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Polyploidy: Occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system

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Objective. **Polyploidy, the state of having greater than the diploid content of DNA, has been recognized in a variety cells. Among these cell types, the megakaryocytes are classified as obligate polyploid cells, developing a polyploid DNA content regularly during the normal life cycle of the organism, while other cells may become polyploid only in response to certain stimuli. The objective of this review is to briefly describe the different cell cycle alterations that may lead to high ploidy, while focusing on the megakaryocyte and the importance of high ploidy to platelet level and function.**

Materials and Methods. **Relevant articles appearing in scientific journals and books published in the United States and in Europe during the years 1910–1999 were used as resources for this review. We selected fundamental studies related to cell cycle regulation as well as studies relevant to the regulation of the endomitotic cell cycle in megakaryocytes. Also surveyed were publications describing the relevance of high ploidy to high platelet count and to platelet reactivity, in normal situations and in a disease state.**

Results. **Different cells may achieve polyploidy through different alterations in the cell cycle machinery.**

Conclusions. **While upregulation of cyclin D3 further augments ploidy in polyploidizing megakaryocytes in vivo, future investigation should aim to explore how normal megakaryocytes may initiate the processes of skipping late anaphase and cytokinesis associated with high ploidy. In humans, under normal conditions, megakaryocyte ploidy correlates with platelet volume, and large platelets are highly reactive. This may not apply, however, to the disease state.** **© 2000 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Keywords: **Polyploidy—Megakaryocyte—Cell cycle—Platelet**

Introduction

Polyploidy, endomitosis, endoreduplication, and acytokinetic mitosis

Polyploidy, the state of having greater than the diploid content of DNA, was once thought to be uncommon; since the advent of DNA cytophotometry techniques, however, polyploidy has been recognized in a large variety of both plant and animal cells. Among these cell types, some may be classified as obligate polyploid cells, developing a polyploid DNA content regularly during the normal life cycle of the organism. As described below, other cells become polyploid only in response to certain stimuli, such as great functional stress or advanced age.

Among all the examples of polyploid cells, megakaryocytes are generally thought to be unique in several respects. Megakaryocytes are clearly obligate polyploid cells in that they all become polyploid during development. Throughout the life cycle of the organism, the pool of 2n megakaryocytic progenitors with proliferative capacity is renewed. At some point in lineage commitment such cells undergo a switch from mitotic to endomitotic cell cycles, no longer dividing but continuing through successive phases of DNA synthesis without either cell or nuclear division, and in so doing becoming both massive and polyploid. Later, through a process that is even less understood, the polyploid megakaryocyte will cease DNA synthesis altogether, undergo a process of cytoplasmic maturation, and eventually fragment to circulating platelets.

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While we who study megakaryocyte development may forget from time to time, megakaryocytes are far from unique in their attainment of polyploidy. In fact, polyploidy was first recognized in plants through the simple observation of polyploid mitoses decades before the DNA content of individual cells could be reliably determined through DNA cytophotometry [1,2]. Outside of the plant kingdom, polyploidy is also far more common than is generally realized. Some of the best-known and best-studied examples occur during embryonic development of insects such as *Drosophila* [3,4]. Among mammalian cells, not only megakaryocytes but also hepatocytes [5,6], arterial smooth muscle cells [7,8], and cardiac myocytes [9], all develop a certain degree of polyploidy during a normal lifespan. In addition, polyploid cells may be found in certain tissues under conditions of stress: in uterine smooth muscle during pregnancy, among thyroid cells in hyperthyroidism, and in the seminal vesicles with aging [10–12]. Polyploid cells also occur in cancer [13], but this instability more often leads to the development of intermediate DNA content values (e.g. 3n, 4.5n, etc.). Aneuploidy is found in a large variety of tumors, often indicating increased malignancy [14]. Flow cytometric analysis of aneuploidy may be used as a prognostic indicator of evolving utility in a growing number of cancers, including tumors of the colon [15], breast [16], and prostate [17]. It should be pointed out, however, that aneuploidy results from deletion or replication of specific chromosomes, a very different process from regulated, repeated replication of all chromosomes.

What are the mechanisms through which these various cell types achieve polyploidy, and how do they relate to one another? The most basic distinction to make is that between processes that involve entry into mitosis, and those that skip mitosis altogether Table 1. Those that do involve M phase entry may be grouped under the umbrella of mitotic poly ploidization. The term "endomitosis," according to Geitler's original definition, involves entry into mitosis but a block at prophase, without dissolution of the nuclear membrane [18]. Likewise, C-mitosis would connote a similar mitotic block but at metaphase, following chromosome condensation and spindle formation. In these two models the cell would proceed from the mitotic block to a G1 state. The resulting cell would contain a single polyploid nucleus and would be ready to proceed through another cycle. In contrast, a third type of mitotic polyploidization, known as acytokinetic mitosis, involves progression through every step of mitosis but for cytokinesis, and, therefore, results in a polyploid but binucleate cell. Unlike each of these examples, the terms endoreduplication and endoreplication refer to a state in which the cell does not enter into mitosis at all, but rather proceeds directly from a gap phase to a phase of DNA synthesis. Polyteny is a special case of endoreplication in which the duplicated chromosomes line up side by side, forming large structures known as polytene chromosomes. The *Drosophila* endocycle is the best studied example of this process (Fig. 1).

An important thing to realize is that these various terms have become rather muddled in the literature, and are used by some authors almost interchangeably. For example, to use the terms endomitosis and endoreduplication to refer to the same event would clearly be incorrect, but this does occur with some frequency. For many of the cell types mentioned, including vascular smooth muscle and cardiac myocytes, for which the fact but not necessarily the mechanism of their polyploidization is known, these terms are also used in a haphazard fashion. As they were originally defined, these terms may, in fact, no longer apply to the very cells for which they are most often used. Endomitosis, for instance, is most often used in reference to the megakaryocyte. As we shall see, however, megakaryocytic polyploidization involves progression through the metaphase, with concomitant chromosome condensation, spindle formation, and initiation of chromatid separation. This clearly does not fit the original definition of endomitosis [18], and yet perhaps a consistent use of a modified definition, such as endomitosis-A to indicate cells that enter mitosis but do not complete Anaphase, may be considered. By this rationale, the term endomitosis may be used to describe all cells that enter mitosis but abrogate this phase at different points, with these stages to be indicated as an index.

Is there a purpose to the development of polyploidy in these various cell types? Perhaps polyploidy is a programmed response to stress in some cell types while a result of normal cellular programming in others, such as megakaryocytes. Polyploid cells are larger, produce greater quantities of mRNA and protein, and presumably can execute more functions per cell than diploid cells. Polyploidization

Table 1. Terms describing mechanisms of polyploidy formation

Term	Definition
Endomitosis	According to Geitler's original definition, polyploidization involving M-phase entry with a block at prophase, without dissolution of the nuclear membrane. Currently used to describe polyploidization in megakaryocytes, which do not fit the original criteria.
Endomitosis-A	Cells enter M phase but do not complete anaphase prior to re-entering the cell cycle, as occurring in megakaryocytes.
C-mitosis	Cells enter M phase but are blocked at metaphase following chromosome condensation and spindle formation
Acytokinetic mitosis	Progression through each step of mitosis but for cytokinesis, resulting in binucleate cells.
Endoreplication	Cells do not enter mitosis but rather proceed directly from a gap phase to a phase of DNA synthesis.
Endoreduplication	See endoreplication
Polyteny	A special case of endoreplication in which the duplicated chromosomes line up side by side, forming large polytene chromosomes.

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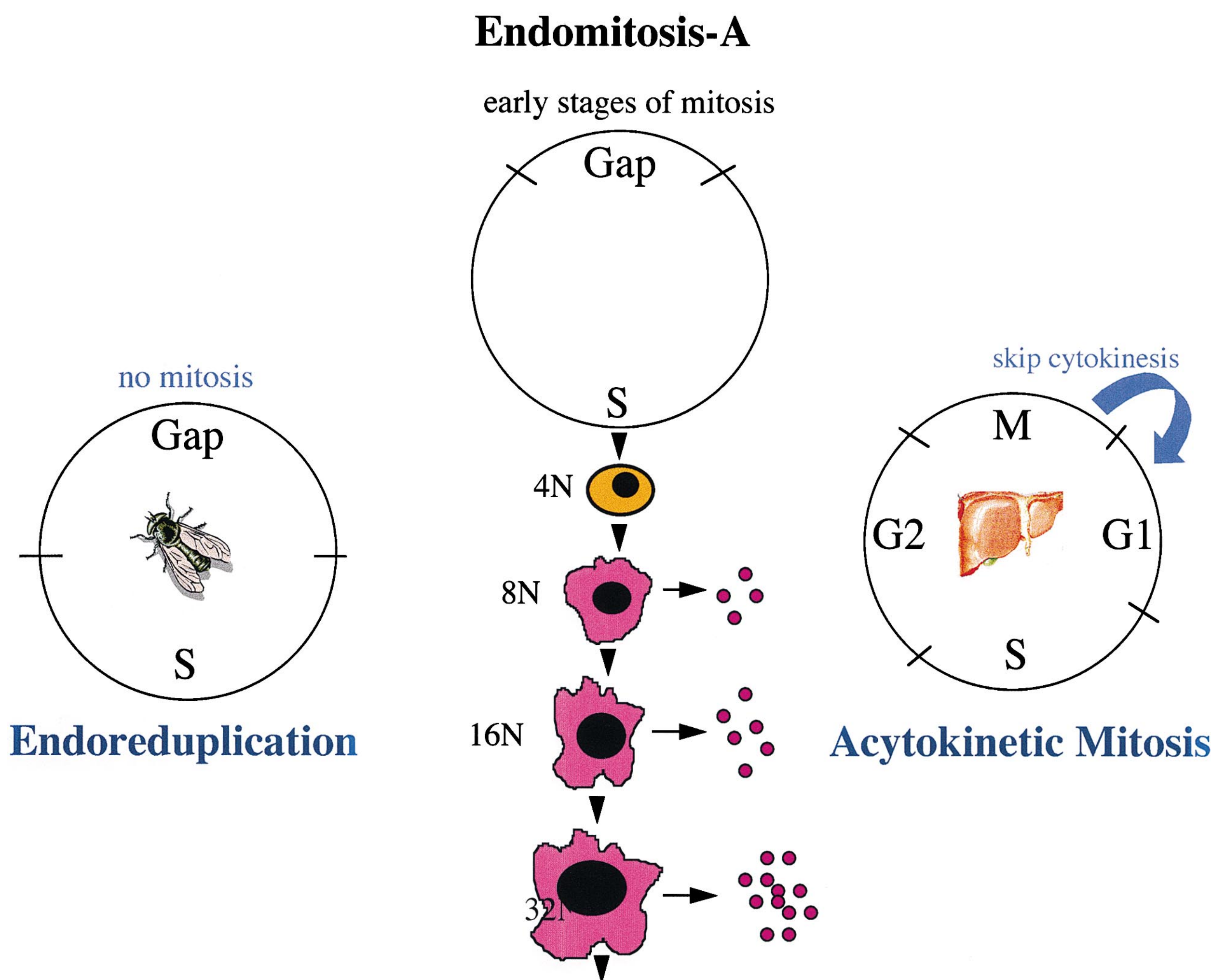

Figure 1. Polyploidy is achieved through a variety of cell cycles. Megakaryocytic endomitosis-A involves progression through the early stages of mitosis during the brief gap separating successive rounds of DNA replication. In contrast, endoreduplication, as occurs in the drosophila embryo, entails alternating synthesis and gap phases without evidence of mitotic entry. The process of acytokinetic mitosis, as occurs in hepatocytes, leads to the formation of binucleate cells through the skipping of cytokinesis.

produces an increase in functional capacity of the cell without the demands and necessity for temporary cessation of function that is involved in full mitosis and cytokinesis.

Normal mechanisms involved in the maintenance of a diploid DNA content

Progression through the normal eukaryotic cell cycle entails replication of the entire complement of DNA exactly once in preparation for mitotic division (for review, see [19]). What mechanisms ensure that this is so? Early experiments using cell fusion established the basic concept of replication competence of a nucleus [20]. Fusion of an S-phase HeLa cell to an interphase nucleus has one of two results: a G1 nucleus is induced to replicate early, whereas a G2 nucleus is unable to replicate at all without going through karyokinesis. This suggests several conclusions. The G2 nucleus is incompetent to replicate, and must go through mitosis in order to regain this competence. Also, the factors present in the S phase cell are able to induce replication only in the competent nucleus. Further fusion experiments between G1 and G2 HeLa cells showed that G1 nuclei were able to enter S phase even in the presence of the nonreplicating G2 nucleus. This demonstrated that replication competence is a property that is autonomous to the nucleus. This is an important point in relation to the development of polyploidy as it indicates that a mechanism, which allows the cells to bypass this restriction and cell cycle arrest, is necessary.

The basic idea that has emerged from these experiments is that nuclear DNA can exist in either replication competent or incompetent states. Chromatin in a G1 nucleus is competent, becomes incompetent upon passage through S phase, and regains this competence through completion of mitosis. Studies in *Xenopus* egg extracts have demonstrated that nuclear integrity is important in maintaining replicated

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nuclei in the incompetent state [21,22]. G1 egg extract allows sperm or G1 HeLa nuclei to replicate once. Transfer of intact G2 HeLa nuclei or replicated sperm to fresh extract is able to initiate re-replication only if the nuclear membranes are first permeabilized with detergent. This suggests that the nuclear membrane normally excludes a factor or factors that are able to confer replication competence upon nuclear chromatin. This activity has come to be known in the literature as "licensing factor," although there are almost certainly multiple factors involved in replication licensing. Such a factor would be essential to DNA replication, and would be inactivated by replication during S phase. It would have to be unable to cross the nuclear membrane, and would only re-enter upon breakdown of the nuclear membrane during mitosis. Thus, DNA replication would occur only once per cycle.

Among the more promising candidates for replication licensing factors are the family of proteins known as minichromosome maintenance (MCM) proteins [23]. MCM proteins from several species have been found to be bound to DNA during G1 phase, displaced during S, and maintained unbound in the nucleus during G2 [24–26]. Binding of MCM proteins to chromatin has been shown to require an additional, incompletely characterized activity known as "loading factor" [24]. This loading factor may be linked to the cell cycle machinery through regulation by cyclin-Cdk kinases [27]. Transition between successive phases of the cell cycle are mediated by successive pulses of Cdk (cyclin-dependent protein kinase) activity. Activation of each Cdk depends upon a distinct type of cyclin (reviewed in [28, 29]. There are at least six classes of cyclins, designated A through G [30,31]. Cyclin B in association with p34Cdc2 (Cdc2 kinase) is fundamental to the regulation of the G2 to M transition in all eukaryotes thus far examined (reviewed in [28]). Cyclin A is also involved in the G2 to M transition and regulates progression through S phase [32,33]. Regulation of the progression through G1 phase and exit from the cell cycle depends on the G1 cyclins. G1 cyclins associate with specific Cdk subunits: cyclin E associates primarily with Cdk2 [34,35]. The D-type cyclins appear to be induced earlier in G1 phase than is cyclin E and are capable of forming complexes, at least in vitro, with Cdk2, Cdk4, Cdk5 and Cdk6 [36–39]. The activity of the different cyclin-Cdk complexes depends on alterations in kinase activity by phosphorylation or dephosphorylation of the kinase complex, the binding of Cdk inhibitors, and the rate of synthesis and degradation of the cyclins in a cell cycle-dependent fashion. It has been shown that Cdc2 is maximally active when phosphorylated at residues Thr 161 by cyclinH-Cdk7, while inactive when phosphorylated in residues Thr14 and Tyr15 by the kinase mik1-wee1-myt1 [40]. At the onset of mitosis these later residues are dephosphorylated by the phosphatase Cdc25C, leading to full activation of Cdc2 kinase [40,41]. Among the Cdk and Cdc2 inhibitors are p21 and p27, which associate with the cyclin-Cdk complex and inhibits kinase activity [42]. The expression of these inhibitors is also cell cycle dependent. As shown below, both Cdc25C and kinase inhibitors have been implicated in regulating the acquisition of a polyploid DNA content in megakaryocytes.

Mechanisms of polyploidy in mammalian cells

Microtubule inhibitors and the spindle checkpoint. The cell cycle contains several checkpoints that ensure orderly progression and allow for arrest in response to insults. Interruption of the mitotic spindle using microtubule inhibiting drugs leads to activation of such a checkpoint, resulting in M phase arrest. The yeast genes Mad2 [43] and Bub1 [44] are components of this checkpoint, and cells harboring mutations in either gene fail to arrest in response to anti-microtubule agents, continuing through multiple rounds of DNA replication. Mammalian cells injected with antibody to the human Mad2 homologue, hsMad2, fail to arrest when treated with nocodazole [45]. In a like manner, cells expressing a dominant negative murine Bub1 arrest with a much lower frequency than normal during nocodazole treatment [46]. Targets of the spindle checkpoint have recently been identified through interactions with Mad proteins in yeast. These targets, the Slp1 gene in fission yeast [47], and the Cdc20 gene in budding yeast [48], are important for M phase progression. The spindle and DNA damage checkpoints are, therefore, hypothesized to enforce mitotic arrest through inhibition of these genes. Thus, Cdc20 overexpression is able to overcome the spindle checkpoint to achieve polyploidy, and Slp1 mutants that are unable to interact with Mad proteins bypass the checkpoint in a similar manner.

In normal mammalian cells, treatment with nocodazole or other microtubule-inhibiting agents also activates the spindle checkpoint, leading to mitotic arrest. Recently, the tumor suppressor genes p53 and pRb have been implicated in this process [49]. Mouse embryonic fibroblasts (MEFs) deficient in p53 or pRb fail to arrest when cultured in the presence of these drugs, continuing through one or more additional round of DNA replication [50,51]. Deficiency in the Cdk inhibitor p21 has a similar effect, suggesting that p53 spindle checkpoint function acts through this effector. Lanni and Jacks [52] have recently examined this phenomenon, and have come to a different conclusion. Following the progress of individual MEFs by time-lapse video microscopy, they showed that all MEFs arrested at mitosis in response to nocodazole, and after a period of several hours progressed into an apparent interphase state, which is termed adaptation. MEFs lacking in p53 or p21 arrested in M phase for the same period of time as wildtype MEFs, but were subsequently able to escape the interphase block and continue with another round of replication. Thus, p53 could not be labeled a true mitotic checkpoint, since cells lacking the gene behave identically to wildtype cells at mitosis. This is in contrast to results with mammalian homologues of the true spindle checkpoint genes such as Mad2 and Bub1,

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where inactivation results in wholesale bypass of the mitotic arrest [45,46].

Kinase inhibition can lead to re-replication A variety of treatments may induce mammalian cells in culture to polyploidize. Usui and colleagues [53] reported that treatment with the protein kinase inhibitor K-252a was able to induce the development of highly polyploid cells in rat fibroblastic cell lines in culture. Similarly, Hall et al. [54] showed that nocodazole-arrested mouse mammary tumor cells can be induced to polyploidize when treated with the protein kinase inhibitor staurosporine. Both groups were able to show at least a partial inhibition of in vivo Cdc2 kinase activity associated with kinase inhibition. Coverley and colleagues [55] returned to the *Xenopus* egg extract system to show that kinase inhibition has a similar effect to nuclear permeabilization on replication-incompetent G2 nuclei. Treatment of G2 HeLa nuclei with the kinase inhibitor 6-dimethylaminopurine (6-DMAP) allowed a further round of DNA replication in *Xenopus* extract without either passage through mitosis or nuclear membrane breakdown. Furthermore, 6-DMAP treatment in G2 was shown to induce MCM proteins to re-associate with chromatin, suggesting that a cell cycle-regulated kinase controls MCM binding. Cell cycle analysis of a rat choriocarcinoma cell line has shown that endoreduplication in these cells is associated with lowered Cdc2 kinase activity, suppression of cyclin B expression, and a switch from cyclin D3 to cyclin D1 expression [56].

Regulators of anaphase and cytokinesis As we shall see below, megakaryocytes are unique in that during the formation of a high ploidy state, these cells skip cytokinesis and likely also forego the late stages of mitosis subsequent to anaphase B. In the search for potential mechanisms leading to high ploidy in this lineage, it is important to review the genes and their products which have been defined as specific regulators of cytokinesis or anaphase in different systems. Studies on potential regulators of these processes in mammalian cells are quite limited at this stage, while valuable information has been obtained in lower organisms. Bhat et al. [57] described the importance of a chromosome-associated protein for anaphase, the barren gene product in *Drosophila*, which interacts with Topoisomerase II in final chromatid segregation at anaphase. In accordance with this observation, it has been demonstrated in a protozoan that a telomerase template mutation causes a block in anaphase [58]. In fission yeast, a mutation in the $cut8^+$ gene, which encodes a protein kinase, was found to cause the cells to bypass anaphase [59]. In mammalian cells, the mitotic kinesin-like protein 1 is colocalized with Plk kinase, an analog of the *Drosophila* polo gene product. This occurs during late M phase, thus implying the potential importance of Plk kinase for late anaphase in mammalian cells [60]. As to cytokinesis, several genes have been reported to be necessary for the proper function of the actin or microtubule cytoskeleton and cytokinesis, including the Pom1p protein kinase in yeast [61] and the human homolog of the tumor suppressor adenomatous *polyposis coli* [62]. Genetic studies in yeast have identified genes crucial for proper cytokinesis to occur, including the genes encoding Myo2p (a myosin heavy chain), as well as a novel protein kinase, a GTP binding protein, a member of the ras superfamily of GTPases, and the multisubunit general transcription factor TFIID [63–65]. These studies indicate that anaphase as well as cytokinesis depend on several regulators, and the mutation of an individual component leads to inability to enter anaphase or to follow cytokinesis. Any one of these above described gene products may be modified in polyploidizing megakaryocytes.

Polyploidy in transgenic models A number of transgenic manipulations have resulted in the artificial production of polyploid phenotypes in vivo. The gene product of the proto-oncogene MDM2 inhibits p53 transcriptional activity by binding the p53 acidic activation domain [66,67]. Lundgren and colleagues [68] expressed MDM2 in mammary epithelial tissue of transgenic mice, resulting in polyploidy in approximately one-third of transgenic cells. Both multinucleated and uninuclear transgenic cells were observed, with ploidy as high as 16n. The phenotype was not dependent upon the interaction of MDM2 with p53, as similar results were obtained on a p53−/− background. Transgenic cyclin D1 overexpression in cardiac myocytes produced a similar picture of a mixed population of diploid and polyploid cells, of which a proportion were binuclear [69]. As we have seen, however, a certain percentage of cardiac myocytes normally develop such polyploidy with age and stress, while mammary epithelial cells do not.

Polyploidy in megakaryocytes

The megakaryocyte is the best-known of the mammalian polyploid cells. It appears in the yolk sac, the fetal liver, spleen, and, finally, the marrow during emryogenesis and fetal development [70]. During its life cycle, the megakaryocyte proceeds through a proliferative 2n stage, during which its progression through the cell cycle is indistinguishable from any other hematopoietic cell. Each cell then undertakes the process of polyploidization, accumulating a DNA content of 8n, 16n, 32n, 64n, and even 128n (median DNA content, 16n) before proceeding with its final maturation and platelet fragmentation. Fetal megakaryocytes, however, are smaller in size and have a lower modal ploidy than those of adults (e.g. [71–73]). The major regulator of megakaryocyte development, thrombopoietin (TPO), acts at each stage of this process, including commitment and proliferation of progenitor cells, polyploidization of developing cells, and maturation of cytoplasmic processes and demarcation membranes in more mature cells [74–78]. With regard to the polyploidization process, TPO has the effect of stimulating cells to achieve higher DNA contents, and

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megakaryocytes in mice deficient either for TPO or its receptor, c-mpl, are found to be small and low in ploidy [79].

The true nature of megakaryocytic polyploidization is still being investigated, but considerable evidence has been accumulated. Do megakaryocytes polyploidize via a process that involves entry into and abrogation of mitosis? Several studies have demonstrated that the cell cycle in polyploidizing megakaryocytes is composed of repeating rounds of DNA replication separated by short gaps [80,81]. This excludes the possibility of polyploidy being achieved by means of a continuous synthesis phase. Several early studies reported morphologic observations of mitotic events in polyploid megakaryocytes, which include entry into mitosis, including chromosome condensation, but lack of late stages of this phase of the cell cycle [82,83]. Given the rarity of megakaryocytes in the marrow and the fact that the gap period in endomitotic megakaryocytes has been estimated to be less than 90 minutes in duration [84,85], the task of finding sufficient polyploid mitotic megakaryocytes for study is difficult indeed. The development of recombinant TPO as a tool for producing polyploid megakaryocytes in culture has eased the situation somewhat. Two recent publications have taken advantage of this agent, and appear to have confirmed the earlier observations of mitotic events in endomitotic cells. Nagata and coworkers [86] have examined large numbers of primary murine megakaryocytes, cultured in the presence of TPO, for features of mitosis. They demonstrate several polyploid cells with condensed chromosomes, spindles originating from multiple spindle poles, and multiple centrosomes. Breakdown of the nuclear membrane is also clear in these cells. They did not, however, observe signs which would indicate outward movement of the spindle poles or other features of late mitosis. The authors' interpretation is that megakaryocytes in the polyploid cell cycle enter mitosis and proceed as far as anaphase A, but are then blocked from proceeding to anaphase B, telophase, and cytokinesis (Fig. 2). Based on this study, identifying regulatory proteins that affect spindle outward movement in megakaryocytes would be of great interest. A similar study published by Vitrat and colleagues [87] took a comparable approach to study the morphological features of human megakaryocytes cultured with TPO. They note the difficulty associated with detailed analysis when fewer than 1% of cultured megakaryocytes are found to be in M phase. To overcome this difficulty, this group resorted to culture in the presence of nocodazole, after which they report approximately 20% of cells are blocked in "pseudometaphase." Consistent with the other study, Vitrat reports observation of DNA condensation, nuclear membrane breakdown, and the formation of a spherical mitotic spindle. Further, chromatid separation and movement towards the spindle poles are demonstrated, suggesting that anaphase is in fact completed in these cells. This report, therefore, contradicts the hypothesis that megakaryocytic endomitosis is due to a block at the metaphase-anaphase transition, proposing instead that only cytokinesis is lacking. While the determination of precisely which events of normal mitosis occur here is obscured by the complexity of the polyploid event as well as by the use of nocodazole, it is, nonetheless, clear that these cells do enter into an abortive mitosis during the polyploid cell cycle.

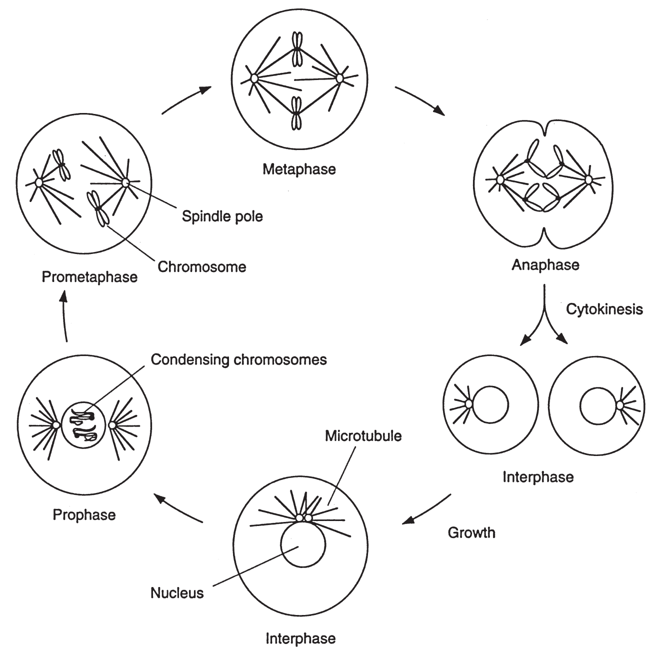

Figure 2. Stages of mitosis during a mitotic cell cycle (adapted from [155]). Anaphase A, which involves movement of sister chromatids into the multiple centrosomes (indicated in the figure by black dotes), is documented in megakaryocytes undergoing endomitosis [86,87]. According to Nagata et al. [86], in megakaryocytes undergoing polyploidization, anaphase B, which involves outward movement of the spindle poles and full chromatide separation, is missing as well as cytokinesis. The spindle checkpoint and the regulation of chromosome condensation occur during the transition from interphase to prophase.

What are the elements that control polyploidization in megakaryocytes? The question of whether or not the standard cell cycle control machinery is sufficient to control this altered process is a complex one. Several groups have suggested that the switch to polyploidization in megakaryocytes depends on a reduction in activity of the cyclin B-dependent Cdc2 kinase [81,88–90]. Zhang et al. [81,85] have demonstrated detectable, but reduced levels of cyclin B1 and its associated kinase activity in megakaryocytic cell lines induced to undergo polyploidization. More recently, they have shown an increased destruction of cyclin B1 by the ubiquitin-proteosome pathway, both in polyploid megakaryocytic cell lines and in high ploidy primary murine megakaryocytes, and suggested that accelerated or premature destruction of cyclin B would allow re-entry into the S phase of the cell cycle [91]. In cells undergoing a mitotic cell cycle, it has been well established that cyclin B destruc-

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tion is associated with entry to anaphase. In accordance with the above-described results in megakaryocytes, it is important to note that in yeast, activation of the degradation system which lowers the levels of cyclin B was found to be essential for re-entry into S phase in cells that do not enter anaphase due to mitotic blockage [92]. This feature would be important in cells that undergo polyploidization by the endomitosis-A cell cycle. Gu et al. [88]have examined non-TPO treated human bone marrow by immunohistochemistry and electron microscopy and reported a significant staining with an antibody to Cdc2 in all megakaryocytes and other bone marrow cells. Anti-cyclin B, on the other hand, showed no or weak staining in megakaryocytes but strong staining in granulocytes, monocytes and macrophages. The work of Datta and colleagues [89], done in a clone of HEL cells, has shown a reduction in Cdc2 kinase activity with polyploidy, although levels of cyclin B1 in this system are apparently unaltered. In two other megakaryocytic cell lines, HEL and MEG-O1, polyploidy was associated with high levels of cyclin B and Cdc2 but downregulation of the phosphatase Cdc25C, which leads to the common endpoint of downregulation of Cdc2 kinase activity [90]. In contrast to some of these studies, the recent work of Vitrat [87] reports the detection of functional cyclin B1 by flow cytometry in polyploid human megakaryocytes treated with TPO. By staining with both propidium iodide and antibody to cyclin B, the authors report detection of cyclin B1 in polyploid megakaryocytes. Immunofluorescence microscopy showed association of cyclin B with the mitotic spindle in polyploid cells. Further, immunoprecipitation of cyclin B1-associated proteins (Cdc2-associated ones were not examined in this study) showed a similar level of H1 histone kinase activity in polyploid cells as compared to the 2n/4n population. In summary, a reduced activity of the mitotic kinase is observed in polyploidizing cell lines as compared to proliferating diploid cells, while no reduction in this activity is detected in primary, polyploid TPO-treated megakaryocytes as compared to low ploidy ones, and no cyclin B is detected in primary polyploid megakaryocytes not treated with TPO. The state of proliferation of the diploid cells in all these distinct systems is likely to be different, and the level of cyclin B and Cdc2-associated kinase activity would be higher in a pool of highly proliferating cells (e.g., the megakaryocytic cell lines). This may contribute to the variance observed in the profile of mitotic kinase activity during the acquisition of a high ploidy state in the cell lines and primary megakaryocytes. A common finding in all these systems, however, is the detection of some level of Cdc2 kinase activity that would allow entry to M phase of that cell cycle. These levels are perhaps kept low enough to allow re-entry into S phase of the cell cycle in the absence of late anaphase, as found in yeast [92].

Another issue in control of polyploidization in megakaryocytes is whether or not there are alterations in the G1/S phase. Examination of the cyclin machinery has shown that cyclin D3 is expressed in both megakaryocytic cell lines and primary megakaryocytes, and is upregulated by ploidy-promoting factors such as TPO and phorbol ester [81,85,93]. Overexpression of cyclin D3 in megakaryocytes of transgenic mice results in an increase in ploidy commensurate with that observed upon in vivo treatment with thrombopoietin [93]. This supports a role for upregulation of a G1 phase component in promoting multiple cycles of endomitotic DNA synthesis and thus allowing the development of high-ploidy cells. In this context, it is interesting to note the study by Datta and colleagues [94] who had shown a significant increase in the activity of G1-associated and S phase-associated Cdk complexes in polyploidizing HEL cells. This was facilitated by a differential loss of Cdks associated with the p21 and p27-inhibitory proteins. In contrast, it has been reported that overexpression of p21 in the human megakaryocytic leukemia cell line UT-7 resulted in an increase in ploidy. It should be pointed out, however, that in this study polyploidy was determined by morphologic examination of cytospun specimens and by counting the number of cells containing more than two nuclei [95], while Datta and colleagues determined polyploidy by flow cytometry analysis [94].

An additional important aspect of the regulation of the cell cycle in polyploidizing megakaryocytes are the mechanisms that lead to potentially abrogated anaphase and to skipping cytokinesis. As detailed in a previous section, several gene products have been implied as crucial regulators of these processes in other cell types. Their relevance to a high ploidy state in megakaryocytes awaits future examination.

Correlation of megakaryocyte ploidy to platelet mass and properties

Considerable effort has been expended in the attempt to relate megakaryocyte ploidy to platelet size, number, and reactivity. The results are not necessarily intuitive. In response to thrombocytopenia, megakaryocytes generally will proliferate and develop higher ploidy. One's first thought, then, would be that ploidy would increase along with platelet count. Under normal conditions, however, megakaryocyte ploidy in humans has been found to follow a direct non-linear relationship to mean platelet volumes [96–98]. As a general rule, the platelet mass (count x volume) is held to fairly steady levels. It has been also reported that there exist an inverse relation between platelet volume and platelet number [99]. It is instructive, however, to examine a number of situations in which these parameters are varied.

Although this relationship between ploidy and platelet volume has been observed repeatedly in populations of normal subjects, we should note that the effects on platelet volume are dissociated from ploidy in the response to acute changes in circulating platelet mass. In response to destruction of platelets by antiplatelet serum, platelet volumes begin increasing by 8 hours, well before the observed increase

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in megakaryocyte DNA content. Corash and colleagues [100] have observed increases in platelet volume in response to a very mild thrombocytopenia, during which no detectable increase in ploidy is seen. They persuasively argue that generalizable conclusions regarding the relationship between ploidy and platelet size are difficult to make [101]. Similarly, Stenberg and Levin [102] have noted a temporal dissociation between changes in platelet size and those in megakaryocyte ploidy in response platelet antiserum-induced thrombocytopenia.

Evidence linking platelet size and reactivity takes several forms. Clinically, patients with severe thrombocytopenia and a high mean platelet volume (MPV) have a lower frequency of bleeding episodes than those with low MPV [103]. The production of large platelets in response to experimentally-induced thrombocytopenia in animals is similarly associated with a decreased bleeding time [104]. In vitro, large platelets aggregate more rapidly in response to agonists such as ADP and collagen [105], contain greater numbers of dense granules, and produce more thromboxane A2 [106], serotonin, and thromboglobulin [107,108] per unit volume than control platelets. Addition of ADP to platelet suspensions results in a preferential aggregation of large over smaller platelets [109].

It is well-established that bone marrow megakaryocytes respond to changes in the mass of circulating platelets. Chronic reductions in platelet count by immunologic mechanisms, such as in idiopathic thrombocytopenic purpura (ITP), in most cases result in increases in megakaryocyte ploidy, as well as frequency and size [110,111]. Megakaryocyte and platelet responses to the acute induction of thrombocytopenia, accomplished either by infusion of anti-platelet serum (APS) or by plasma exchange, is also well-characterized. The first measurable change occurs in platelet size, which begins to increase 8 to 12 hours after the induction of thrombocytopenia and peaks at approximately 24 hours [100,112]. Increases in megakaryocyte ploidy are measurable by 24 hours, with a peak between 40 and 62 hours. Platelet production increases by 36 hours, and megakaryocyte number is increased by approximately 5 days [113]. Conversely, infusion of platelets to artificially induce thrombocytosis results in a decrease in both megakaryocyte number and size [114,115]. Kuter and Rosenberg [116] used a rat model to attempt to demonstrate the existence of a true “feedback loop” between the mass of circulating platelets and ploidy of the megakaryocytes. In this model, the induction of thrombocytopenia through the use of APS was accompanied by increases in megakaryocyte ploidy that were proportional to the decrease in platelet count. The demonstration that these increases in ploidy could be blocked by replenishing platelets by transfusion within several hours of APS treatment provided convincing evidence for a platelet-mediated control of megakaryocyte ploidy. In response to acute changes in platelet number, then, megakaryocyte ploidy varies inversely with and proportionally to platelet change.

What are the mediators of the apparent feedback mechanism between platelets and megakaryocytes? A wealth of evidence implicates thrombopoietin, the mpl ligand, in this process. The response to thrombopoietin, in vitro and in vivo, involves both a stimulation of megakaryocyte proliferative activity and augmentation of differentiation and polyploidization [74,117,118]. Induction of thrombocytopenia in mice results in an increase in circulating thrombopoietin levels, and this increase does not appear to be mediated at the transcriptional level [119]. Significant binding of labeled TPO to platelets has been demonstrated, suggesting that platelets control plasma thrombopoietin levels directly though this binding [120]. Binding of TPO to megakaryocytes has also been shown [121]. Chang et al. [122] have demonstrated that TPO levels are increased in patients with thrombocytopenia following myeloablative therapy but remain low in patients with ITP. More recent studies performed in mice by this group suggest that high TPO levels in subjects with thrombocytopenia due to megakaryocyte hypoplasia result from decreased uptake and degradation of TPO, while the lack of a similar increase in immune-mediated thrombocytopenia may be due to increased TPO uptake by the high platelet turnover and increased megakaryocyte mass seen in this condition [123]. Thus, TPO is likely the mediator of ploidy changes in response to changes in platelet and megakaryocyte mass, although other mechanisms have not been ruled out.

The issue of correlation between megakaryocyte ploidy and platelet levels and volumes was also addressed in the C3H mouse. Murine megakaryocytes generally have a modal ploidy of 16n, and similar ploidy profiles have been noted in normal individuals from the majority of mammalian species, including man [124]. Despite that, the rodent platelets are much smaller than human platelets, and there is conservation of platelet mass between species. Megakaryocytes from the C3H strain have been noted to have a higher modal DNA content, 32n. Examination by two groups has shown that, in comparison to up to 11 other strains of mice, C3H mice are similar in platelet count, mean platelet volume, and megakaryocyte size [125,126]. Megakaryocyte frequency was found to be marginally increased in one study, and not changed in the other. This provides one more piece of evidence that neither platelet count nor volume depend on very high ploidy. Indeed, old studies have indicated that cells with a ploidy as low as 8n produce platelets (reviewed by [127]).

The in vivo use of vincristine provides an additional system which is instructive on the issue of correlation between ploidy and platelet level. Vincristine is a vinca alcaloid drug used as an anticancer chemotherapeutic agent in the treatment of a variety of leukemias, lymphomas, and solid tumors [128]. It acts as an antimicrotubule agent, similar in mechanism to drugs such as colchicine and nocodazole, which prevents cells from completing mitosis. At low doses, vincristine has been noted to lead to a mild but significant thrombocytosis without a preceding thrombocytopenia

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[129–131]. Megakaryocyte ploidy is also significantly enhanced, but without an increase in MPV. Megakaryocyte ploidy has been shown by Harris and Penington to be increased at 2 and 3 days following treatment, preceding the rise in platelets which peak at days 4 and 5 [132]. It is interesting to note that although there are detectable abnormalities in platelets produced in vincristine-treated animals [133], both polyploidization and platelet fragmentation are able to occur in the presence of this drug.

Megakaryocyte ploidy in pathology and disease

Megakaryocyte ploidy in myeloproliferative disorders

The myeloproliferative disorders, which include essential thrombocythemia (ET), polycythemia vera (PV), and chronic myelocytic leukemia (CML), are considered to be diseases involving clonal expansion of the hematopoietic stem cell. In the majority of cases, the frequency of bone marrow megakaryocytes is elevated, although this is not uniformly true in CML [134,135]. The peripheral platelet count is of course always elevated in ET. PV and CML are frequently associated with markedly elevated platelet counts, and either of these diseases may be mistaken for ET, especially early in the course of the illness. Each of these disorders displays a megakaryocyte ploidy profile that is markedly different from that seen in healthy individuals [136]. Patients with ET show a marked shift towards higher ploidy number, with a large increase in the percentage of 32n megakaryocytes [137,138]. PV patients show a similar increase in ploidy, but to a lesser extent.

CML is a special case of megakaryocytopoiesis, in which the ploidy profile is actually shifted markedly to the left, towards low ploidy [139]. The striking thing about the disease is that this low ploidy profile is in most cases associated with a platelet count that is either within the normal range or high at the time of presentation, although occasional patients present with low platelet counts. Most eventually develop thrombocytopenia with progression of the disease, however. A recent study has suggested that cases of CML with marked thrombocythemia represent a distinct subtype, expressing a particular form of the bcr-abl transcript [140]. The consistency with which this unique low-ploidy profile is observed in CML argues against a need for high ploidy megakaryocytes in the production of high platelet counts. In this case, it appears that large numbers of small low-ploidy cells are able to produce large numbers of fully functional platelets. Whether or not these platelets differ significantly in activity from a normal population will require further investigation. It is instructive to also note here a recent study in which human granulocyte colony-stimulating factor (G-CSF) was overexpressed in transgenic mice [141]. Although G-CSF had the effect of reducing mean megakaryocyte ploidy to 8n, both platelet count and megakaryocyte number were found to be unchanged. This further suggests that very high ploidy is not essential for achieving normal peripheral platelet count.

Megakaryocyte ploidy in leukemias

It is often reported that different types of leukemias are associated with aberrant platelet counts and megakaryocyte shape, but a reliable examination of megakaryocyte ploidy is usually missing. In the case of chronic granulocytic leukemia, it was suggested that the abnormal platelet production observed is due to the prevalence of low ploidy megakaryocytes, as determined by cytophotometric evaluation of DNA content per cell [139]. The blastic crisis of chronic myelogenous leukemia, with rearrangement of 3q26, was reported to induce high levels of circulating platelet fragments and megakaryocytic nuclei. In these patients, ploidy analysis indicated that the megakaryocytes are predominantly 2N [142]. As to acute megakaryoblastic leukemia (AML M7), karyotype analysis pointed to some patients having Down syndrome, some with a t(1;22)(p13,q13) translocation or other cytogenic abnormalities involving 22q13, while the third group includes a variety of cytogenic changes such as rearrangement of 3q21 and 3q26-27 [143]. These leukemias show a wide morphologic spectrum. In some cases, small cells expressing megakaryocytic markers are dominant, while in others the dominant cells are large with a high nucleus to cytosol ratio, resembling ALL-L2 blasts [144].

Megakaryocyte ploidy and cardiovascular disease

Recent investigations have revealed a correlation between megakaryocyte ploidy and cardiovascular disease. Patients with atherosclerosis show an increase in megakaryocyte ploidy over normal controls, with a corresponding increase in platelet mass [145]. A similar increase is seen in patients with diabetes, and a further enhancement occurs in patients diagnosed with both disorders. Megakaryocyte size, which correlates with ploidy, has been found to be increased in patients who have suffered a recent MI as compared to normal controls. Increases were also seen in a population of men suffering sudden cardiac death, compared with age-matched victims of traumatic death [146]. The increase in megakaryocyte ploidy is presumed to have preceded the ischemic event, and may be hypothesized to have a causal role. In a rabbit model, the development of large, high-ploidy megakaryocytes following platelet destruction by anti-platelet serum was found to herald a significant increase in atherosclerotic lesions of the aorta [147]. Platelets derived from these large megakaryocytes are presumed to have a causative role in atheroma formation. In animal models of atherogenesis, including guinea pigs and rabbits, increased megakaryocyte size and ploidy may actually be induced by extended feeding of high cholesterol diets [148,149]. MPV, however, was actually lower in the cholesterol-fed animals. On the other hand, others have reported that platelet and megakaryocyte size were smaller in rabbits fed high cholesterol diet, while

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ploidy was not increased [150]. It was also reported that Watanabe rabbits, which have hereditary hyperlipidemia, also have small platelets and megakaryocytes with increased platelet numbers. Modal ploidy in both normal and Watanabe rabbits was 32n [151]. Further confusing the issue, shorter-term high cholesterol diets have been found to actually decrease megakaryocyte size [152]. All these studies differ somewhat by the length and exact content of the high fat diet used during the study, which may impact on the results in terms of platelet level and/or megakaryocyte ploidy. Moreover, the genetically hyperlipidemic animal may induce megakaryocyte abnormalities in a different fashion from the short-term dietary assaults with the high cholesterol feeding.

The available evidence suggests, then, that increased megakaryocyte ploidy is associated with atherosclerotic disease, and may be a risk factor for the development of cardiovascular complications. The effects of increased ploidy are likely to be mediated through the production of highly reactive platelets, whose role in acute coronary syndromes and in atheroma formation is already clear [153].

Summary

Polyploidy occurs in nature in a variety of organisms and cell types. Clearly, while these systems share many features in common, and much may be learned through comparisons, it is dangerous to group them together mechanistically. Some achieve polyploidy through complete skipping of mitosis, while others abrogate mitosis at some stage. Even within these classifications, however, different cell types surely achieve polyploidy through different alterations in the cell cycle machinery. Future investigation should aim to explore the mechanism by which megakaryocytes may skip late anaphase and particularly cytokinesis, leading to the formation of polyploid cells. During a mitotic cell cycle, check points assure that the cell does not progress to a new phase before completing a preceding one, and yet, in polyploidizing megakaryocytes a potential abrogation of late mitosis and the lack of cytokinesis do not result with cell cycle arrest. One then may hypothesize that the simultaneous downregulation of components of late mitosis and/or cytokinesis and a modified G1 checkpoint are needed to achieve high ploidy in megakaryocytes. The mechanisms regulating ploidy in non-megakaryocytic systems, such as vascular smooth muscle, will need to be explored more fully in the future, aided by principles discovered in megakaryocytes. A very recent study involving the use of a microarray-based gene expression analysis identified clusters of genes showing ploidy-dependent expression in a yeast strain that varied in ploidy from haploid to tetraploid only [154]. In this study, mating produced the desired ploidy, while ploidy drove the expression of specific groups of genes. While the specific genes inducing high ploidy in megakaryocytes are still to be fully explored, the state of ploidy is likely to determine the expression of other genes, which may alter the property of the cell. This is a relevant point in relation to the frequently asked question, what are the advantages of polyploidy in the megakaryocytic lineage?

In human under normal conditions, megakaryocyte ploidy correlates with platelet volume, and large platelets are highly reactive. This may not apply, however, to the disease state and to some experimental animal models, such as the C3H mice described above. There may be mechanisms acting at the level of the megakaryocyte that have a causative role in coronary disease.

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