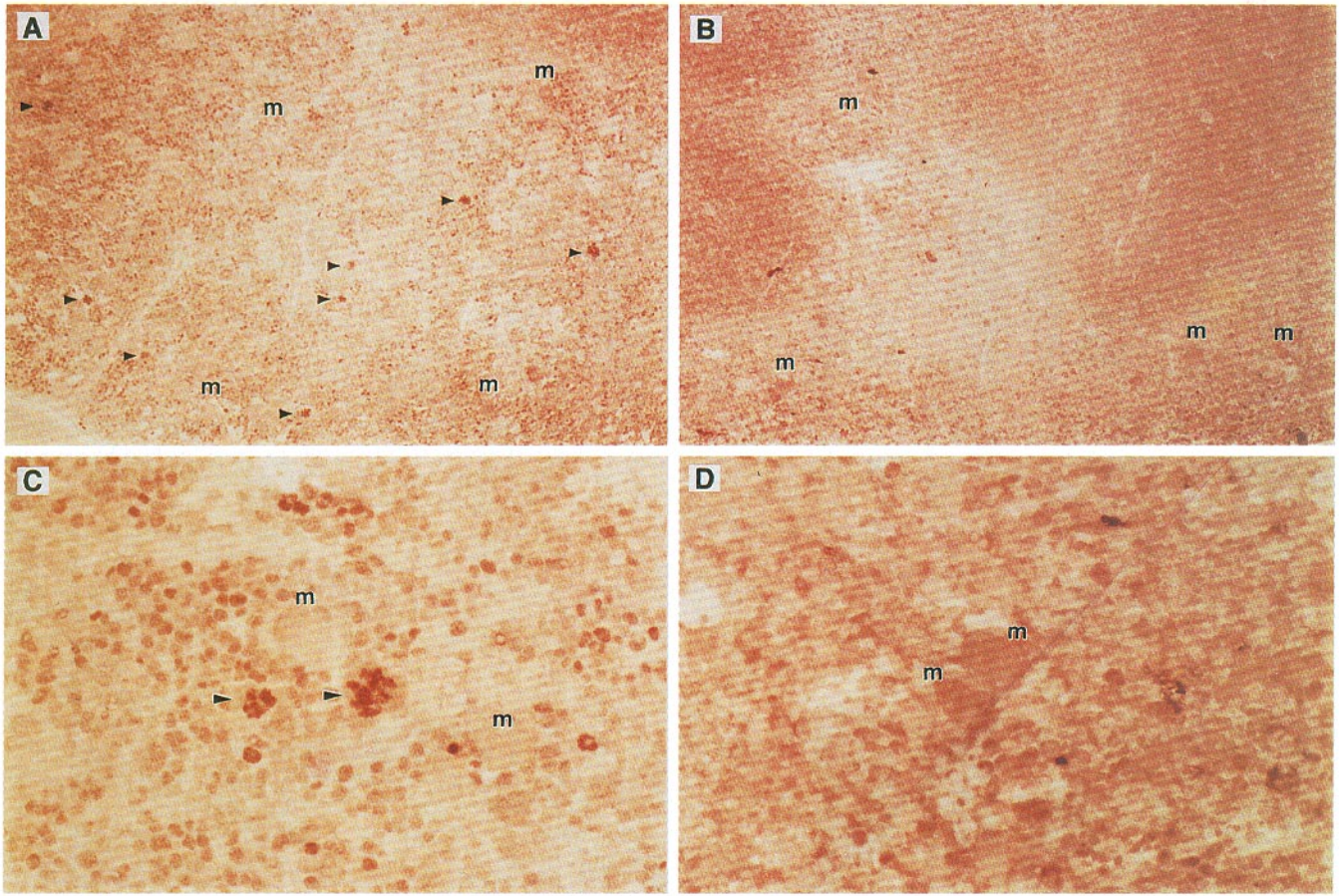
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