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Overexpression of cyclin D1 moderately increases ploidy in megakaryocytes

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Background and Objectives. Megakaryocytes undergo a unique cell cycle by which they replicate their complete genome many times in the absence of cytokinesis. In the search for regulators of the endomitotic cell cycle, we previously produced mice transgenic for cyclin D3 to identify this cyclin as able to enhance ploidy and to increase the number of differentiated cells in the megakaryocytic lineage. Of the D-type cyclins, cyclin D3 and to a much lesser extent cyclin D1, are present in megakaryocytes undergoing endomitosis and these cyclins are, respectively, markedly and moderately upregulated following exposure to the ploidy-promoting factor, Mpl-ligand. Our objective was to explore whether cyclin D1 can mimic the effect of cyclin D3 on ploidy in megakaryocytes.

Design and Methods. We generated transgenic mice overexpressing cyclin D1 in megakaryocytes and analyzed megakaryocyte ploidy, number and platelet levels in these mice and control mice.

Results. We show that transgenic mice in which cyclin D1 is overexpressed in megakaryocytes display higher ploidy level than the control mice, with no change in the number of differentiated cells of the megakaryocytic series, or in platelet level.

Interpretation and Conclusions. Our models support a key role for D-type cyclins in the endomitotic cell cycle, and also indicate that although cyclin D3, from among the D cyclins, is unique in its high levels of expression in megakaryocytes, it is not unique in its ability to promote polyploidization.

Key words: cyclin D1, megakaryocytes, ploidy

Megakaryocytes, precursors of circulating platelets, originate from committed progenitors derived from pluripotent hematopoietic stem cells. At a still undefined point during differentiation, megakaryocytes cease to proliferate and enter endomitotic cell cycles, by which cellular DNA content can increase to 128N.[1,2] Polyploidization has been viewed as a marker of megakaryocytic differentiation, aside from several other membrane-associated glycoproteins that are also abundantly expressed in platelets.[3,4] The major cytokine, thrombopoietin (TPO) or megakaryocyte growth and differentiation factor (MGDF), that stimulates megakaryocyte differentiation was isolated in 1994.[5-9] The effect of TPO on megakaryocytopoiesis is mediated through its cognate receptor c-Mpl.[10,11] Signal transduction originating from c-Mpl leads to stimulation of megakaryoblastic proliferation and megakaryocytic differentiation, including polyploidization.

Preparation for DNA replication in a mitotic cell cycle commences in G1 phase in which the cyclin-dependent protein kinase 4/6 (CDK4/6), in association with its regulatory partner, the D-type cyclin, phosphorylates the retinoblastoma protein, pRb. Hypophosphorylated pRb complexes with the transcription factor E2F and holds E2F/DP1 in check.[12] E2F is released from the CDK4/6-phosphorylated pRb and is free to activate the transcription of target genes that are necessary for DNA replication.[13] We previously reported the existence of a gap phase prior to the synthesis phase during the megakaryocytic endomitotic cell cycles and that cyclin D3 is highly abundant in these cells.[14] Studies by Datta and colleagues indicated that there is a significant increase in G1-associated Cdk complexes (with D-type cyclins) in a polyploidizing megakaryocytic cell line (HEL).[15] We hypothesized that a G1 cyclin may be the target of TPO-induced signal transduction that leads to high ploidy. This hypothesis is supported by the finding that overexpression of cyclin D3 induced polyploidization of megakaryocytes in transgenic mice.[16] In fact, the increase in polyploidization induced by overexpressing cyclin D3 is similar to that induced by TPO.[16] Most interestingly, TPO itself was able to increase cyclin D3 levels substan-

tially in megakaryocytes *in vivo* (about 5 fold), and to a much lesser extent the level of cyclin D1 (1.7 fold), while cyclin D2 was not detectable in these cells.[16] These observations indicate that cyclin D3 is one of the physiologic effectors of TPO. However, they do not exclude the possibility that the role of cyclin D3 in megakaryocyte polyploidization could be covered by other D-type cyclins. In this study, we addressed the question of whether the polyploidization-promoting effect of cyclin D3 is unique to this D-type cyclin, i.e., whether the effect could also be produced by cyclin D1? This question is particularly relevant in view of the speculated unique functions of different D-type cyclins in specific cell types.[17] We report that although cyclin D3, among D cyclins, is unique in being expressed at high levels in megakaryocytes, it is not unique in its ability to promote polyploidization, as overexpression of cyclin D1 resulted in an increase in ploidy in megakaryocytes.

Design and Methods

Plasmid

Mouse genomic cyclin D1 clones, D1-ϕ6, (containing the first 4 exons of the gene) and D1-6X-XX5 (containing exons 4 and 5), were kindly provided by Drs. Peter Sicinski and Robert Weinberg. The Eag1-XbaI fragment of D1-ϕ6 containing cyclin D1 exons II and III was joined to exon V of D1-6X-XX5 using a Not I linker. The XbaI fragment containing exon IV and the Eag I fragment containing exon I were inserted sequentially (to the appropriate position) to generate the full-length mouse cyclin D1 genomic clone. During this engineering , an additional intron fragment was inserted between these exons IV and V. This full-length D1 genomic DNA was introduced to platelet factor 4-human growth hormone polyA (PF4-hGH) plasmid using a Sac I-Eag I linker and a Hind III-Nhe I linker to generate PF4-cyclin D1-hGH recombinant plasmid. All cloning manipulations were confirmed by DNA sequence analyses.

Transgenic mice

The Not I fragment of PF4-cyclin D1-hGH containing full-length mouse cyclin D1 genomic DNA placed under the control of the rat PF4 promoter was microinjected into pronuclei of one-cell FVB/N strain mouse embryos to produce transgenic mice, as described previously.[18] Ten micrograms of tail DNA were digested with Bam HI and electrophoresed on an agarose gel. Fractionated DNA was transferred to a nylon membrane, blocked, and hybridized with a ^{32}P-labeled cyclin D1 probe, yielding a 2.2 kb endogenous and a 1.6 kb exogenous band. Repeated Southern blot analyses identified eight PF4-cyclin D1 founder lines. The mice were bred to F1 and F2 generation and used for analyses at the age of 6-10 weeks.

Acetylcholine esterase assay and immunohistochemistry

Bone marrow was harvested from femora of control or transgenic mice, as described before.[18] Megakaryocytes that displayed acetylcholine esterase activity were identified as described elsewhere.[19] Immunohistochemistry with anti c-myc or anti-cyclin D1 (Oncogen Science, Uniondale, New York, USA) were performed as described before.[20]

Ploidy and statistical analyses

Ploidy analysis was performed as already described.[16] Briefly, bone marrow cells were suspended in CATCH buffer, and were allowed to react with rat monoclonal antibody 4A5 21 and subsequently with a fluorescein-conjugated goat anti-rat IgG (Fab')$_2$ (Biosource International, Camarillo, CA, USA). Cells were stained with propidium iodide and the cellular DNA content was determined using a statistical package on a FACScan flow cytometer (Becton Dickinson, Rutherford, NJ, USA), as described before.[20]

Semi-quantitative polymerase chain reaction (PCR)

Total RNA isolated from control, PF4-cyclin D3 mice,[16] or PF4-cyclin D1 mice was reversely transcribed and the first-strand cDNA was used in PCR reactions containing 0.5 μCi γ-^{32}P-dATP. The PCR was stopped at the indicated cycle and labeled products were separated on a native 10% polyacrylamide gel. The gel was dried and autoradiographed. The D1 sense primer 5'-CCAGGAACAGATTGAAGCC-3' and hGH antisense primer 5'-TCCTGACGACGTCCCCCTTG-3' were designed to distinguish products amplified from either the first-strand cDNA or from the possible contaminating genomic DNA. Genomic DNA contamination was not detected in any case.

In situ hybridization

Tissues were collected and subjected to *in situ* hybridization using mouse sense or antisense cyclin D1 cDNAs as riboprobes, as we described in our previous papers.[16]

Histologic analysis of tissues

Samples from the sternum, femora, spleen, liver, and thymus were placed in a fixative (Optifix, American Histology, Lodi, CA, USA) immediately after their removal from mice. Fixed samples were sent to the Transgenic Histopathology Laboratory, University of California, Davis, USA. There, the samples were dehydrated, embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin for further interpretation. Dr. Robert Cardiff, an expert in mouse pathology, assisted in analysis of the samples (at least three sections for each mouse line).

Results

PF4-cyclin D1 transgenic mice express various levels of transgene mRNA

To examine the effects on megakaryocytic polyploidization elicited by overexpressing cyclin D1, we created cyclin D1 transgenic mice in which the expression of the exogenous mouse cyclin D1 gene is driven by the rat PF4 promoter. It has been documented that transcription from the PF4 promoter is specific for the megakaryocytic lineage.[17] Eight founder lines were identified by tail genomic DNA Southern blot analysis (Fig-

ure 1). As suggested by Southern blot, multiple copies of D1 transgene were inserted into the genome and the level of expression varied among founder lines. In all lines, expression of D1 transgene could be detected by reverse transcription PCR of bone marrow cells derived from F1 generation mice (not shown). Immunohistochemistry involving a reaction with anti-cyclin D1, followed by staining with anti-IgG conjugated to a fluorescent substrate indicated a very moderate difference in fluorescence in cytospun bone marrow transgenic megakaryocytes, as compared to controls, but only in founder line 2. In order not to rely on a subjective estimation, cells were also analyzed by a more quantitative assay involving *in situ* hybridization with cyclin D1 riboprobe. Counting grains in each megakaryocyte subjected to this examination revealed an approximately two fold induced expression of the D1 transgene in megakaryocytes of founder lines 2 > line 9, i.e., for slides exposed for four weeks, in control megakaryocytes we counted 85±17 grains, while in megakaryocytes of comparable size from line 2 we counted 203±41 grains and in line 9 we counted 156±11 grains per megakaryocyte (n=15) (Figure 2). The level of cyclin D3 mRNA in megakaryocytes of cyclin D1 mice was similar to that in control cells.

Increased abundance of cyclin D1 in D1 transgenic mice correlates with increased ploidy

We examined the ploidy level of megakaryocytes from all the PF4-cyclin D1 mice (sex and age-matched) by FACS analysis of 4A5-positive (megakaryocyte specific) cells of different ploidy classes. A mild but significant increase in ploidy was observed in founder lines 2 and 9. The proportion of 32N cells was significantly increased (p <0.05, line 2) as analyzed by the two-sample rank test (Table 1). The level of increase in polyploidization was, however, lower than that observed in the PF4-cyclin D3 mice, i.e., there was an equivalent increase in 32N cells in both models (about 1.4 fold), but no increase in 64N cells in cyclin D1 mice while a 10 fold increase in 64N cells was observed in cyclin D3 mice.[16] It should be pointed out, however, that the level of induction of cyclin D3[16] was much greater (about 8 fold) than that of cyclin D1 (about 2 fold) (Figure 2). The increase in polyploidization was restricted to lines that displayed increased cyclin D1 level (Table 1), suggesting a cause-effect relationship between the level of cyclin D1 and that of polyploidization. In other founder lines, in which low-level cyclin D1 expression was observed, there was in fact a trend toward a decrease in ploidy, but only line 21 showed a significant decrease (p <0.05) in 32N cells.

Neither the number of mature megakaryocytes nor platelet number changed in PF4-cyclin D1 mice

As reported previously, the number of megakaryocytes increased in PF4-cyclin D3 mice by about two fold without affecting the number of circulating platelets.[16] Also,

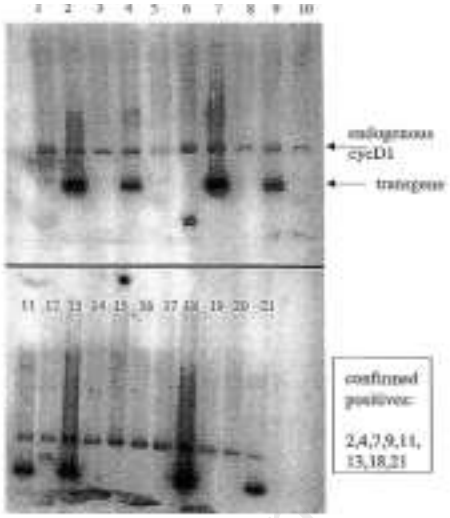

Figure 1. Southern blot analysis of the PF4-cyclin D1 mice. Tail genomic DNA isolated from the PF4-cyclin D1 mice was digested by restriction enzymes as indicated, electrophoresed, transferred to a nylon membrane and probed with ^{32}P-labeled mouse cyclin D1 cDNA. The designated number of each potential founder line is indicated at the top of each lane. The positions of endogenous and exogenous signals are indicated.

several models of transgene expression driven by the PF4 promoter indicated that transgene expression is restricted to the megakaryocytic lineage, with no effect on the expansion of other cell types such as the erythroid or myelomonocytic lineages.[16,20] Histologic analysis of all four compartments in the sternum of each transgenic line indicated that the marrow of cyclin D1 overexpressing mice, as that of c-myc overexpressing cells,[20] appeared hypercellular and was populated by small immature myeloid-type cells (Figure 3). As expected from the restricted expression driven by the PF4 promoter, there was no increase in the number of cells expressing markers of the erythroid (TER-119) or monocytic (Mac-1) lineage (*data not shown*, and refs. #16, 20). Hypercellular marrow in cyclin D1 transgenic mice was also noted during histologic analysis of spleens (compare cellularity in panel C to A in Figure 2 and details under *Methods*). An average of thirty morphologically identifiable megakaryocytes was determined in each sternum compartment of a control mouse, as we also reported before.[20] A similar number was counted in the sternum of the cyclin D1 transgenic lines 2 or 9 (analysis of four mice). Bone marrow derived from control or from PF4-cyclin D1 mice was cytospun (100,000 cells/slide) and subjected to *in situ* staining for acetylcholine esterase. This analysis revealed that the number of acetylcholine esterase-positive cells (megakaryocytes) in these preparations was not significantly different (60-80 megakaryocytes per slide, as concluded from three

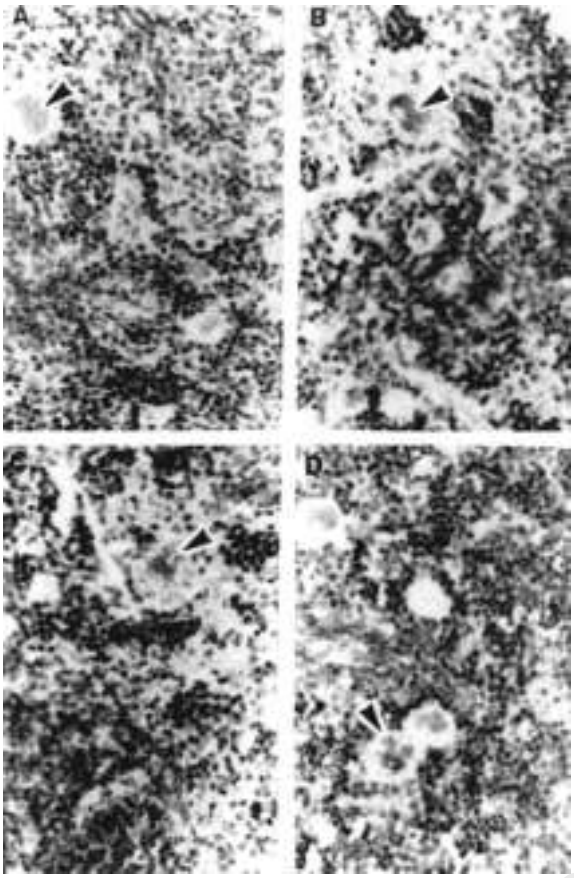

Figure 2. Expression of cyclin D1 in PF4-cyclin D1 mice. *In-situ* hybridization analysis revealing the abundance of cyclin D1 message in spleen sections of control and PF4-cyclin D1 mice. Bone marrow sections of control mice were hybridized with ^{35}S-labeled sense (A) or antisense cyclin D1 riboprobe (B). Bone marrow sections of PF4-cyclin D1 mice line 2 (C) or line 9 (D) were hybridized with ^{35}S-labeled antisense cyclin D1 riboprobe, or sense (results not shown, as in A). Slides were autoradiographed after a two-week exposure. In a parallel experiment (data not shown), the level of cyclin D3 in megakaryocytes of cyclin D3 transgenic mice[16] was about 8 fold higher than that in the controls, while that of cyclin D1 in cyclin D1 transgenic mice was about 2 fold higher. The level of cyclin D3 mRNA in megakaryocytes of cyclin D1 mice was similar to that in control cells (not shown). This was evaluated by grain counting in several cells (n=20) subjected to *in situ* hybridization with appropriate riboprobes, using the techniques we have already described.[16] The arrows point to some megakaryocytes (magnification x150).

determinations), in accordance with the lack of increase in 4A5-positive cells in the transgenic lines (Table 1). Since the number of acetylcholine esterase-positive cells did not increase in the transgenic mice, we conclude that the specific level of cyclin D1 reached in the transgenic cells induced expansion of immature cells that did not reach the differentiation stage at which acetylcholine esterase is expressed. This phenotype was also observed in c-myc overexpressing transgenic megakaryocytes.[20] The number of circulating platelets was unaltered in the low-expressing and high-expressing transgenic lines, as compared to control (Figure 4).

Discussion

We have previously reported that from among the D-type cyclins, cyclin D3 and to a much lesser extent cyclin D1, but not cyclin D2, are present in polyploidizing megakaryocytes.[14] We also demonstrated that overexpression of cyclin D3 led to a precipitous increase in ploidy in megakaryocytes,[16] and reported that TPO induced the expression of cyclin D3, and to a lesser extent of cyclin D1, *in vivo*.[16] In this study, we report a change in ploidy level in megakaryocytes depending on the level of cyclin D1. An increase in ploidy correlated with an increase in cyclin D1 level in transgenic megakaryocytes, suggesting a cause-effect relationship between the level of cyclin D1 and that of polyploidization. Interestingly, a previous study in the Dami megakaryocytic cell line indicated that overexpression of cyclin D1 enhanced the ability of phorbol esters to induce polyploidization in these cells, while by itself, cyclin D1 caused cell cycle arrest.[22] There was no indication of cyclin D1-induced cell cycle arrest in our transgenic mice, suggesting that the results in Dami cells are due to either a cell line specific property and/or the immense overexpression of cyclin D1 achieved in this system. We do recognize that the level of increase in polyploidization in PF4-cyclin D1 mice was less than that observed in PF4-cyclin D3 mice, but so also was the induction of transgene expression. Interestingly, we observed a tendency for reduced level of polyploidization in cyclin D1 transgenic mice that displayed a low level of transgene expression. This could have been due to an increase in immature cells (see below), as also noted for c-myc overexpressing transgenic mice.[20] This leads us to conclude that tight regulation of cyclin D1 level may be needed to maintain a balance between a shift to low ploidy versus high ploidy in this lineage. As both cyclin D1 and cyclin D3 were placed under the control of the rat PF4 promoter, and multiple copies of transgene were detected in both transgenic lines, we speculate that D1 messenger RNA might be relatively unstable in mouse megakaryocytes. Although further analysis is warranted, this hypothesis is consistent with the fact that the level of the endogenous D1 transcript is much lower than that of the D3.

In terms of expansion of the megakaryocytic lineage, it is interesting to note that overexpression of cyclin D3,[16] or myc[20] in megakaryocytes of transgenic mice increased the megakaryocytic population, suggesting that activation of positive regulators of the G1 phase may lead to megakaryocytic expansion. Expansion of the PF4-expressing immature cells could be induced by a moderate elevation of cyclin D1, yielding a marrow with increased number of immature, myeloid-type cells. There was not, however, an increase in acetylcholine esterase-positive or 4A5-positive cells, indicating that at the level of cyclin D1 reached, the expanded population

Table 1. Megakaryocyte DNA content distribution in transgenic mice.

		% of cells in ploidy class*						
Mouse Group	*Sample*	*2N*	*4N*	*8N*	*16N*	*32N*	*64N*	*128N*
Control	n=4	21.1±1.8	9.9±0.5	5.2±0.8	39.8±2.3	21.2±3.0	2.0±0.1	0.7±0.2
Line 2	n=3	23.4±3.4	10.8±1.7	4.4±0.8	30.9±2.3	27.9±2.6	2.1±0.4	0.4±0.3
Line 7	n=3	20.7±0.1	11.0±1.3	6.1±2.7	39.9±3.1	20.5±4.4	1.3±0.2	0.5±0.3
Line 9	n=3	17.4±2.6	10.9±3.0	7.2±1.6	37.1±7.1	24.1±6.5	2.7±1.2	0.7±0.3
Line 13	n=2	17.4±2.8	7.5±2.8	10.2±0.1	47.3±4.1	15.5±2.4	1.6±0.9	0.6±0.1
Line 18	n=3	20.8±6.6	10.3±3.3	7.9±1.3	44.1±5.6	15.4±2.7	1.0±0.3	0.4±0.1
Line 21	n=3	22.0±3.9	9.2±2.2	7.9±1.3	42.8±4.9	15.8±0.4	1.6±0.2	0.6±0.0

**Bone marrow cells derived from femurs of control or PF4-cyclin D1 transgenic mice (except for line 11 which was not expanded as a line) were incubated with the rat monoclonal antibody 4A5 and subsequently with a florescein-conjugated anti-rat antibody. The percentage of 4A5-positive cells was similar in all samples tested (not shown). Cells were stained with propidium iodide and the cellular DNA content was determined by flow cytometry analysis gated for florescein-positive cells, as described under Methods. The percentage of cells in each ploidy class is presented as means±standard errors of the means.*

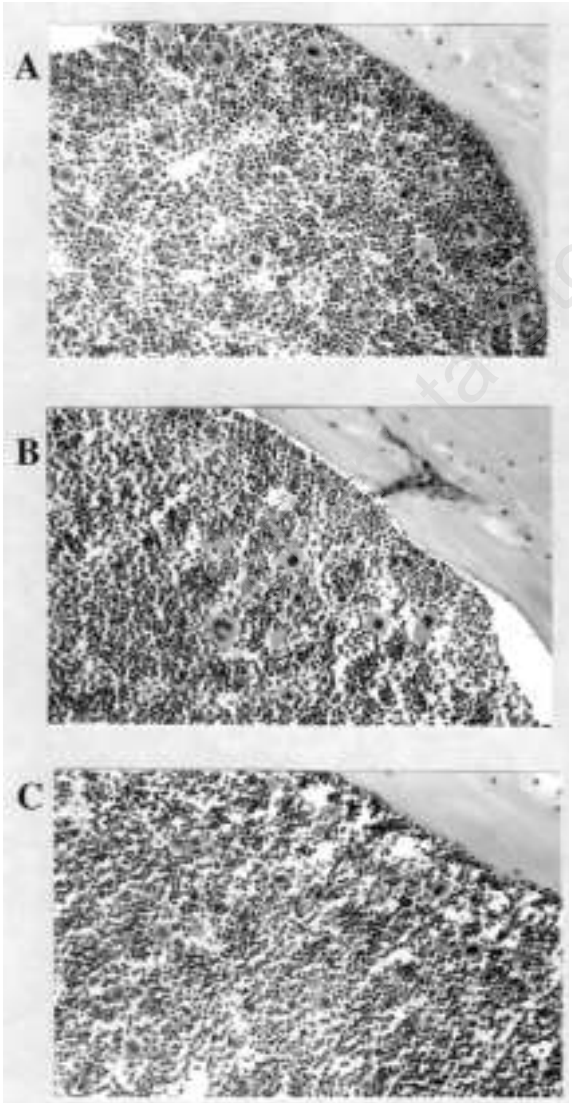

Figure 3 (left). Bone marrow samples from PF4-cyclin D1 mice are hypercellular. Sternum sections from control (A) or PF4-cyclin D1 mice line 2 (B) or line 9 (C) were stained with hematoxylin and counter-stained with eosin to view bone marrow cells. A representative compartment in the sternum of each mouse is shown (magnification x150).

of cells was not further recruited to the pool of differentiated cells. This expansion of the immature myeloid-type cell population was not seen in the cyclin D3 mice, but is reminiscent of overexpression of low levels of c-myc in megakaryocytes.[20] Since cyclin D1, but not cyclin D3, was reported to induce c-myc expression,[23] we raised the possibility that the effect of cyclin D1 on the cellularity of the marrow is partially mediated via c-myc upregulation. Because of the rarity of megakaryocytes in the marrow, we resorted to immunohistochemistry with anti c-myc. This revealed that there was not a significant difference in c-myc levels in control megakaryocytes and cyclin D1 megakaryocytes (line 2), at least using the above method of detection. It is still possible, however, that low level upregulation (not detectable by *in situ* examination) of c-myc is partially responsible for cellular expansion.

As to ploidy, we conclude that the function of cyclin D3 in this regard can be partially covered by cyclin D1. Of particular interest is the repeated finding that cyclin D3 is upregulated in myoblasts upon terminal differentiation to myotubes.[24,25] In the same system, cyclin D1 is predictably downregulated. The suggestion of a distinct and unique role for cyclin D3 in differentiated or differentiating cells has been offered in these reports.[17] Since we were unable to attain, in any of the many D1 transgenic lines produced, very high levels of cyclin D1 (comparable to those found in cyclin D3 transgenic

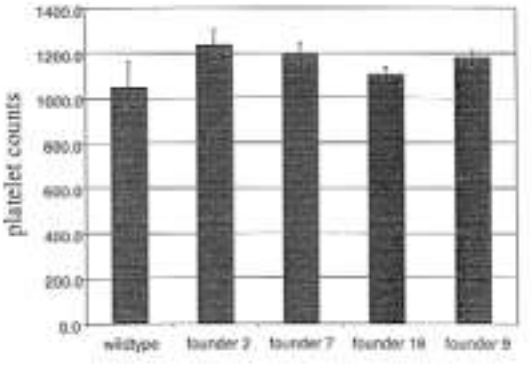

Figure 4. The circulating platelet number did not change in PF4-cyclin D1 mice. Platelets were counted from whole blood of four mice from each of four founder lines, as well as from non-transgenic littermates used as controls. Error bars represent standard deviations. Although the platelet values are higher for each transgenic founder line than for the non-transgenic controls, this difference does not reach statistical significance (two-sample rank test).

megakaryocytes) (Figure 2), we cannot exclude the possibility that in our system, the recruitment of cells to the differentiated pool depends on the level and not on the type of D cyclin involved. We conclude, however, that a two fold augmentation in cyclin D1 levels in megakaryocytes can induce a moderate increase in ploidy and in the level of immature cells, but has no ability to recruit these cells further to the differentiated pool.

Contributions and Acknowledgments

SS genotyped the transgenic mice and subjected them to bone marrow analyses; JMZ generated the transgenic construct used to produce transgenic mice and performed platelet analyses as well as initial bone marrow analyses; PT performed all the in situ hybridization studies; AT performed transgene expression studies and more bone marrow analyses; CWJ performed all the megakaryocyte ploidy analyses; KR performed immunohistochemistry analyses. All authors, led by KR, contributed to the conception, design and interpretation of the data. The order of authorship was decided based on the level and duration of contribution to the project.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Potential implications for clinical practice

3 Conditions that may deregulate the expression of cyclin D1 in megakaryocytes may lead to a myeloproliferative syndrome.

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