New Roles for Cyclin E in Megakaryocytic Polyploidization*§

Alexia Eliades‡, Nikolaos Papadantonakis‡§, and Katya Ravid‡

From the ‡Departments of Medicine and Biochemistry, Evans Center for Interdisciplinary Biomedical Research, Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts 02118 and the ‡§Graduate Program on Molecular Basis of Human Disease, School of Medicine, University of Crete, Crete 71003, Greece

Megakaryocytes are platelet precursor cells that undergo endomitosis. During this process, repeated rounds of DNA synthesis are characterized by lack of late anaphase and cytokinesis. Physiologically, the majority of the polyploid megakaryocytes in the bone marrow are cell cycle arrested. As previously reported, cyclin E is essential for megakaryocyte polyploidy; however, it has remained unclear whether up-regulated cyclin E is an inducer of polyploidy in vivo. We found that cyclin E is up-regulated upon stimulation of primary megakaryocytes by thrombopoietin. Transgenic mice in which elevated cyclin E expression is targeted to megakaryocytes display an increased ploidy profile. Examination of S phase markers, specifically proliferating cell nuclear antigen, cyclin A, and 5-bromo-2-deoxyuridine reveals that cyclin E promotes progression to S phase and cell cycling. Interestingly, analysis of Cdc6 and Mcm2 indicates that cyclin E mediates its effect by promoting the expression of components of the pre-replication complex. Furthermore, we show that up-regulated cyclin E results in the up-regulation of cyclin B1 levels, suggesting an additional mechanism of cyclin E-mediated ploidy increase. These findings define a role for cyclin E in promoting megakaryocyte entry into S phase and hence, increase in the number of cell cycling cells and in augmenting polyploidization.

Megakaryocytes (MKs) are bone marrow precursor cells responsible for the production of platelets, which are renewed on a daily basis (1). In a process termed megakaryopoiesis, multipotent hematopoietic stem cells commit toward becoming megakaryocyte progenitors. This is followed by differentiation of progenitors into mature MKs while concomitantly undergoing polyploidization (reviewed in Ref. 2). The major regulator of megakaryopoiesis is thrombopoietin (TPO), which signals through the c-mpl receptor (reviewed in Ref. 3–4). The maturation of MKs includes both endoreplication (also known as endomitosis or polyploidization) and cytoplasmic maturation (reviewed in Ref. 5). The mature MK grows in size severalfold the diameter of a normal diploid (2N) cell and can obtain a DNA content of up to 128N, with the average being 16N (6–7). During this process, MKs increase the production of proteins necessary for platelet biogenesis and function (8). Mature MKs form proplatelet extensions that fragment and give rise to platelets (9).

The mechanism by which MKs become polyploid is still not well understood. Following a series of normal cell divisions, MKs enter a cell cycle with a brief G1 phase, followed by a typical S phase and a very short G2 phase (6). Next, MKs undergo endoreplication, which represents a mitotic cell cycle that is terminated at the late anaphase stage. Repeated rounds of endoreplication eventually give rise to a polyploid megakaryocyte. The regulatory mechanisms that control polyploidization have been partially explored, with a major focus on the regulation of mitotic phase and cytokinesis (10–13). As to mitotic regulation, it became clear that it is different in MK cell lines and primary MKs, as the former show decreased levels of cyclin B in polyploid MKs (14–15), while primary MKs display cyclin B during endomitosis (16–17). Other studies have focused on the G1 phase of the MK cell cycle as a regulatory phase of polyploidy. The first study in this area showed that cyclin D3, which is highly expressed at early G1 phase, is up-regulated in MKs (18). Cyclin D3 transgenic mice indicated that overexpression of this cyclin results in an increase in polyploidy (19). In addition, in vitro studies using the HEL megakaryocytic cell line showed that G1 phase CDK/cyclin complexes (Cdk2/cyclin D3 and Cdk2/cyclin E) exhibit increased activity during G1 to S phase transition as well as S phase progression (20). Cyclin D1 overexpression in vivo was also shown to correlate with an increase in MK ploidy level, although in a less pronounced manner than cyclin D3 (21). Moreover, overexpression of cyclin D1/cdk4 in GATA1-deficient MKs rescues their growth and polyploidization (22).

Cyclin E is a 52-kDa protein that is highly expressed in the G1/S boundary of the cell cycle. Its expression is regulated by members of the E2F-transcription factor family (reviewed in Ref. 23). The mammalian cyclin E family is composed of two highly related proteins, cyclin E1 and E2 (24–25). These cyclins serve as regulatory components that bind and activate cell cycle-dependent kinases. Members of this family primarily associate with cyclin-dependent kinases Cdk2 and Cdk3 (26–27). The most prominent role of cyclin E is to complete phosphorylation of retinoblastoma protein (Rb), a process initiated by the cyclin D/Cdk4 complex in early stages of G1 phase (28–29). Hyper-
Cyclin E in Megakaryocytic Polyploidization

phosphorylated Rb loses affinity for E2F transcription factors and results in the release and activation of E2F factors. In turn, activated E2F factors lead to up-regulated S phase regulators, such as b-myb and cyclin A (26). Recently, studies indicated that cyclin E has an additional role besides Cdk-mediated functions. Cyclin E was shown to facilitate the loading of minichromosomal maintenance factors (Mcm) to the origin of replication, thus enabling the formation of the pre-replication complex (pre-RC) and the subsequent entry of cells to S phase (30).

Cyclin E1 and E2 double knock-out mice show impairment of endoreplication of trophoblast giant cells and MKs (31). Apart from these two cell types, cyclin E is overall excessive for mouse embryonic development. An additional study also supports the requirement of both cyclin E1 and E2 for endoreplication of trophoblast giant cells of the placenta (32). These findings suggest that in the absence of cyclin E, cell cycle functions can be carried out by other G1 and S phase cyclins, with the exception of MK and trophoblast polyploidy. Therefore, S phase re-entry during polyploidization is a rate-limiting step. However, while cyclin E is essential for MK polyploidy, it remained unclear whether up-regulated cyclin E is an inducer of polyploidy in vivo.

In the current study, we examined the inducibility of cyclin E by TPO in primary murine MKs and the role of this cyclin in polyploidization. We generated a transgenic (Tg) mouse model that specifically overexpresses cyclin E1 in MKs. Our results clearly demonstrate a role for cyclin E in promoting S phase entry and polyploidy. Furthermore, we show that up-regulated cyclin E results in accumulation of cyclin B1, suggesting an additional mechanism in cyclin E-mediated polyploidy increase.

EXPERIMENTAL PROCEDURES

Bone Marrow Culture—Femoral and tibial bone marrow was isolated as previously described (19). Briefly, bone marrow was collected from femurs and tibias of transgenic and control mice using a 23-gauge needle and CATCH buffer (1× Hank’s balanced salt solution, 0.38% sodium citrate, 1 mM adenosine, 1 mM theophylline, 5% fetal bovine serum). Cells were centrifuged for 5 min at 500 × g at 4 °C. Red blood cell lysis was performed using 10 ml of lysis buffer (17 mM Tris, 14 mM phosphatase, Invitrogen, cat. M0290S). The cDNA sequence corresponding to the human cyclin E1 (CCNE1), contained in the pCMV6-XL4 vector, was amplified via PCR using the following primers: forward primer 5′-ATGCCAGGAGGCGCCAGG-3′, reverse primer 5′-TCAAGCTATTTCCCGGCGC-3′ and cloned into a pcR2.1 vector (Invitrogen, cat. K200001). DNA sequencing was performed to confirm the cDNA sequence of the insert. Upon amplification of the plasmid, EcoRI digestion was performed followed by Klenow reaction. The cyclin E fragment was ligated with the blunted PF4-plasmid. The resulting plasmid was digested with Xhol and AatII to release the transgene from the vector backbone. The PF4-cyclin E fragment was purified via electrophoresis and microinjected into the pronuclei of fertilized FVB/N strain mouse oocytes to produce transgenic mice, as previously described (34).

Transgene Integration—Possible cyclin E litters from three different founder lines were screened for the human cyclin E1 gene using tail genomic DNA isolation as follows: tails were digested in 300 μl of digestion buffer (100 mM NaCl, 10 mM Tris pH 7.6, 25 mM EDTA pH 8.0, 0.5% SDS) and 6 μl of 10 mg/ml proteinase K (American Bioanalytical, Natick, MA, cat. AB00925). Tails were incubated overnight at 55 °C. The digested tails were spun down at 16,000 × g for 5 min. The supernatant was collected and treated with 1.5 μl of 100 mg/ml RNase A type III (Sigma, cat. R5125) for 20 min at 37 °C. After cooling the samples to room temperature, 100 μl of Protein Precipitation Solution (Qiagen, Valencia, CA, cat. 1045697) was added and incubated on ice for 5 min followed by centrifugation at 16,000 × g, at 4 °C for 10 min. Supernatants were collected and transferred to a new tube and 300 μl of 100% isopropanol was added. After mixing, the samples were spun down at 16,000 × g at 4 °C for 10 min and washed with 300 μl of 70% EtOH. The final pellet was resuspended in 30 μl of TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). The human cyclin E1 transgene was screened by polymerase chain reaction using 100 ng of genomic DNA, sense primer 5′-CCCTCTGGAAAAAT-TCCA-3′ and antisense primer 5′-TGACAGTTGAGTTTG-GGTA-3′ for 30 cycles at 57 °C to generate a fragment of 684 bp as detected by agarose gel electrophoresis.

Megakaryocyte Enrichment by MACS® Magnetic Bead Purification System—MKs were prepared for analysis by Western blot and qRT-PCR analysis. BM cells were washed twice with staining buffer (0.5% bovine serum albumin, 2 mM EDTA, and PBS, pH 7.2). MKs were labeled with a MK- specific antibody, anti-CD41-FITC, (BD Pharmingen, Franklin Lakes, NJ, cat. 553848). Following two washes with staining buffer, BM cells were resuspended in staining buffer and anti-FITC labeled microbeads (10 μl per 10 × 106 cells, Miltenyi Biotech, Auburn, CA, cat. 37-048-701). Cells were loaded onto an equilibrated large cell separation column (Miltenyi Biotech, cat. 130-042-202) supplied with a 23-gauge needle for flow resistance and processed as described by the manufacturer. The bound CD41-FITC labeled MKs were eluted with 1 ml of staining buffer. These cells were further analyzed for purity using Flow cytometry, based on CD41-FITC labeling (BD-FACSscan flow cytometer using a CellQuest software, BD Biosciences).

mRNA Preparation and Transcript Analysis—Homogenization of primary murine MKs was performed using Qiagen columns (Qiagen, cat. 79654). RNA isolation was carried out.
using the RNeasy Mini kit (Qiagen, cat. 74104) following the manufacturer’s instructions. For cDNA preparation, reverse transcription was achieved using the QuantiTect Reverse Transcription kit (Qiagen, cat. 205310). The cDNA was used for quantitative reverse transcriptase PCR, performed using mouse cyclin E1 (Mm01266311_m1 Ccne1) and human cyclin E1 (Hs00233356_m1 Ccne1) TaqMan® gene expression primers and probes (Applied Biosystems). Samples were run on an Applied Biosystems Sequence Detection System 7300. Data were normalized to GAPDH (Applied Biosystems, 4352339E) and 18 S rRNA (Applied Biosystems, 18 S rRNA 4319413E) and analyzed using the ΔΔCT method.

**Western Blot Analysis**—The MK-enriched fraction was collected, and total protein was extracted using radioimmuno precipitation assay (RIPA) buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with proteinase inhibitor mixture (Roche, Complete, Indianapolis, cat. 11697948001), 1 mM Na3VO4, 10 mM NaF, 5 mM Na4P2O7, 125 nM ocdiac acid, and vortex at 4 °C for 10 min followed by centrifugation at 16,000 × g for 10 min at 4 °C. Protein concentration was assayed using Bradford protein assay (Bio-Rad, cat. 500-1612). Western blotting was performed using a PVDF membrane. Proteins were detected by Western blotting with rabbit polyclonal anti-cyclin E1 (diluted 1000-fold) (Santa Cruz Biotechnology, cat. sc-26404), mouse monoclonal anti-CD41 (BD-Pharmingen, cat. 553848) or FITC rat-IgG1 κ isotype control (BD-Pharmingen, cat. 554684) labeled antibody was added at a dilution of 1:200 (0.625 μg/106 cells) and incubated on ice for 30 min. After washing twice with PBS, cells were treated with 0.1 mg/ml ribonuclease A (Sigma, cat. R51), followed by DNA staining with 0.05 mg/ml propidium iodide (Invitrogen, cat. P1304MP). Cells were incubated for 30 min at 37 °C prior to analysis on a BD-FACSscan flow cytometer using a CellQuest software (BD Biosciences).

**DNA Synthesis Assay**—For S phase labeling, 5′-bromo-2′-deoxyuridine (Sigma, cat. B9285) was injected intraperitoneally (1 mg/400 μl PBS) into 7-week-old wild-type and cyclin E transgenic mice. The mice were euthanized via CO2 asphyxiation 2 h after injection. Bone marrow was collected and MKs were fixed in 70% ethanol. Cells were treated with 2 N HCl/0.5% Triton X-100 solution followed by neutralization with sodium tetraborate decahydrate (Sigma-Aldrich cat. 71999). DNA was stained with DAPI (Vector Laboratories, Inc., cat. H-1200) and FITC-BrdU antibody (BD Biosciences, cat. 347583) was used to detect BrdU incorporation. Bone marrow from a non BrdU-injected mouse (concomitantly processed) was used to determine FITC-BrdU antibody specificity. MKs were identified with a fluorescence microscope and determined based on size and morphology. Bone marrow slides were prepared, observed, and processed as described above for immunofluorescence analysis.

**Blood Cell Counts**—Blood (0.5–0.6 ml) was collected via cardiac puncture. Blood was analyzed using the Hemavet multispecies hematology analyzer (Drew Scientific, Dallas, TX, cat. HV950FS) for platelet, red blood cells, and lymphocytes as well as mean platelet volume.

## RESULTS

**Effect of TPO on Endogenous Levels of Cyclin E in MKs**—To explore a possible role of cyclin E during polyploidization, we first determined the effect of the ploidy-promoting cytokine, TPO on MKs. Bone marrow cells were isolated from wild-type (WT) mice and cultured with TPO for 0, 6, and 12 h. MKs were purified using a MACS® magnetic bead purification system and purity of the MK fraction was estimated at ~83% based of FACS analysis (Fig. 1A). This is an underestimation in terms of mass, considering the large size of MKs compared with other contaminating diploid cells. qRT-PCR indicated a 2.5-fold increase of cyclin E1 after 12 h of TPO stimulation, as compared with non-stimulated MKs (Fig. 1B). In addition to GAPDH, expression was normalized to 18 S rRNA (supplemental Fig. S1A). Western blot analysis for murine cyclin E also showed a 2.5-fold expression increase after 12 h of TPO stimulation, compared with non-stimulated MKs (Fig. 1B). FACs analysis confirmed the effect of TPO on MK polyploidy (Fig. 1D). These results suggest a role for cyclin E during MK polyploidization.

**Analysis of Cyclin E Transgene Expression**—To further investigate the role of cyclin E in MK polyploidy, transgenic mice specifically overexpressing cyclin E1 in MKs were generated...
with 1.1 kb of the rat PF4 gene promoter driving the expression of the transgene exclusively in the megakaryocytic lineage (19, 34, 36–37). Three transgenic (Tg) founder lines were generated (nos. 11, 38, 39), of which founder line 11 and 38 were used for continued analysis (Fig. 2A). Transgene expression at the mRNA and protein levels were examined using real-time PCR, Western blot and immunofluorescence microscopy, respectively (Fig. 2B and supplemental Fig. S1B, Figs. 2C and 3). Endogenous cyclin E1 levels remained largely unaltered (Fig. 2B, lower panel) between Wt and cyclin E Tg MKs. Lack of transgene expression in non-megakaryocytic cells confirmed its MK-specific targeting (Fig. 2C, right panel). The level of total cyclin E1 (endogenous and transgene) was increased in the transgenic mice, further confirming the overall up-regulation of cyclin E1 in our transgenic mouse model (Fig. 2, D and E). Furthermore, transgenic cyclin E was highly expressed in interphase MKs, as depicted in immunostained cells in Fig. 3 (example of MK in the middle panel).

Cyclin E and MK Polyploidy—Next, we explored the relationship between cyclin E and polyploidy. For this reason, bone marrow cells were harvested from Wt and cyclin E transgenic mice. Flow cytometry analysis was applied to determine the DNA content in CD41-positive MKs (Fig. 4). Data indicated that cyclin E overexpression results in a significant increase in polyploidy, as compared with the Wt ploidy profile distribution. Data shown here are for transgenic line 11. A similar ploidy profile was obtained in line 38 (supplemental Fig. S2). TPO-stimulated bone marrow cultures showed no difference in MK ploidy distribution between Wt and transgenic cells. This suggested that TPO dominates over the effect of cyclin E overexpression, possibly via its ability to stimulate other factors, including endogenous cyclin E gene (supplemental Fig. S3).

MK and Platelet Number in Cyclin E Transgenic Mice—Given the fact that the ploidy of MKs is increased in the cyclin E transgenic mice, we sought to investigate whether cyclin E affects the number of circulating platelets in the peripheral blood. Platelet counts and mean platelet volume (MPV) showed no difference between the two groups (supplemental Fig. S4, A and B). This suggested that MK fragmentation and platelet production are highly regulated processes involving a number of factors besides cyclin E. The percentage of CD41 (MK marker) positive bone marrow cells was not significantly changed in cyclin E Tg mice, as compared with wild type (supplemental Fig. S4C). Platelet function was also tested via ADP-induced platelet aggregation and no significant difference was found between Wt and cyclin E Tg

FIGURE 1. Effect of TPO on cyclin E levels in MKs. A, left panel, MKs were isolated from freshly derived wild-type bone marrow cells using MACS® magnetic bead purification system (Control, MK-depleted bone marrow fraction; MK, megakaryocyte-enriched fraction) as described under “Experimental Procedures.” Purity of the MK-enriched fraction was estimated by flow cytometry analysis of the CD41-positive population. Data represent the average of two independent experiments. Right panel, example of enrichment of CD41-FITC labeled MKs as assessed by fluorescence microscopy. B, wild-type MKs were stimulated for 0, 6, and 12 h with 25 ng/ml TPO. RNA was extracted from the MK cultures and quantitative RT-PCR analysis was applied to measure the levels of cyclin E. GAPDH was used as an internal control. Data represent the mean of three experiments and statistical analysis was applied using the Student’s t test for paired values, n = 3, *, p < 0.05. C, left panel, Western blot analysis was applied to determine the levels of cyclin E. Wild-type MKs were stimulated for 0 and 12 h with 25 ng/ml TPO, and protein was extracted and subjected to SDS-PAGE as described under “Experimental Procedures.” Cyclin E was detected with a rabbit polyclonal anti-cyclin E antibody, and β-actin was used as loading control. One representative, out of three experiments, is shown. Right panel, total cyclin E protein expression was normalized to β-actin and presented as fold change. Quantification was performed by using the NIH ImageJ software (version 1.41o). The data shown represent the averages ± S.D. of three experiments using the Student’s t test, *, p < 0.05. D, ploidy distribution upon stimulation of megakaryocytes with 25 ng/ml TPO (Control, unstimulated MKs, TPO, 3-day-stimulated MKs). One representative out of three experiments is shown.
mice (data not shown). As predicted from the tissue-specific expression of cyclin E, other hematopoietic lineages, such as red blood cells, white blood cells and lymphocytes were unaffected (supplemental Fig. S4D).

**DNA Synthesis in Cyclin E-overexpressing MKs**—It has been reported that cyclin E is highly expressed during late G1 and early S phase of the cell cycle (26). However, only a maximum of 15% of the MK population in fresh bone marrow cells is engaged in S phase (18). Therefore, we hypothesized that up-regulation of cyclin E serves to increase the percentage of MKs undergoing DNA synthesis. To address this question, we measured protein levels of the S phase markers proliferating cell nuclear antigen (PCNA) and cyclin A (Fig. 5, A and B). PCNA and cyclin A are up-regulated 2-fold in cyclin E Tg MKs, as compared with Wt (Fig. 5B). Furthermore, the fraction of cell cycling MKs was determined via *in vivo* BrdU staining of cells. The percentage of BrdU-positive MKs was ~2-fold higher in the cyclin E Tg MKs, as compared with Wt. In accordance, we observed a similar fold increase in the protein levels of PCNA and cyclin A (Fig. 5, C and D). Overall, these data show that cyclin E promotes G1/S phase cell cycle progression, which in MKs also promotes polyploidy.

**Effect of Cyclin E Overexpression on S Phase Entry Regulators**—In some cell types, cyclin E is known to promote progression to S phase by facilitating the formation of the pre-RC (30–31). This requires a number of factors and the up-regulation of Cdc6 and Mcm2–7 proteins are a hallmark of this process (38). Because cyclin E overexpression promotes an increase in polyploidy, we asked whether cyclin E also facilitates the process of pre-RC formation. For this purpose, MK protein extracts were collected from Wt and cyclin E transgenic mice and subjected to Western blot analysis for different S phase regulators (MCM2, Cdc6) (Fig. 6). Data showed that Cdc6
and Mcm2 levels are up-regulated in the cyclin E Tg MKs, compared with Wt.

**Overexpressed Cyclin E Up-regulates Cyclin B1**—Recent in vitro studies in non MKs showed that overexpression of cyclin E leads to accumulation of cyclin B1 and polyploidy, as elevated cyclin B could delay chromosome segregation (39). Given the fact that cyclin B1 is associated with polyploidy in primary MKs, and that the level of cyclin B in G2/M MKs increase linearly with polyploidy (39–41), we tested the contention that overexpression of cyclin E causes an elevation of cyclin B1 in primary MKs. Because immunofluorescence is not ideal for quantitation and cyclin B has already been shown by immunostaining to be localized to the mitotic spindle in metaphase in primary MKs (16–17), we resorted to quantitation of cyclin B by Western blotting. Western blot analysis showed that non-synchronized, as well as synchronized transgenic MKs exhibit elevated levels of cyclin B1, compared with control (Fig. 7). MKs were synchronized with monastrol, following a protocol described by Geddis and Kaushansky (11). Hence, under conditions of overexpression of cyclin E there is also a significant up-regulation of cyclin B1, which is associated with polyploidy in other cell types.

**DISCUSSION**

During MK endomitosis, repeated cell cycles are composed of G1, S, G2, and M phases, where M phase is halted at late anaphase (17, 42). Numerous studies have centered on the regulation of mitotic events in MKs. For instance, the chromosome passenger protein Survivin is localized to the chromosomes in primary murine MKs undergoing early anaphase, but is diffused or not properly localized in the midzone of some high ploidy MKs (33). In cultures of human MKs (which typically have lower ploidy) chromosome passenger proteins are reported to be properly localized (43). Furthermore, a recent study indicates that ex vivo ablation of Survivin in mouse BM cells leads to increased MK polyploidy (13). Additionally, it was reported that inhibition of RhoA, a small GTPase that belongs to the Rho family with a known role in cytokinesis, caused an increase in MK polyploidy (12). In MK cell lines, mitotic regulators, such as cyclin B are reduced in polyploid MKs, compared with diploid ones (15, 44) while in primary MKs cyclin B is readily detectable in polyploidizing MKs (16, 40), highlighting the importance of also studying MK polyploidy in primary cultures and/or in vivo.

Whereas much attention has been placed on the regulation of mitosis, an important limiting factor in the promotion of polyploidy is the ability of the cells to re-enter the cell cycle and proceed though S phase. This is particularly important, because the majority of MKs in vivo are cell cycle-arrested. Therefore, it is necessary to better understand the molecular mechanisms of cell cycle regulation in all aspects that control endomitosis in MKs.

**In vitro** studies in megakaryocytic cell lines showed high expression of cyclin E, and that ectopic expression of cyclin E allows non-endomitotic MKs to undergo endoreplication (20, 45–46). Previous in vivo studies using cyclin E1, E2 double knock-out mouse model showed that cyclin E is necessary for MK polyploidy (31). Recently, we reported that up-regulation of cyclin E partially restores the ploidy levels in NADPH oxidase-inhibited MKs, further supporting a role for cyclin E as an inducer of polyploidy (47). Here, we show that TPO stimulation of MKs highly up-regulates cyclin E. This finding suggests a role for cyclin E in TPO-mediated MK polyploidization.
To investigate a possible role for cyclin E in MK endoreplication, we generated a transgenic mouse model where cyclin E1 gene expression is driven by the PF4 promoter. Flow cytometry analysis revealed a significant increase in polyploidy in cyclin E transgenic MKs, with 16N MKs composing the most prominent fraction of the whole. Platelet number, as well as platelet mean volume remained the same in cyclin E transgenic and wild-type mice. This is reminiscent of similar findings with cyclin D3-overexpressing MKs (19), suggesting that up-regulation of a cyclin alone is not sufficient to promote an increase in platelet release. It is possible that other TPO-mediated factors, in synergy with cyclin E-induced polyploidy, are involved in platelet production.

As cyclin E promotes progression into S phase (48–50), we confirmed that up-regulated cyclin E drives MKs to undergo DNA replication. Based on PCNA and cyclin A protein levels, as well as BrdU incorporation (a marker of an active S phase) we concluded that a higher fraction of cyclin E Tg MKs undergo cell cycles, as compared with Wt. A number of studies implicate cyclin E in facilitating the formation of the pre-replication complex (pre-RC) (51–52). In addition, Sicinski and colleagues showed that cyclin E-deficient mouse embryonic fibroblasts have defective loading of minichromosomal maintenance factors (Mcm) into the pre-RC (30–31). Cdc6, a component of the pre-RC complex (38, 53–54), is stabilized when phosphorylated by the CDK/cyclin E complex and subsequently promotes pre-RC assembly (55). Hence, increased level of Cdc6 is another indicator of a stable pre-RC complex. Western blot analysis in whole-cell extracts from MK-enriched bone marrow showed up-regulation of Cdc6 and Mcm2 in cyclin E transgenic mice. It is known that Mcm2 is a target for the E2F family of transcription factors (56). Furthermore, in vitro studies in REF52 cells have shown that all

FIGURE 5. Analysis of S phase markers in Wt and cyclin E-overexpressing MKs. A, Western blot for PCNA and cyclin A was performed in purified MKs (as under “Experimental Procedures”). B, protein expression for PCNA and cyclin A was normalized to β-actin and presented as fold change. Quantification was performed by using the NIH ImageJ software (version 1.41o). The data shown represent the averages ± S.D. of 4 and 3 experiments for PCNA and cyclin A, respectively using the Student’s t test, *, p < 0.05. C, summary of BrdU incorporation in MKs. A total of 3 mice per group (Wt, cyclin E transgenic) were used. Non-MK bone marrow cells had similar percentage of BrdU incorporation in both groups (data not shown). D, example of BrdU positive staining of MK (I) and non-MK bone marrow cells (II). Immunofluorescence microscopy was applied using FITC-BrdU antibody as described under “Experimental Procedures.” Cells were also stained with DAPI to visualize DNA.

FIGURE 6. Analysis of S phase entry regulators in isolated MKs from Wt and cyclin E transgenic mice. A, MK enriched fraction was collected, and total protein was extracted and subjected to SDS-PAGE. Proteins were detected by Western blotting with the indicated antibodies. B, protein expression of Mcm2 and Cdc6 was normalized to β-actin and presented as fold change. Quantification was performed by using the NIH ImageJ software (version 1.41o). The data shown represent the averages ± S.D. of 4 and 5 experiments for Cdc6 and Mcm2, respectively, using the Student’s t test, *, p < 0.05.
Cyclin E in Megakaryocytic Polyploidization

In summary, the results of our present study indicate that cyclin E is up-regulated in MKs upon TPO stimulation. Cyclin E overexpression alone is sufficient to increase the MK ploidy profile when expressed in already committed cells. Our data also support a mechanism for cyclin E driving MKs to progress to S phase via the up-regulation of different components of the pre-RC. Finally, our study suggests a possible novel role of cyclin E-mediated accumulation of cyclin B in mitotic MKs that results in polyploidy.

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