ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΙΑΤΡΙΚΗ ΣΧΟΛΗ

EX-VIVO ΑΠΕΙΚΟΝΙΣΗ ΤΗΣ ΜΕΓΑΚΑΡΥΟΚΥΤΤΑΡΙΚΗΣ ΕΝΔΟΜΙΤΩΣΗΣ: ΔΙΑΦΟΡΕΤΙΚΗ ΡΥΘΜΙΣΗ ΤΟΥ ΧΡΩΜΟΣΩΜΙΚΟΥ ΔΙΑΧΩΡΙΣΜΟΥ ΣΤΑ ΧΑΜΗΛΟΥ ΚΑΙ ΥΨΗΛΟΥ ΒΑΘΜΟΥ ΠΛΟΕΙΔΙΑΣ ΚΥΤΤΑΡΑ

ΝΙΚΟΛΑΟΣ ΠΑΠΑΔΑΝΤΩΝΑΚΗΣ

B.Sc (Hons), ΠΑΝΕΠΙΣΤΗΜΙΟ ΤΗΣ ΓΛΑΣΚΩΒΗΣ, 1999

ΠΤΥΧΙΟ ΙΑΤΡΙΚΗΣ,ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ, 2005

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ΠΕΡΙΛΗΨΗ

Ο ενδομιτωτικος κυτταρικός κύκλος στα μεγακαρυοκυτταρα (ΜΚ) ο οποίος περιλαμβάνει επαναλαμβανόμενη αντιγραφή του DNA χωρίς κυτταροκίνηση, είναι σημαντικό κομμάτι της ωρίμανσης του ΜΚ. Βάση μελετών ανοσοφθορισμου, είχε υποτεθεί ότι η ρύθμιση της διαδικασίας είναι ίδια για όλα τα ΜΚ ανεξάρτητα πλοειδιας. Πάραυτα, η δυναμική της χρωμοσωμικης κινητικότητας στα ενδομιτωτικα ΜΚ δεν είχε παρατηρηθεί ποτέ in vivo. Χρησιμοποιήσαμε, ένα πρόσφατα κατασκευασμένο στο εργαστήριο μας, διαγονιδιακο μοντέλο ποντικού στο οποίο η ιστονη H2B είναι ενωμένη εν σειρά με την πράσινη φθορίζουσα πρωτεΐνη και έχει στοχευόμενη έκφραση στα ΜΚ. Τα ποντίκια αυτά εμφανίζουν έκφραση της GFP και αλληλεπίδραση με το πυρηνικό DNA μόνο στην μεγακαρυωτικη σειρά. Κύτταρα μυελού των οστών, η κύτταρα από καλλιέργειες εμβρυϊκών κύτταρων ήπατος απομονώθηκαν από τα διαγονιδιακα ποντίκια και χρησιμοποιήθηκαν για μικροσκοπία επικεντρωμένη στα ΜΚ. Ένα κύριο ερώτημα ήταν εάν τα κύτταρα ακολουθούν όλα τα σταδία της μίτωσης περιλαμβανόμενου και του σχηματισμού μεσωζωνης. Η ανάλυση μας καταδεικνύει χρωμοσωμικη συμπύκνωση στην πρώιμη μίτωση σε όλα τα ΜΚ κύτταρα ανεξάρτητα πλοειδιας. Στα υψηλής πλοειδιας κύτταρα

(>8M-16N), η συμπύκνωση ακολουθήθηκε από μια δακτυλιοειδή διάταξη των χρωμοσωμάτων και ένα διαχωρισμό άνισων ποσοτήτων DNA που σχημάτισαν περιοχές που έμοιαζαν με μεσοζωνη, χωρίς να ακολουθήσει κυτταροκινηση. Σε αντίθεση, παρατηρήσαμε 8N κύτταρα που προήλθαν από 2 ομάδες 4N DNA τα οποία αρχικά κινήθηκαν στους αντίθετους πόλους με σχηματισμο καθαρής μεσοζωνης ανάμεσα τους, όπως συμβαίνει στα διπλοειδη κύτταρα, πριν ξαναενωθούν σε ένα πυρήνα απουσία κυτταρικής διαίρεσης. Επίσης, μπορέσαμε να καταγράψουμε τα τελικά σταδία του κατακερματισμού των MK σε αιμοπετάλια.

UNIVERSITY OF CRETE

SCHOOL OF MEDICINE

Master Thesis

EX-VIVO IMAGING OF MEGAKARYOCYTIC ENDOMITOSIS: DIFFERENTIAL REGULATION OF CHROMOSOMAL SEGREGATION IN LOW AND HIGH PLOIDY CELLS

NIKOLAOS PAPADANTONAKIS

B.Sc (Hons), University of Glasgow, 1999

M.D, University of Crete, 2005

Submitted in partial fulfillment of the requirements for the degree of Master in the graduate program “Cellular and Genetic Etiology, Diagnosis and Treatment of Human Disease”

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Approved by

First Reader _____

Dr Helen Papadaki, MD, Ph.D

Professor of Hematology

Second Reader _____

Dr Dimitrios Boumpas, MD, MACP

Professor of Medicine

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My graduation from the University of Glasgow with a B.Sc (Hons) in molecular biology offered me with the opportunity to pursue research in many different and exciting fields. However, I decided to study medicine because I always wanted to expand the knowledge I gained in cellular level to that of the whole organism.

After graduation from the medical school, I faced the dilemma whether to pursue clinical career or try to combine both clinical and research backgrounds. I decided to apply to the graduate program of our medical school and was privileged to be granted the opportunity through the program to join Dr Katya Ravid's laboratory.

Under Dr Ravid's guidance I was exposed and submerged into the field of megakaryocytes. A rare population of blood cells with many and enigmatic aspects. Through her constant encouragement and mentorship I started exploring aspects of megakaryocyte physiology and was privileged to be one of the first to witness a well kept secret of their physiology, that is part of this master thesis. Although for most medical doctors, megakaryocytes and platelets are viewed only in the context of hemostasis, I am very excited every time I discover that platelets and megakaryocytes are implicated in many other and different aspects of human physiology.

Rare cells pose rare problems, with the feelings of discouragement and disappointment emerging at times that problems seemed overwhelming. At that time, apprentices turn to their mentors for insights and guidance. Dr Ravid

always finds time, despite her very busy and hectic schedule, to listen, recommend solutions but above all explain proposed strategies and offer insights. I never saw Dr Ravid discouraged by negative results or experiments that did not work. Through her example I learned that experiments have always information to give us, things to teach us. As many young researchers, I become excited with several "ingenious ideas" of mine. Here, I was taught that every idea must be backed with sufficient data and that every step must be meticulously examined before it is proposed. Dr Ravid constantly request from all members of our lab to come forward with ideas and insights. This is a very important training and learning process, because we learn to think and plan independently. It gives me great pleasure to be able to plan my own experiments and test my own ideas.

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Dedicatory Page

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EX-VIVO IMAGING OF MEGAKARYOCYTIC ENDOMITOSIS: DIFFERENTIAL REGULATION OF CHROMOSOMAL SEGREGATION IN LOW AND HIGH PLOIDY CELLS.

NIKOLAOS PAPADANTONAKIS

University of Crete School of Medicine, 2007

Abstract

The endomitotic cell cycle in megakaryocytes (MKs), which involves repeated DNA replication in absence of cytokinesis, is an important part of MK development. Based on immunofluorescence, it has been assumed that the regulation of this process is uniform in all MK cells regardless of ploidy. However, the dynamics of chromosome movement in endomitotic MKs has never been observed in vivo. We utilized, a recently engineered in our laboratory, transgenic mouse model in which histone H2B fused in frame to green fluorescent protein (GFP) was targeted to MKs. These mice display linage specific expression of GFP associated with nuclear DNA. Bone marrow or fetal liver cells isolated from the transgenic mice were subjected to time-lapse microscopy with a focus on MKs. A central inquiry at hand has been whether the cells undergo all steps of mitosis, including a formation of a proper midzone. Our analysis clearly indicated chromosome condensation at early mitosis in all MK cells regardless of ploidy status. In high ploidy MKs (>8N-16N), the condensation was followed by a ring-

type alignment of chromosomes and a separation of an uneven amount of DNA to form midzone-like territories, followed by chromosome pulling to one side with no cytokinesis to follow. In contrast, we observed 8N that resulted from two sets of 4N DNA pulling apart to opposite poles with a clear midzone area, as typical of mitotic cells, before re-gathering into one nucleus in absence of cellular division. Furthermore, we were able to monitor and document chromosomal DNA localization during terminal stages of MK fragmentation into platelets.

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Chapter I. INTRODUCTION

Outline of platelets and Megakaryocytes in their functional context

Platelets and their precursors the megakaryocytes (MKs), were considered for many years to have a role limited only in the context of hemostasis. MKs constitute only a small fraction of cells residing in bone marrow and are responsible for the daily production of 1 x 10^{11} platelets in human adults[1], that can be further increased 10-fold under conditions of stress[2]. Platelets are one of the most numerous element of the blood and recent studies revealed their intriguing role in immunity, angiogenesis[3] in the context of neoplasias and as carriers of prognostic markers related to cardiovascular diseases[4]. The role of platelets and their precursors is less unexpected when viewed in the context of evolution.

Overview of MK and platelets evolution

Fish were the first vertebrates to evolve approximately half a billion years ago. By sharp contrast to mammals, fish hemostasis is carried out by thrombocytes (Greek *Clotting Cells*) that are nucleated cells and their progenitor remains elusive. Thrombocytes is the second most abundant cell population in fish circulation but their number and size varies among species[5]. Intriguingly, thrombocytes, similarly to platelets, contain granules but the degree of granulation is highly variable between different species It remains controversial whether thrombocytes have true phagocytic properties[6].

Seminal studies using the zebra fish model system have expanded our knowledge regarding thrombocytes[7,8]. Zebra fish thrombocytes can be detected at 36 hours post fertilization (hpf) and several transcription factors with a key role in mammalian thrombopoiesis have been also identified in zebra fish.

More studies are available for avian thrombocytes that play important role both in hemostasis and immunity. Chicken has emerged as the model system for the study of avian thrombopoiesis. Chicken thrombocytes are nucleated cells and are produced by antecedent mononucleated cells. Although chicken thrombocytes contain scarce quantity of Golgi apparatus and rough endoplasmatic reticulum, they can synthesize proteins[9].

Chicken thrombocytes contain several proteins associated with immunity such as leukotriene B4, CD40 ligand and several chemotactic factors. Moreover, a recent study demonstrated that thrombocytes can be stimulated by LPS through a signaling cascade involving Toll-Like Receptor 4, MAP and NF-Kβ pathways[10]. In addition, stimulated thrombocytes produce prostaglandin E2 and the expression of pro-inflammatory cytokine IL-6 is significantly increased. The reason that thrombocytes, with a dual role in hemostasis and immunity, were not retained in mammalian species remains enigmatic.

Megakaryopoiesis

Even though, the hematopoietic system has been studied rigorously for several decades many aspects of MK physiology remain elusive. MK studies are inherently difficult, because MK population is very scarce precluding large scale

isolation experiments. Furthermore, intermediate stages of maturation exhibit variation in size and phenotypic characteristics that further subdivide MK population in smaller groups. In order to overcome these limitations, other sources of MKs apart from bone marrow can be utilized such as murine fetal liver cultures obtained at days 12-15 post coitum. In addition, MKs can also obtained from $CD34^+$ cells isolated from cord blood that are induced to differentiate towards MK lineage by growth factors such as Thrombopoietin (TPO). MK structure and morphology were primarily studied though immunocytochemistry and bone marrow specific staining methods such hematoxylin & eosin. This knowledge was later complemented with ultra structural details obtained by electron microscopy, Flow Assortment Cell sorting (FACS) analysis and recently with advanced imaging modalities[11].

MKs are generated by Hematopoietic Stem Cells (HSC) that reside in specialized areas of the bone marrow called niches. The most well studied is in the vicinity of endosteum where HSC can interact with osteoclasts and osteoblasts[12]. Recently, evidence is mounting for the existence and role of the vascular niche that is placed around the bone marrow sinusoids.

Arterial vessels penetrate the bone and branch into a capillary network that intermingles with bone marrow. Capillaries form sinusoids that are highly permeable blood vessels, and their wall is composed by a single layer of endothelial cells. This unique structure allows the cells in the vicinity of sinusoids to be directly bathed by the growth factors and nutrients of blood circulation[13].

An evolving concept is that stem cells in the endosteum niche are in maintenance state while those that are located towards the vascular bed of bone marrow are differentiating towards more mature forms. Similarly, the great majority of mature MKs are found in the vicinity of vascular niche and can through sinusoids a) shed platelets in the blood stream or b) gain entrance to blood circulation[14]. The MKs that gained entrance in the blood circulation are trapped in the capillary network of the lungs with increased numbers reported in biopsies of patients suffering from diffuse alveolar damage[15]. During megakaryopoiesis HSC produce MKs through the generation of progressively more differentiated progenitors. HSC give rise to Megakaryocyte/Erythrocyte Progenitor (MEP) population which is common for megakaryocytic and erythroid lineage[16]. The most definite primitive progenitor of MKs are the high proliferate potential-colony-forming unit-megakaryocyte (HPP-CFU-MK). A more differentiated progenitor is the burst-forming unit-megakaryocyte (BFU-MK) that gives rise to colony-forming unit-megakaryocyte (CFU-MK)[17]. The CFU-MK then give rise to Megakaryoblasts, (alternatively designated group I MKs), the most primitive cell of thrombopoiesis that can be recognized in histological preparations of bone marrow and has a size of 8-30 microns. It is characterized by a large, oval to round, nucleus with several nucleoli which is surrounded by a basophilic cytoplasm that lacks granules. Chromatin appears dense in small cells but assumes a reticular pattern in larger cells.

Immature MKs (alternatively designated group II MKs or pro-megakaryocytes) are produced after 7 divisions of the megakaryoblast. Immature MKs have a larger size and cytoplasm to nucleus ratio than megakaryoblasts. Their single and with multiple lobes nucleus has a horse - shoe shape. In addition, scarce amount of azurophilic granules are dispersed in their cytoplasm.

Eventually, cells with large nucleus that do not proliferate further are generated and are called Mature MKs (alternatively designated group III MKs or granular megakaryocytes). The MKs comprise the 0.002-0.005% of Bone marrow nucleated cells and their number is estimated to be 4×10^7. MKs are large cells with a median size 30-70 μm (range 20-100 μm), a single large polyploid nucleus that is multi segmented with coarse grained chromatin. The cytoplasm of MKs can be divided in 3 zones. The perinuclear zone remains attached to the nucleus after release of platelets and contains several organelles such as ER, Golgi apparatus and centrioles. The intermediate zone contains the demarcation membrane system which plays a critical role in the formation of proplatelets. The marginal zone involves the megakaryocytic cell membrane and is composed mainly by cytoskeletal filaments .

MK developmental staging is based predominantly on studies with flow cytometry that detect characteristic cluster of differentiation (CD) patterns (reviewed in[18] [2]). The most widely surface markers for the study of megakaryopoiesis are integrin GPIIIa (CD61), von Willebrand factor (CD42),

GPIIb/IIIa alternatively designated as integrin a_{IIb} β_3(CD41a), GPIb (CD41b), Platelet Factor 4 (PF4) as well as CD34 and CD38.

Growth factors affecting Megakaryopoiesis

Several cytokines are involved in the thrombopoiesis[19] and many of them exert a positive effect such as TPO,EPO,SCF,GM-CSF,IL-3,IL-6,IL-12,IL-11. An inhibitory effect is exerted by TGF-B, PF4 and IL-4. The Src inhibitors were recently reported to have a detrimental effect in thrombopoiesis.

Furthermore, bone marrow endothelial cells (BMECs) can interact with MK progenitors and through the action of stromal derived factor -1 (SDF-1) and fibroblast growth factor – 4 (FGF-4) rescue thrombopoiesis of TPO or it's receptor (mpl) knock out mice. The evolving concept is that SDF-1 and FGF-4 promote localization of MKs in the vascular niche where the microenvironment can sustain their proliferation maturation and ultimately the release of platelets[20].

However, the capital role on MK physiology has the Thrombopoietin (TPO) or mpl ligand that belongs to the GP 130 family of proteins (reviewed in[21,22]). Under normal conditions TPO levels in the human plasma are in the range of 95 pg/l and can increase several fold under conditions of stress. Human TPO gene is located on chromosome 3q26.3-3q27 and encodes a 353 amino acid precursor protein that contains a 21 amino acid signal peptide. Modification of the precursor leads to production of the acidic and highly glycosylated 95 KD TPO protein. TPO protein can be divided into the N and C terminal domains. The N terminal domain is consisted of 153 amino acids and shares 23% homology with

Erythropoietin. Importantly, N-terminal domain has two interaction sites with TPO receptor, one conferring high and the other low affinity binding. N-terminal domain is sufficient for binding in the TPO receptor and promote downstream signaling cascades.

On the other hand, the C terminal domain has 179 amino acids and is rich on O- and N- linked carbohydate sites and it does not share homology to any known protein. C-terminal is involved in protein stability and secretion. Murine TPO shares extensive homology (93%) with human TPO in the N terminal domain although in the C terminal domain homology is less pronounced (74%).

The TPO acts on the HSC and is very important for their homeostasis. TPO exerts its effect on HSC by upregulating gene expression or by facilitating nuclear localization of transcription factors such as homeobox proteins. Furthermore, TPO increase the percentage of cells committed to megakaryocytic lineage and increase the size, ploidy status and augments proplatelet formation of MKs.

The action of TPO in platelets is exerted in many different levels. TPO augment the action of thrombin by inducing the secretion of α granules through activation of PI3K pathway. Another action of TPO is that reduces the amount of collagen and ADP for the activation and aggregation of platelets.

TPO shares extensive homology, especially in the N terminus, with EPO a cytokine with very important role in erythropoiesis. Disruption on the production of TPO or its receptor mpl reduces by 85% production of platelets[23]. However,

this finding indicates that other redundant pathways exist that can promote thrombopoiesis in the absence of TPO / mpl.

TPO is produced constitutively by two pathways and the effect in the megakaryopoiesis is largely regulated by the level of circulating platelets. Under steady state conditions TPO is produced in the liver and kidneys and is captured by circulating platelets through their mpl receptors followed by internalization and proteolytic digestion. The free (unbound) TPO that reaches bone marrow will exert its effect on MKs. In the second pathway TPO is produced by the stromal cells of bone marrow and is released in the vicinity of MKs. Under normal conditions the TPO production under this pathway is minimal but it can increase significantly in states of thrombocytopenia. It has been reported PDGF and FGF-2 have a positive effect while PF4 and TGF-b a negative effect in the production of TPO by stromal cells of human bone marrow.

In addition, hepatic production of TPO can be significantly upregulated in inflammatory disorders such as rheumatoid arthritis and Crohn's disease through the action of IL-6.

Intriguingly, the regulation of megakaryopoiesis by TPO is not straightforward. For example, the number of mpl receptors in endothelial cells surpass those found on platelets and MKs, but endothelial cells are not implicated in the regulation of TPO levels. Furthermore, in patients suffering from idiopathic thrombocytopenic purpura, characterized by massive consumption of platelets, the levels of TPO are not increased proportionately to platelet destruction.

MK ploidy correlation with size

Although the mean ploidy of MKs in the bone marrow of humans and most mice strains is 16N, some MKs can acquire a DNA content of up to 128N, where 2N is the DNA content of a diploid somatic cell. The increase in MK ploidy is also followed by increase in MK size[18] with the diameter of human 2N MK being 21 -/+ 4 μm compared to 55.80 -/+ 8.1 μm of 64 N MK. However, estimating MK ploidy based on size is difficult since human 16 N MK have a diameter of 37.80 -/+ 30.2 μm.

MK endomitosis

The MKs are responsible for the production of daily production of 1 x 10^{11} platelets in human adults[1]. The production of this vast amount of platelets is achieved through an elaborate process of megakaryocytic maturation[24]. The hallmarks of MKs maturation are the accumulation of ribosomes, proteins, platelet specific granules coupled with reorganization of the cytoskeleton[25] and an increase in DNA ploidy status[26]. Mature MKs have 4N-128N content and achieve this state of high ploidy through endomitosis[27]. Many aspects of endomitosis remain enigmatic and its role and significance in platelet production has not been clearly elucidated[27,28].

The relation between MK ploidy level and platelets remains controversial, with some studies linking platelet quality to MK ploidy level (reviewed in[26]) while other studies demonstrated production of platelets by 2N or 4N MKs[29]. Endomitosis is not unique to MKs and has been documented in cardiomyocytes, in vascular

smooth muscle cells of ageing or hypertensive animals[30], and also in the giant cells of trophoblast[27]. Hence, deciphering the mechanisms that govern endomitosis in MKs may serve as model for further understanding of the endomitotic related phenomena in other cell types.

During endomitosis S-phase is followed by mitosis that anaphase and telophase are skipped and a gap phase before cell proceeds to the subsequent round of DNA synthesis. The endomitotic process is characterized by nuclear breakdown and the presence of multiple spindle poles[31].

The duration of endomitotic cycle is estimated to be approximately 9 hours with G1 phase lasting 1 hr, S phase approximately 7 hr and rest allocated to G2 and mitosis. The majority (greater than 85%) of murine polyploid MKs isolated from bone marrow are cell cycle arrested. Moreover, only 60 % are positive for cyclin D3 or p21 which are markers of G1 phase. Taken together these data indicate the majority of polyploid MKs are arrested at G1 while the rest at Go. However, cell cycle arrested MKs may be recruited into endomitotic cycles by TPO treatment. This is suggested by the fact that 15% of freshly isolated bone marrow MKs are in S phase and this percentage is doubled after 12 hours of TPO treatment. This relatively fast increase of MK population in S phase may arise from the recruitment of polyploid MK cells that are cell cycle arrested in parallel with diploid MKs entering endomitotic cycles.

Approximately 50 % of diploid MKs cultured in the presence of TPO are in G1 phase. However, in the presence of TPO, as the MKs increase their ploidy state

up to 32N the percentage of cells in G1 phase is reduced with concomitant increase of cells found in G2/M and S phase. In contrast, in the MK population going from 32N to 64N the percentage of cells in G1 increase with a parallel decrease of cells in the G2/M phase. Taken together these data indicate that MKs that reach ploidy states greater 32N tend to exit endomitotic cycles.

Studies of phenomena related to MK endomitosis are predominantly based on immunofluorescence microscopy, with only one study in the literature pursuing ex-vivo time lapse microscopy. However, this study focused only on the microtubule network of MKs without visualizing the chromosomes.

During Interphase MK DNA is replicated but becomes visible only during prophase. In early prophase chromosomes are seen as threads in the nucleus and the centrioles are migrating towards the poles of the cell. At that time also, microtubule filaments start emanating from centrioles surrounding the nucleus. At late prophase, the nuclear membrane is disturbed and the microtubule filaments form a network that connect through a specialized area –the kinetochore- sister chromatids with the centrioles that are positioned at the opposite poles of the cell.

In metaphase, the sister chromatids through the action of microtubules migrate towards cell equator and gradually align. At this point chromosomes have a tight, compact and lobulated form and dense arrays of microtubules connect them to the centrioles.

During early anaphase the sister chromatids separate and depart towards the poles of the spindle network that they are attached consistent with Anaphase A. An integral part of anaphase is the formation of spindle midzone. The spindle midzone is formed by a set of anti parallel microtubules that are not attached in the kinetochores and are placed between the departing chromosomes. Although anaphase A appears to occur the anaphase B which involves spindle elongation and further movement of chromatids towards the poles is not observed in MKs.

Nevertheless, Central spindle midzone is instrumental for the concentration of an array of proteins involved in cytokinesis. One of them is Centralspindlin complex, which composed by MKLP1 and CYK-4, a Rho family GTPase protein and have a key role in furrowing. During furrowing of diploid cells, a contractile actomyosin network under the regulation of Rho1 protein is formed that partitions the cell into two territories. In last steps of cytokinesis the contractile ring is constricted generating two daughter cells but the terminal steps of cytokinesis require the addition of membrane vesicles to cover the area between the edges of the contractile rings and also to surround with membrane surface area the daughter cells. Although the position of furrowing is dictated by midzone, the role of midzone in ingression of furrowing during cytokinesis remains, at least for diploid cells, controversial.

In the MKs the late events of mitosis related to telophase, such as formation of nucleus around each group of chromatids and generation of two daughter cells are aborted. Although phenomena related to telophase had not been reported in

MKs based on data obtained from fixed cells[31], recent ex-vivo studies indicated that furrowing occurs at least in MKs of low ploidy[32]. High ploidy MKs (designated as those with 4 or more spindle poles) exhibited transient and attenuated furrowing. Moreover, the generation of midbody formation is claimed to occur[33] but this is not certain as analysis is based on fixed cells, and live imaging has not been pursued. Rapidly occurring phenomena may have been missed and thus, crucial details of these dynamics may not yet have been revealed. Taken together these data clearly demonstrate that time lapse microscopy can have a paramount role in answering these questions.

Key proteins of Endomitosis

Despite the fact that during endomitosis the mechanisms that refrain diploid cells from attaining polyploidy status[3435] are circumvented, the whole process remains tightly controlled[36]. Several layers of regulation have been described involving cyclins/Cdk, chromosomal passenger complex and microtubule organizing proteins.

Cyclins interact with Cdks and regulate progression through cell cycle[37]. In the regulation of the mitotic cycle in megakaryocytes cyclins of type D (D3 and in lesser extent D1) and type E (E1 and E2) have a significant role. Type D cyclins interact with Cdk 4 and 6 and phosphorylate pRB and pRB related proteins (p107 and p130) priming progression to S-phase[38]. Our Laboratory has previously shown that overexpression of cyclin D3 in MKs has as result increase in endomitosis[39]. Type E cyclins associate with Cdk 2 and 3 and

hyperphosphorylate pRB promoting progression to S-phase. Importantly, knock out models of type E cyclins indicate that are absolutely required for progression of cells from Go to S phase and that have a significant role in endoreplication of megakaryocytes and trophoblast giant cells[40]. Although the significance of cyclins has been documented, their mediators or mechanisms that result in high ploidy MKs are poorly described.

Microtubule organization and spindle assembly checkpoints have an important role on preventing cells to attain polyploidy/aneuploidy state[41]. MKs must overcome these regulatory circuits when switching from normal mitosis to endomitosis. Chromosomal passenger proteins have attracted attention as important mediators of endomitosis because they are involved in the association of microtubules with chromosomes, in the spindle checkpoint and late events of telophase[42]. Aurora-B, INCENP, Borealin/Desra and Survivin are members of the Chromosomal passenger complex[43].

Survivin is also member of the IAP (Inhibitor of Apoptosis) family of proteins but its precise role in the context of apoptosis remains elusive[44] with several cancer clinical trials targeting Survivin in progress[45]. Survivin has a firmly established role in cell cycle regulation in the context of chromosomal passenger proteins[46]. Survivin mouse knockout models were embryonic lethal at day 5.5 post coitum. Gross anomalies included small number of cells with abnormal and large nuclei coupled with defects in microtubule formation and cytokinesis[46]. A study adopting a siRNA approach[47] indicated that Survivin is essential for the

proliferation of megakaryocyte – erythroid progenitor and is also required for the differentiation of the more committed progenitors of erythroid lineage. Furthermore, reduction of Survivin levels through RNA interference in vitro augmented megakaryopoiesis. Localization of Survivin in murine models during all phases of endomitosis has not been precisely defined [47,48].

Another member of the chromosomal passenger complex is Aurora B/AIM-1[49]. Aurora B is implicated in cytokinesis and segregation of chromosomes[50]. It interacts with Survivin and INCENP and by phosphorylating Vimentin exerts control in Vimentin mediated cytokinesis. Our laboratory has previously shown in transgenic model of Aurora B that small MKs have higher levels of Aurora B compared to large MKs[48]. Excess Aurora B was removed with an internal mechanism and high levels had an inhibitory effect in endomitosis. Furthermore, knock out models of Aurora B had mitotic defects and formation of polyploid cells[51]. Immunofluorescence studies focused on Aurora B pattern of localization are difficult due to staining and antibody reactivity issues. However, the Aurora B localization was reported to be similar with that of diploid cells in MKs of human [49] and murine[52] origin.

Recently, new players were implicated in endomitosis adding another layer of complexity. Stathmin[53] is a protein involved in microtubule depolymerization and high levels are necessary in early steps of megakaryocyte maturation. However, downregulation of Stathmin protein levels may be required for the terminal stages of megakaryopoiesis[54]. Another potential player is the EVI proto-oncoprotein[55].

EVI protein levels are undetectable in normal bone marrow but expression of Evi protein was detected in patients suffering from 3q21q26 syndrome[56]. The 3q21p26 syndrome is an aggressive form of leukemia characterized by dystrophic MKs and associated with poor prognosis. MKs isolated from patients suffering from this syndrome have relatively small size and their ploidy level is low. Studies in cell lines indicated that Evi protein may have a role in polyploidization[57].

The ability to monitor and track fluorescently tagged proteins with a key role in endomitosis could address the questions regarding their presence and localization throughout the endomitotic cycle.

Biogenesis of proplatelets

The terminal steps of MK maturation involve the production of platelets. The mechanism underlying platelet production has been controversial for several years. One hypothesis, based predominantly on electron microscopy studies, proposed that preformed platelets were produced in the cytoplasm of MKs that where subsequently released. The second model proposed that platelets are generated by proplatelets; an array of cytoplasmatic processes that extend from the surface of MKs with a highly variable pattern and can reach up to 60 nm length. The proplatelet model was further strengthened with pioneering ex-vivo real time studies pursued by Italiano and coworkers which demonstrated that microtubules is the main component of proplatelets and their sliding action is responsible for the proplatelet elongation [58]. Proplatelets are formed in most

cases from a single broad elongating pseudopodium. The pseudopodium continues to elongate for 4-10 hours and the proplatelet are formed with a diameter of 2-4 μm and have numerous bulges, which are interconnected with a thin bridge of cytoplasm. Furthermore as the proplatelets extend from the MK surface the thick bundle of microtubules proximal to MK cell membrane gradually thins out to approximately 20 microtubules at the tip of proplatelet. Platelets are released only form nascent tips of proplatelets. An elegant mechanism that increase the number of nascent tips that can shed platelets has been documented that implicates rearrangement of actin.

The generation of proplatelets continues until almost all cytoplasm is converted to proplatelet formations. Ultimately, proplatelets are either extruded in the bone marrow sinusoids or the mature MK gains entrance in the blood circulation for release.

Proplatelet release from mature MKs heralds the formation of senescent (apoptotic) MKs, which are removed by macrophages. The apoptosis of MKs is very complex and involves tight regulation through Bcl-2, Bcl-xL, and caspase 3 and 9 proteins. Apoptosis is integral part of proplatelet formation but the events related to the fate of nucleus have not been completely elucidated. For example, activation of caspase 3 during proplatelet formation appears localized but in the senescent MK the pattern is diffuse. Ultrastructural studies of human MKs using TEM demonstrated the existence of micronuclei and cytoplasmatic vacuolization in apoptotic MKs. The precise timing of the apoptotic events in nucleus in

relation to proplatelet formation may require time lapse imaging in order to be elucidated.

The PF4-H2B-GFP mouse model

Studies of endomitosis related phenomena are based predominantly on immunocytochemistry data of fixed cells and data from imaging of unfixed living megakaryocytes are very limited and do not visualize directly chromosomal dynamics. We decided to study endomitosis by devising a strategy of in vivo cell imaging based on fetal liver derived MKs. For the in vivo imaging studies we took advantage of a mouse model in which Rat PF-4 promoter is driving expression of GFP fused to human Histone 2B[59]. Histone 2B is an essential component of the chromosomal scaffold[59] and constructs based on the PF-4 promoter have already successfully utilized in previous studies to drive expression of genes of interest only in MKs[60,61]. It has been previously demonstrated that fluorochrome tagging of Histones does not affect cell viability[62] and has been used for the visualization studies in murine embryos[63]. Histone tagging is particularly useful in studies of endomitosis because MKs are readily identified by fluorescence circumventing purification procedures. Furthermore, DNA can be visualized during all phases of cell cycle regardless of nuclear envelope integrity. This aspect is particularly useful since it alleviates potential problems related to fixation methods commonly faced on immunochemistry studies. In our experience and reported elsewhere[31] obtaining fixed MKs at anaphase for

immunocytochemistry studies may require screening of a large number of samples.

These properties of our model are of key importance in the study of MK polyploidization because for the first time we have the ability in vivo to visualize, track and monitor chromosome segregation during endomitosis. Our model allowed us to observe in vivo that the endomitotic process is different in high and low ploidy MKs. These findings may have significant implications in our understanding of regulatory mechanisms governing endomitosis.

Chapter II. Materials and Methods

Bone marrow culture

Bone marrow cells were isolated from femurs of transgenic mice as described previously[39]. Mice were sacrificed according to institutional regulations using CO_2 and cervical dislocation. Femurs were isolated using sterile surgical instruments and the bone marrow was flushed with CATCH buffer (calcium-free Hanks' buffered salt solution supplemented with 1 mM adenosine, 0.36% sodium citrate, 5% bovine Calf Serum, 2 mM theophylline and distilled H_2O up to 65% of final volume). Bone marrow cells in CATCH buffer were spun at 500 g for 5 minutes at 4 ^oC. The pellet was resuspended in prewarmed, at 37 ^oC, lysis buffer (0.205% Tris (w/v), 0.75 % NH4Cl (w/v) dissolved in distilled H_2O) for exactly 10 minutes to eliminate red blood cells.

Cell suspension was centrifuged at 500xg for 5 minutes at 4 ^oC and the pellet was reconstituted with CATCH buffer and another centrifugation was performed again at 500 g for 5 minutes at 4 ^0C. The pellet was reconstituted with IMDM supplemented with 10% Bovine Calf Serum (BCS), 50 U/ml penicillin and 50 µg/ml streptomycin. Bone marrow cells suspended in IMDM were then filtered through a 250 µm mesh to remove clots and connective tissue remnants. After filtration, cells were spun at 500 g for 5 minutes, at 4 ^0C and supernatant was discarded. The pellet was resuspended in IMDM supplemented with 10 % BCS, 50 U/ml penicillin & 50 µg/ml streptomycin and recombinant TPO was added to a

final concentration of 25 ng/ml followed by incubation at 37 ^{0}C and 5% CO_2 conditions.

Fetal liver cells cultures

Fetal liver cells can be obtained 13-15 days post coitum and can be induced to differentiate towards megakaryocytic lineage through stimulation with TPO. Fetal liver cells were isolated as described elsewhere[64] with modifications. Female PF4-H2B-GFP mice 13.5 days post coitum were sacrificed according to institutional regulations using CO_2 and cervical dislocation. The umbilical sac was surgically removed and placed on prewarmed, at 37 ^{0}C, DMEM medium supplemented with 10% BCS and 50 U/ml penicillin & 50 µg/ml streptomycin. The murine fetuses were removed from their yolk sac and the fetal liver was excised and cleaned from the surrounding connective tissue. The liver tissue was homogenized and single cell suspensions were obtained by passing the fetal liver tissue progressively through 18 up to 25 g needle gauges. The single cell suspensions were spun at 500 g for 5 minutes at 4 ^{o}C and were resuspended in DMEM supplemented with 10% BCS, 50 U/ml penicillin & 50 µg/ml streptomycin and 25 ng/ml TPO. The volume of medium was adjusted to achieve a cell number of 5-10 X 10^6 cells/ml. The cells were then incubated at 37 ^{0}C with 5% CO_2.

Fluorescence and Ex-vivo cell imaging

After 24 to 72 hours of incubation at 37 ^{o}C in 5% CO_2 incubator, fetal liver or bone marrow cells were spun at 500 g for 5 minutes at 4 ^{0}C. The pellet was

resuspended in DMEM without phenol red supplemented with 10% BCS, 50 U/ml penicillin & 50 µg/ml streptomycin and 25 ng/ml TPO. Cells were plated onto, pretreated with 3% BSA in PBS, incubation video chambers containing DMEM without phenol red supplemented with 10% BCS, 50 U/ml penicillin & 50 µg/ml streptomycin, 25 ng/ml TPO and 20% Leibowitz's medium. The incubation video chambers were mounted on the microscope and held at 37 ^{0}C for the duration of observation.

We employed two different microscope settings for the in vivo imaging. In the first setting bright field and fluorescence imaging was performed by time-lapse video microscopy as described elsewhere[65] [58] on a Nikon Eclipse TE-2000E microscope using a 60X 1.4 NA objective lens and Metamorph imaging software (Universal Imaging Corporation, Molecular Devices, Downington, PA). Images were captured using an Orca-II ER cooled CCD camera (Hamamatsu, Hamamatsu City, Japan) every 90 seconds, however time interval could be manually adjusted in order to capture rapidly occurring phenomena. Imaging data were analyzed, compiled and exported in Apple Quick time video format utilizing Metamorph software.

In the second setting Bright field and fluorescence imaging of cells plated on onto Delta T micro-observation chambers (Bioptechs) was performed on Olympus IX70 microscope using 60x 0.9 NA objective lens and Image pro software (Media Cybernetics, Inc). Images were captured using a C4742-95CCD camera (Hamamatsu, Hamamatsu City, Japan) every 121 seconds. Imaging data

were analyzed, compiled and exported in Microsoft AVI format using ImagePro Software.

MK isolation

In order to study MK terminal maturation phenomena, we isolated an enriched fraction of large MKs by employing two different methods a) Flow Assortment Cell Sorting (FACS) sorting and b) BSA gradient. For the FACS sorting, isolation was based on GFP gating since only MKs of the PF4-H2B-GFP mouse model are GFP positive (Data not Shown). Briefly, fetal liver cells isolated from PF4-H2B-GFP mouse model were cultured for 24 hours in DMEM medium in the presence of TPO as described above. After 24 hour incubation, fetal liver cells were centrifuged at 500 g for 5 minutes at 4 ^{0}C. The pellet was reconstituted in 3 ml of DMEM without phenol red supplemented with 50 U/ml penicillin & 50 μg/ml streptomycin.

Flow sorting was performed with gating at GFP region. Cells were collected in tubes containing DMEM supplemented with 10% BCS, 50 U/ml penicillin & 50 μg/ml streptomycin. Cells were spun at 500g for 5 minutes at 4 ^{0}C and the pellet was DMEM supplemented with 10% BCS, 50 U/ml penicillin & 50 μg/ml streptomycin. Cells were then incubated on 37 ^{0}C and 5% CO_2 conditions.

In the BSA gradient method fetal liver cells were placed on the surface of a discontinuous gradient of 3% and 1.5% albumin followed by incubation at 37 ^{0}C to allow MKs to form a sediment. Supernatant was discarded and the pellet and small volume of BSA above the sediment were centrifuged at 500g for 5 min at 4

^{0}C. The pellet was resuspended in DMEM supplemented with 10% BCS, 50 U/ml penicillin & 50 µg/ml streptomycin and was incubated at 37 ^{0}C and 5% CO_2 conditions followed by Ex-vivo Imaging.

Immunofluorescence

Fetal liver cells 13.5 days post coitum and bone marrow cells were obtained both from PF4-H2B-GFP transgenic and wild type mice. Cells were cytospun and fixed overnight at 4 ^{0}C with 4% parafolmadehyde. Slides were washed with Phosphate Buffer Saline (PBS) and PBS supplemented with 0.25% Triton-X (PBST) and blocked with Image-iT FX signal enhancer (molecular probes, Invitrogen). Slides were then incubated with mouse anti α-tubulin antibody 1:500 dilution (Sigma) at 4 ^{0}C overnight followed by PBST wash and incubation with Alexa 594 highly adsorbed goat anti-mouse antibody at 1:1250 dilution (molecular probes, Invitrogen) for 1 hr at room temperature. PBS wash was performed before addition of vectrashield with DAPI mounting medium (Vector Laboratories,Inc). Images were obtained with an Olympus IX70 inverted fluorescence microscope (Melville,NY) at 60X 0.9 NA using ImagePro software.

Chapter III. Results

Implementation of the PF4-H2B-GFP transgenic mouse model in Ex-vivo imaging

Our aim was to document endomitotic related phenomena in living MKs. Our lab had already engineered a transgenic mouse model in which human histone 2B fused to GFP was expressed only in MKs (Figure 1A). The PF4 promoter confers megakaryocytic specific expression and has been used successfully for the construction of similar transgenes[60]. The number of MKs, their ploidy status and platelet levels had been already shown not to be affected by transgene expression. The PF4-H2B-GFP transgenic mouse model is particularly useful for ex-vivo imaging studies because it allows direct and specific visualization of MK chromosomes (Figure 1B). Visualization of DNA was evident also during proplatelet formation and nuclear fragmentation that occur at the terminal stages of the MK life cycle (Figure 1C). In order to reduce phototoxicity, imaging was performed with fluorescence exposure time kept in the lowest possible levels.

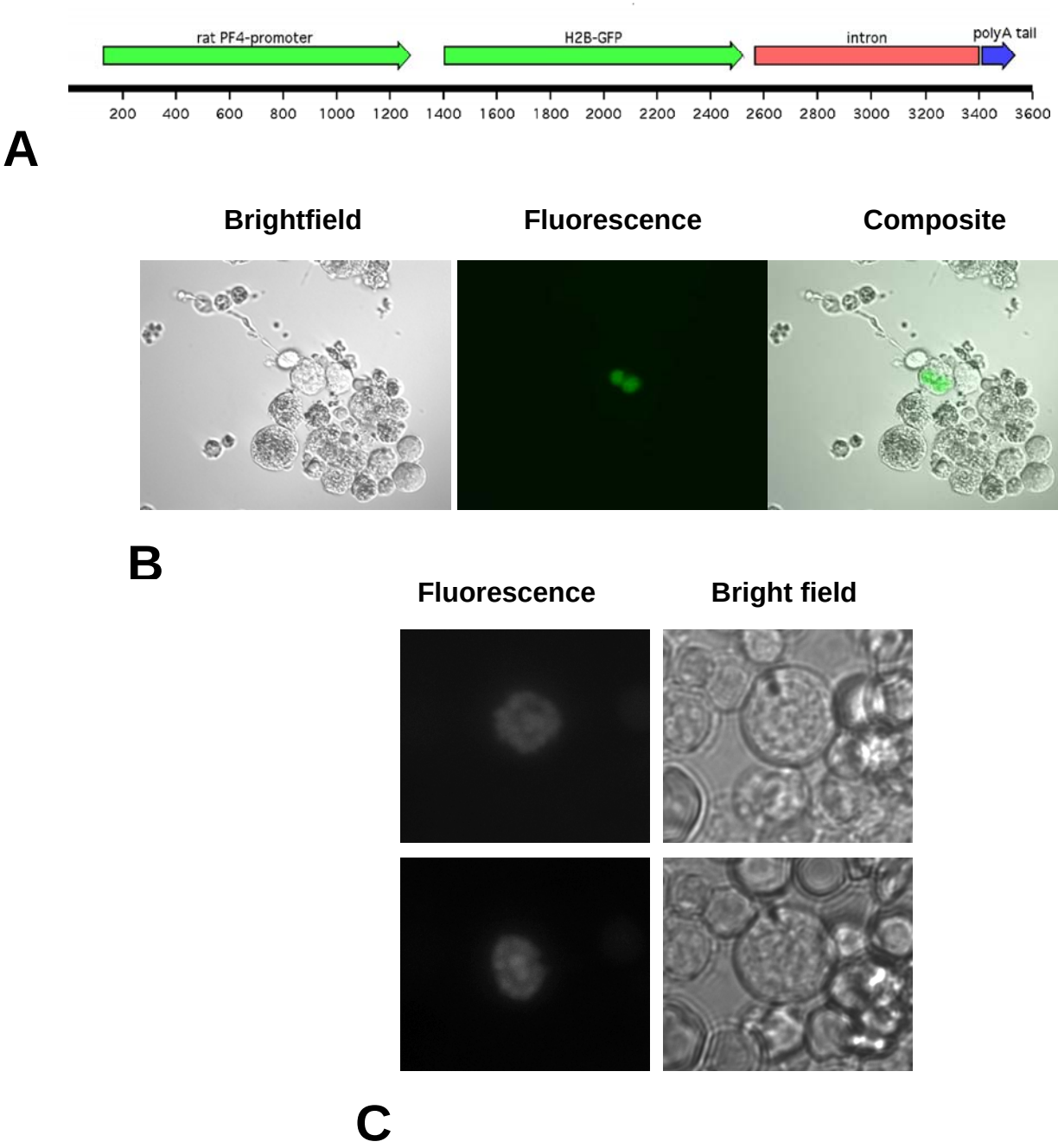


Figure 1. PF4-H2B-GFP transgenic mouse model & Ex-vivo imaging.

A. Schematic representation of the transgenic mouse model expressing H2B-GFP under the control of PF4 promoter. Human H2B cDNA fused to GFP is

preceded by PF-4 promoter conferring specificity of expression only in MKs. The untranslated region containing β-globin intron and polyA tail stabilizes mRNA levels of H2B fused to GFP. **B.** Fetal liver cells derived from PF4-H2B-GFP transgenic mouse viewed under in vivo imaging conditions. **C**. DNA of MKs is labeled during all stages of the cell cycle including terminal stages of MK maturation such as proplatelet formation.

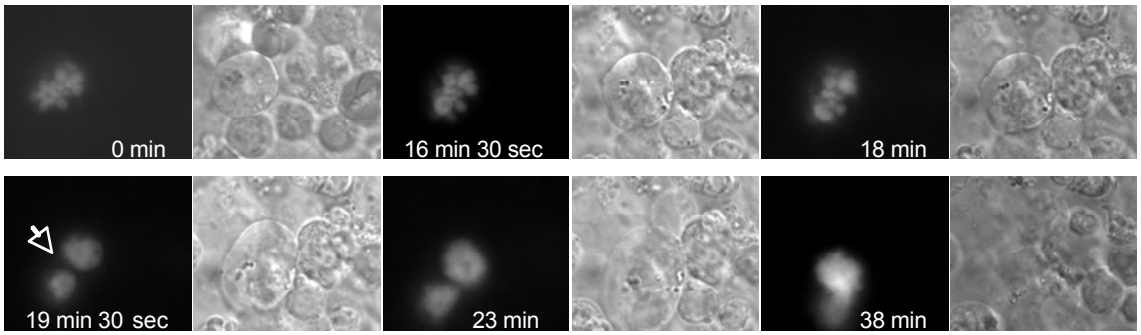
Ex-vivo MK imaging

Ex-vivo MK imaging was performed as described in Materials and Methods section. We had to trace several MKs in order to record endomitotic related phenomena because the whole process is rare, and as documented, in the case of low ploidy MKs of short duration.

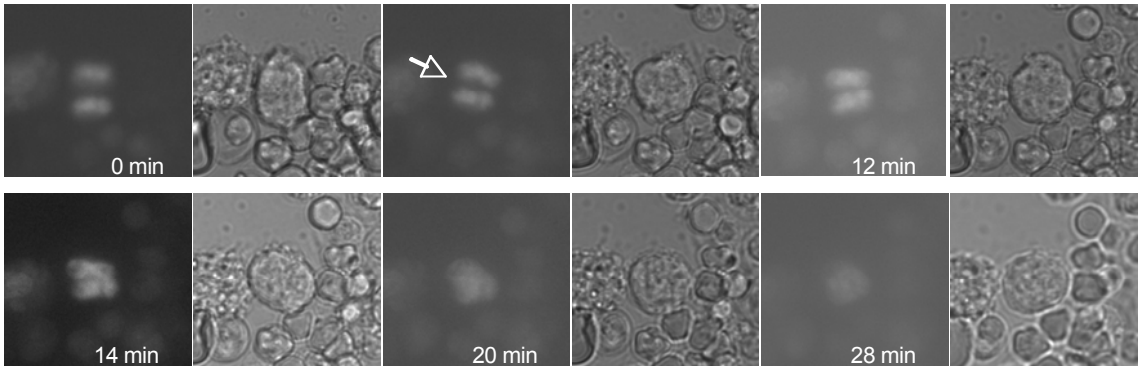
We observed that endomitosis is not uniform between MKs of high and low ploidy. Namely, during endomitotic metaphase MK chromosomes assume a compact, tight and lobulated form regardless of ploidy. However, at anaphase of low ploidy MKs the chromosomes were separated on two groups with formation of a territory between them consistent with a clear midzone as typically documented on anaphase A of diploid cells (Figure 2). In the next step, the chromosomal groups did not move towards poles of the cell, as typical of anaphase B, but instead they gradually rejoined into a single nucleus. The whole process had duration time of approximately 30 minutes, and it was accompanied by cell shape changes. More specifically, low ploidy MKs had initially a spherical shape which during chromosomal segregation changed to lancet, but finally reverted back to spherical.

On the other hand, the endomitosis of high ploidy MKs was significantly different (Figure 3). The first steps of endomitosis were retained with DNA content assuming a compact lobulated form consisted with metaphase. However, during anaphase A chromosomes did not segregate into two groups. Instead, chromosomes assumed a ring type alignment and territories resembling

midzones were formed. We estimated that the timing was significantly prolonged compared to low ploidy MKs and ranged approximately 60 minutes.



A



B

Figure 2. Ex-vivo cell imaging of low ploidy MKs undergoing endomitosis.

Living low ploidy MKs were followed during endomitosis. **(A)** At time point zero MK chromosomes are condensed. At the time point of 19 minutes, two groups of DNA were observed to segregate to opposite poles with a clear midzone area. In the finals steps the 2 chromosomal sets re-gathered into one nucleus in absence of cellular division. **(B)** Low ploidy MK undergoing final steps of endomitosis. The two chromosomal groups were initially separated by a clear space. The distance between the chromosomal groups was gradually reduced and finally the two groups rejoined into a single nucleus.

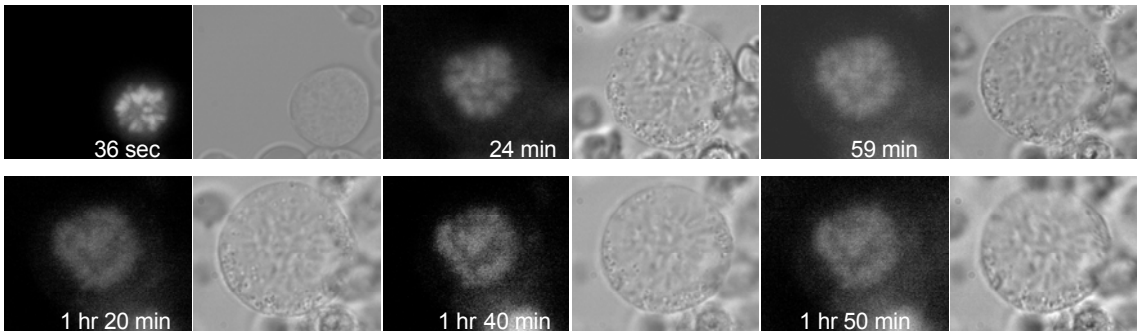
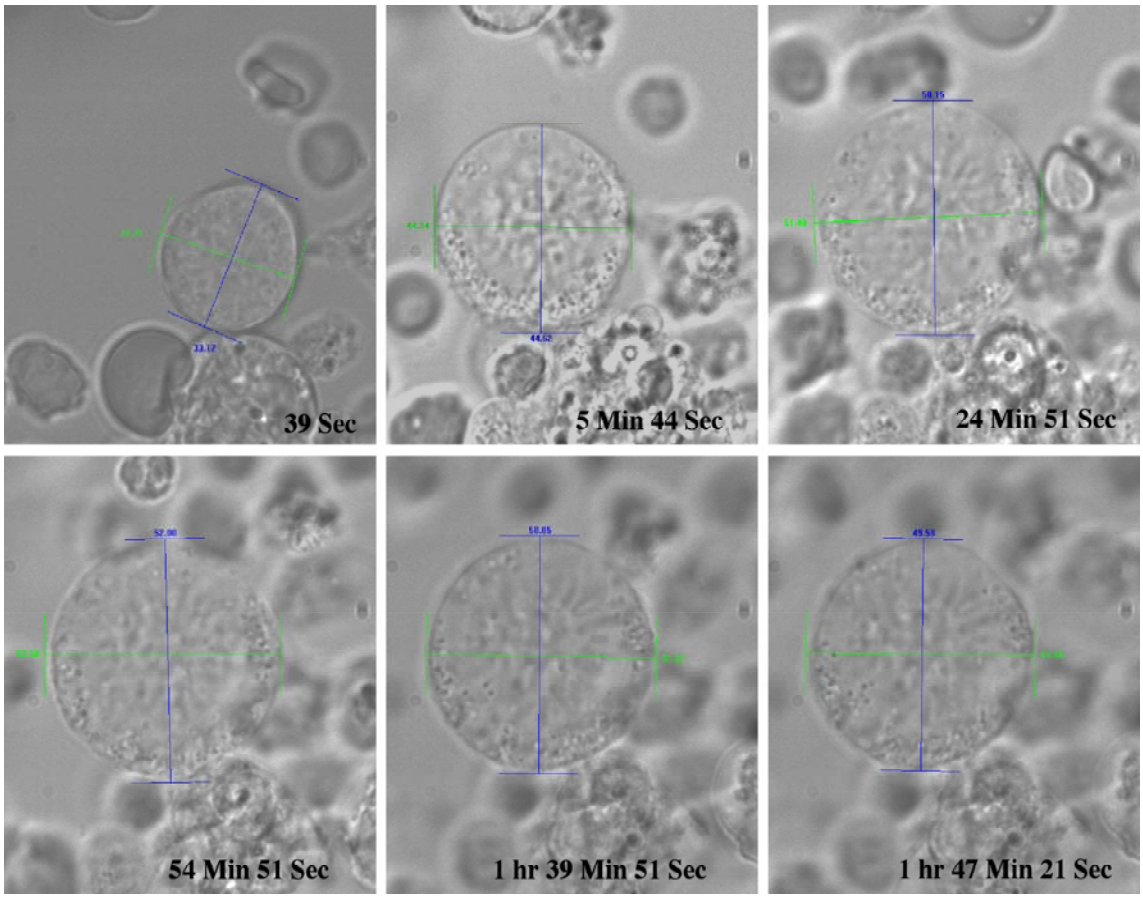


Figure 3. Ex-vivo imaging of high ploidy MK undergoing endomitosis.

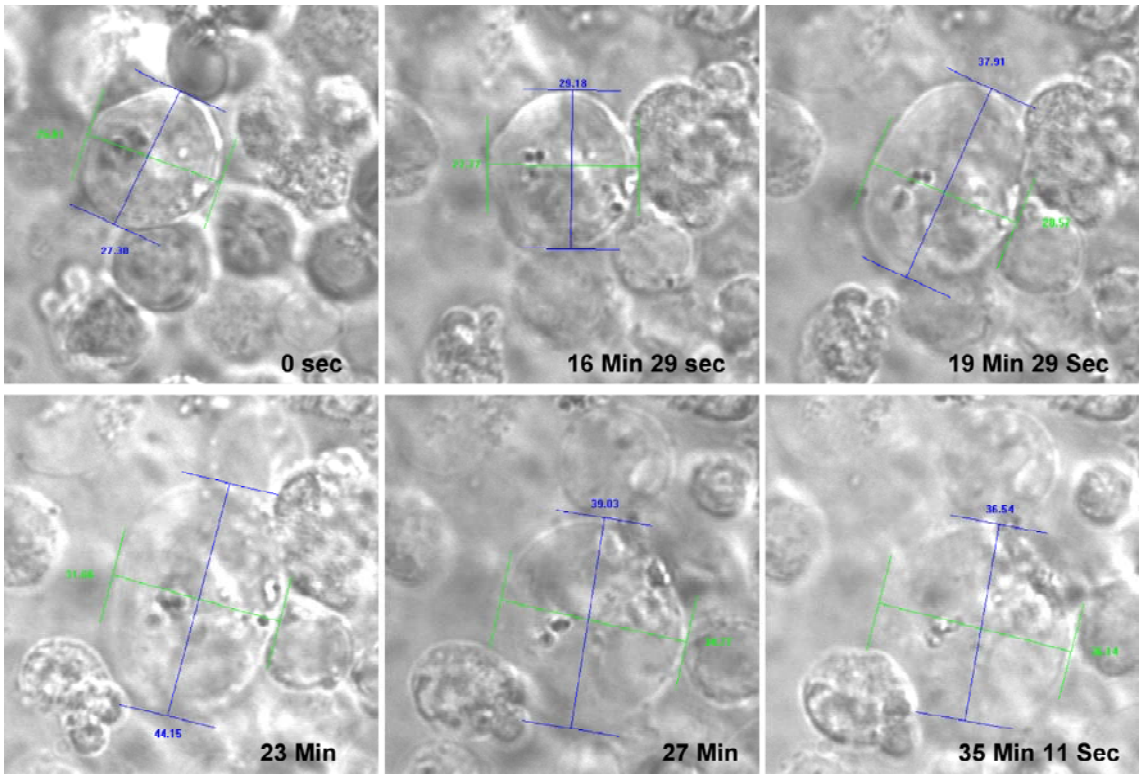
The endomitosis of a high ploidy MK was followed by obtaining images both in bright field and in fluorescence phase at 90X magnification for 96 minutes. Chromosomal condensation was evident at the beginning of monitoring (up to 12 minutes). It was followed by a ring-type alignment of chromosomes and a separation of an uneven amount of DNA to form territories resembling midzone. In the final steps chromosomal pulling to one side was observed without the events associated with telophase.

Importantly, differences in endomitotic process between low and high MKs were also associated with changes in cell morphology (Figure 4). Low ploidy MKs had spherical shape that during anaphase A changed to lancet and reverted back to spherical. The magnitude of elongation was considerable. For example, In one of the time lapse movies the diameter of the MK changed from 27 microns to 44 microns in approximately 20 minutes. The diameter gradually decreased to 36 microns in parallel with last steps of endomitosis.

By contrast, high ploidy MKs did not assume a lancet morphology and their shape was retained nearly spherical during endomitosis. In one of the movies the diameter of a high ploidy MK changed from 33 microns to 52 microns in 24 minutes and remained constant for up to approximately 2 hours of observation. During this period the cell diameter changed, but the morphology remained spherical.


39 Sec
5 Min 44 Sec
24 Min 51 Sec
54 Min 51 Sec
1 hr 39 Min 51 Sec
1 hr 47 Min 21 Sec

A



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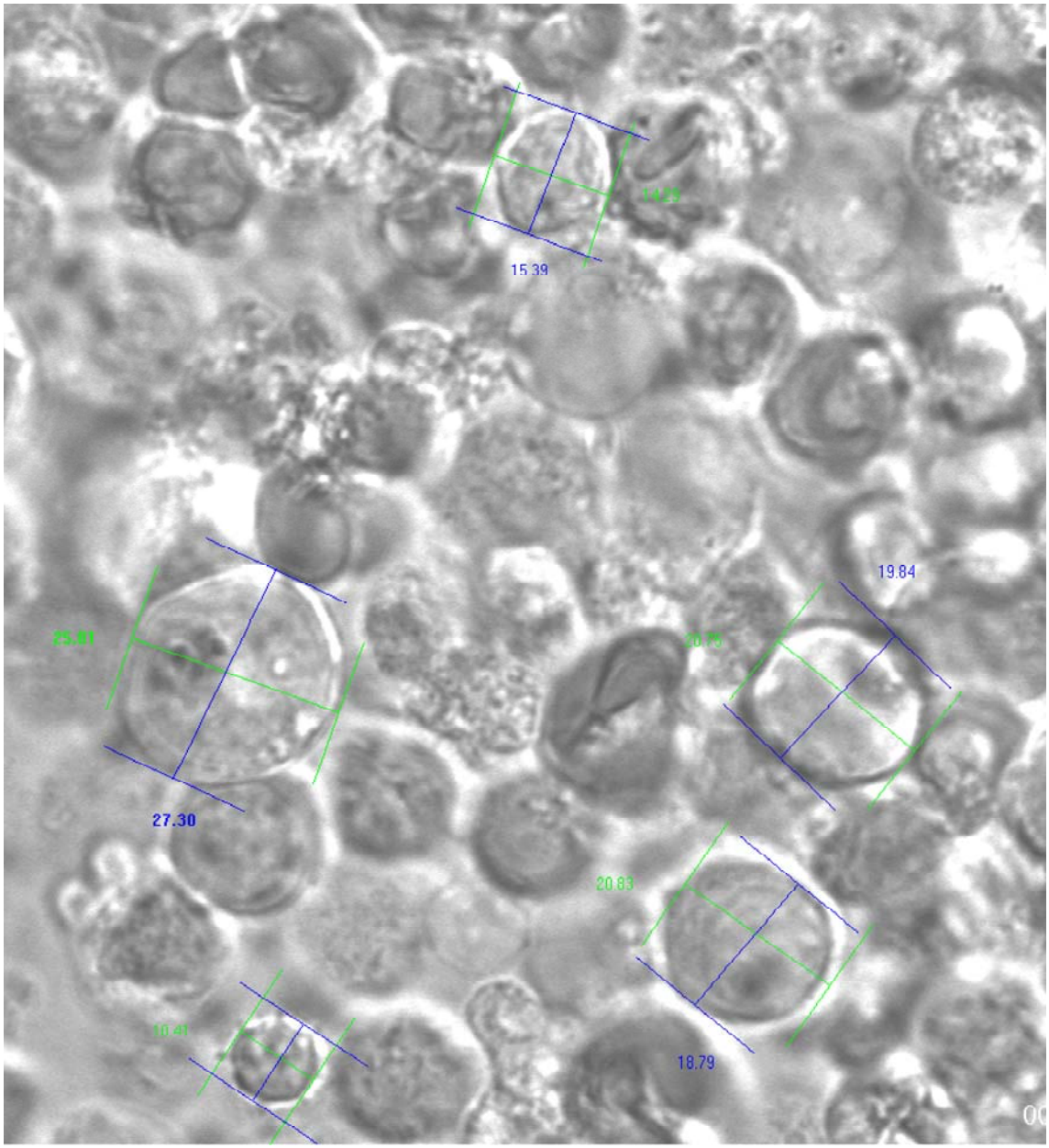


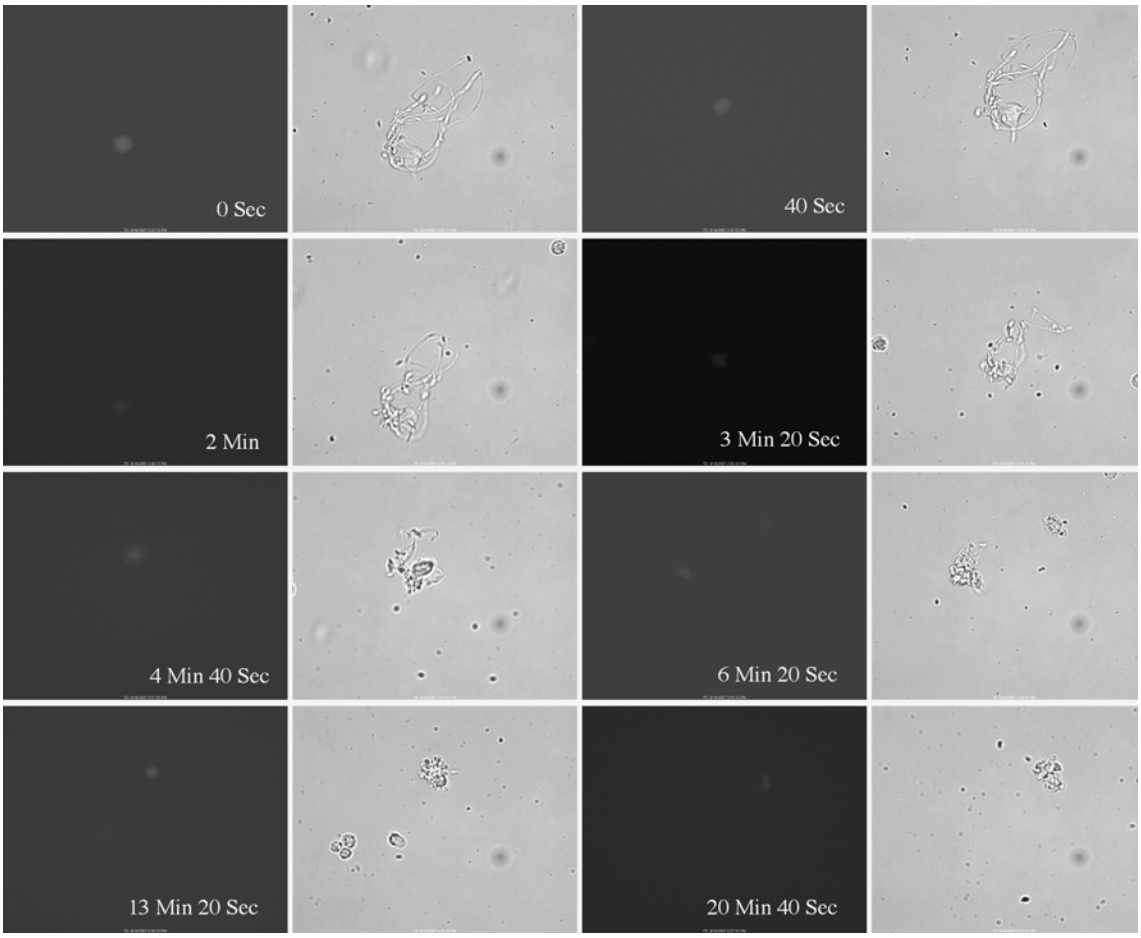
C

Figure 4. Cell morphology and diameter measurements during endomitosis in low and high ploidy MKs.

(A) Diameter measurements of the low ploidy MK depicted on figure were performed. The size of the cell changed from spherical to elongated and reverted back to spherical in a time frame of 30 minutes. **(B)** Diameter measurements of the high ploidy MK depicted on figure . The MK had a spherical shape that was retained during the whole period of observation. The diameter of the cell increased significantly in short time frame of approximately 10 minutes while the diameter was reduced in parallel with last steps of endomitosis. **(C)** Representative measurements of other cells surrounding low ploidy MK of figure 2. Their diameter is smaller compared to that of low ploidy MK.

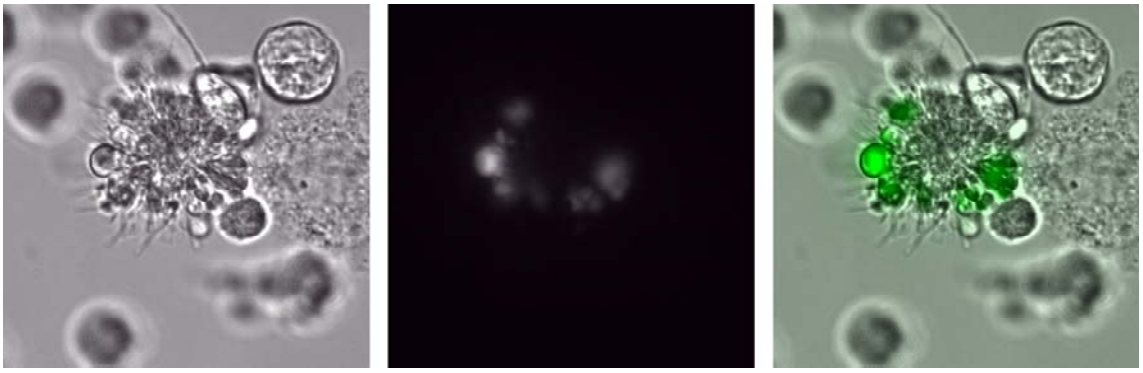
Ex-vivo imaging of MK fragmentation

MK fragmentation and proplatelet formation was also documented. MKs obtained after four to six days of culture in the presence of TPO (see Materials and Methods) were documented to undergo fragmentation through formation of proplatelets (Figure 5). The pattern of proplatelet formation was variable, with some MKs displaying only few long extensions, while in other cases MKs with shorter, multiple, proplatelet formations were observed (Figure 6). Proplatelet formation is a highly dynamic process with MK shape and position changing rapidly. In all cases documented, the nucleus of MKs was retained in the cytoplasm and it was not expelled during proplatelet formation. The cytoplasm of MKs that underwent fragmentation appeared highly granular with apoptotic morphology. Moreover, in some cases MKs that have undergone fragmentation, DNA was packaged into vesicles that decorated the periphery of MK (Figure 6).


0 Sec
40 Sec
2 Min
3 Min 20 Sec
4 Min 40 Sec
6 Min 20 Sec
13 Min 20 Sec
20 Min 40 Sec

A

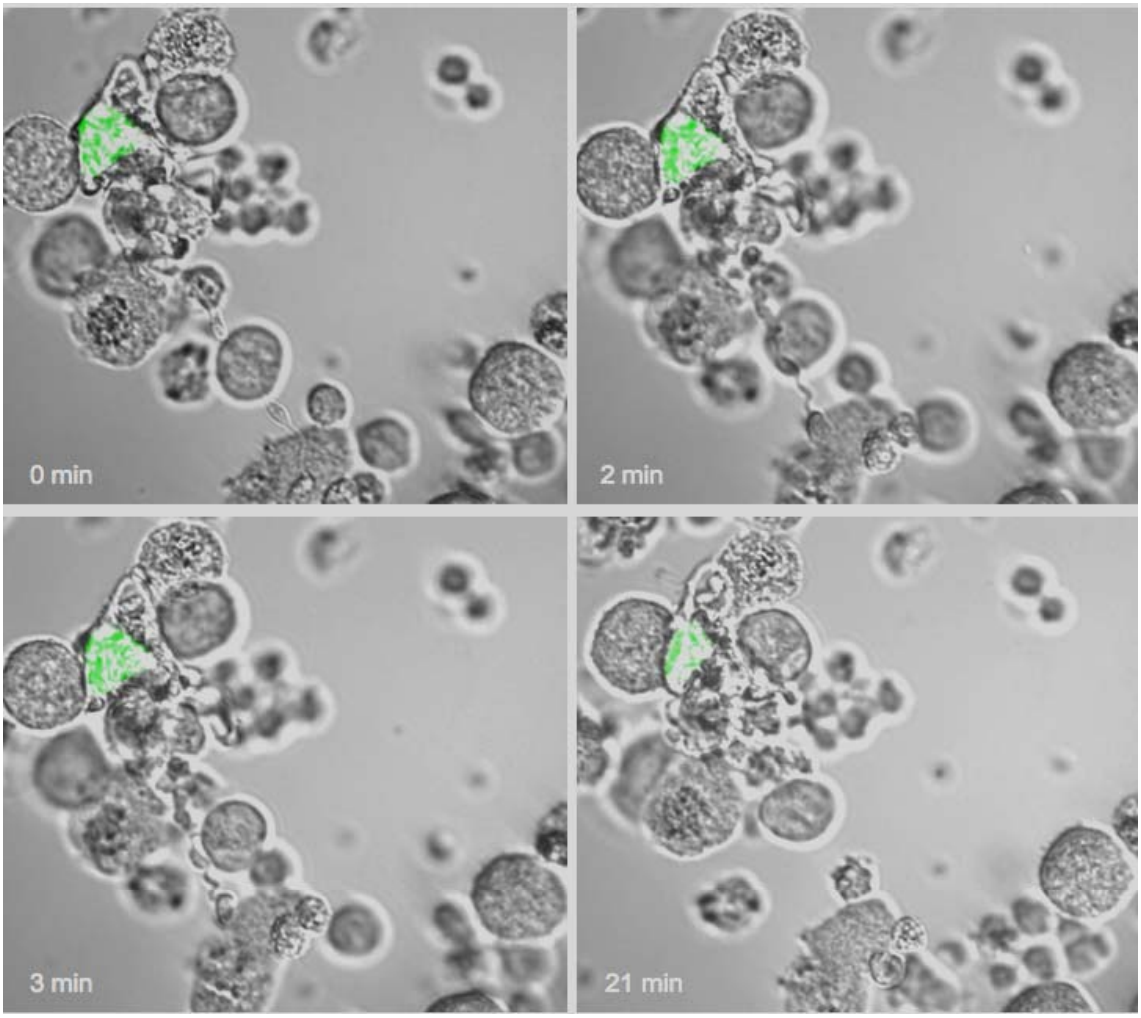
Brightfield **Fluorescence** **Composite**



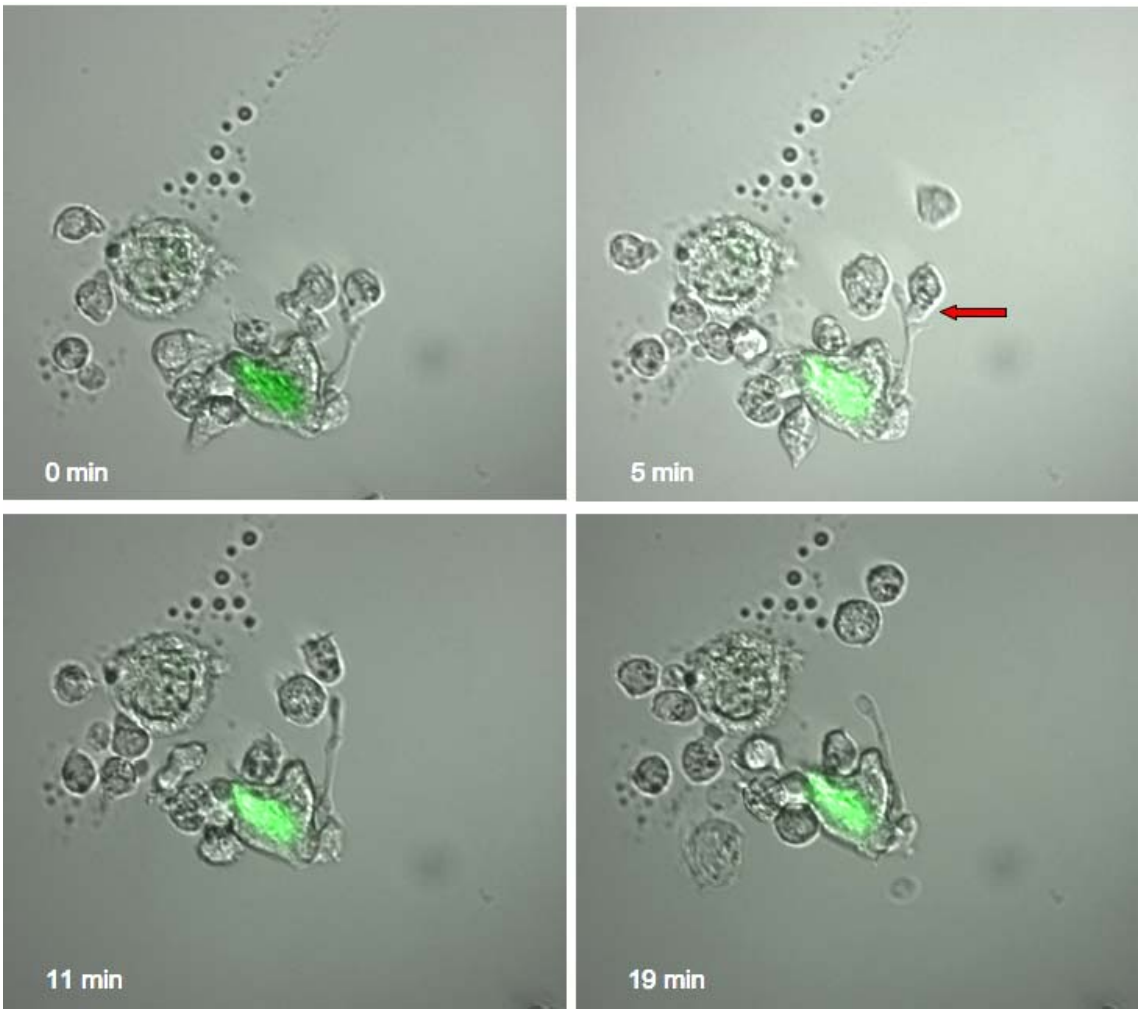
B

Figure 5. MK fragmentation.

(**A**) Time lapse imaging of an MK fragmentation. An MK with multiple long, thin proplatelet formations was monitored for 20 minutes. The length of proplatelet formations gradually diminished and finally only a small amount of cytoplasm surrounded the nucleus. (**B**) Images of an MK that has undergone fragmentation as portrayed from the multiple long thin cytoplasmatic extensions consistent with proplatelet remnants. Vesicles with DNA, as depicted by the GFP fluorescence, decorated the periphery of the MK. Original magnification 600X.



A



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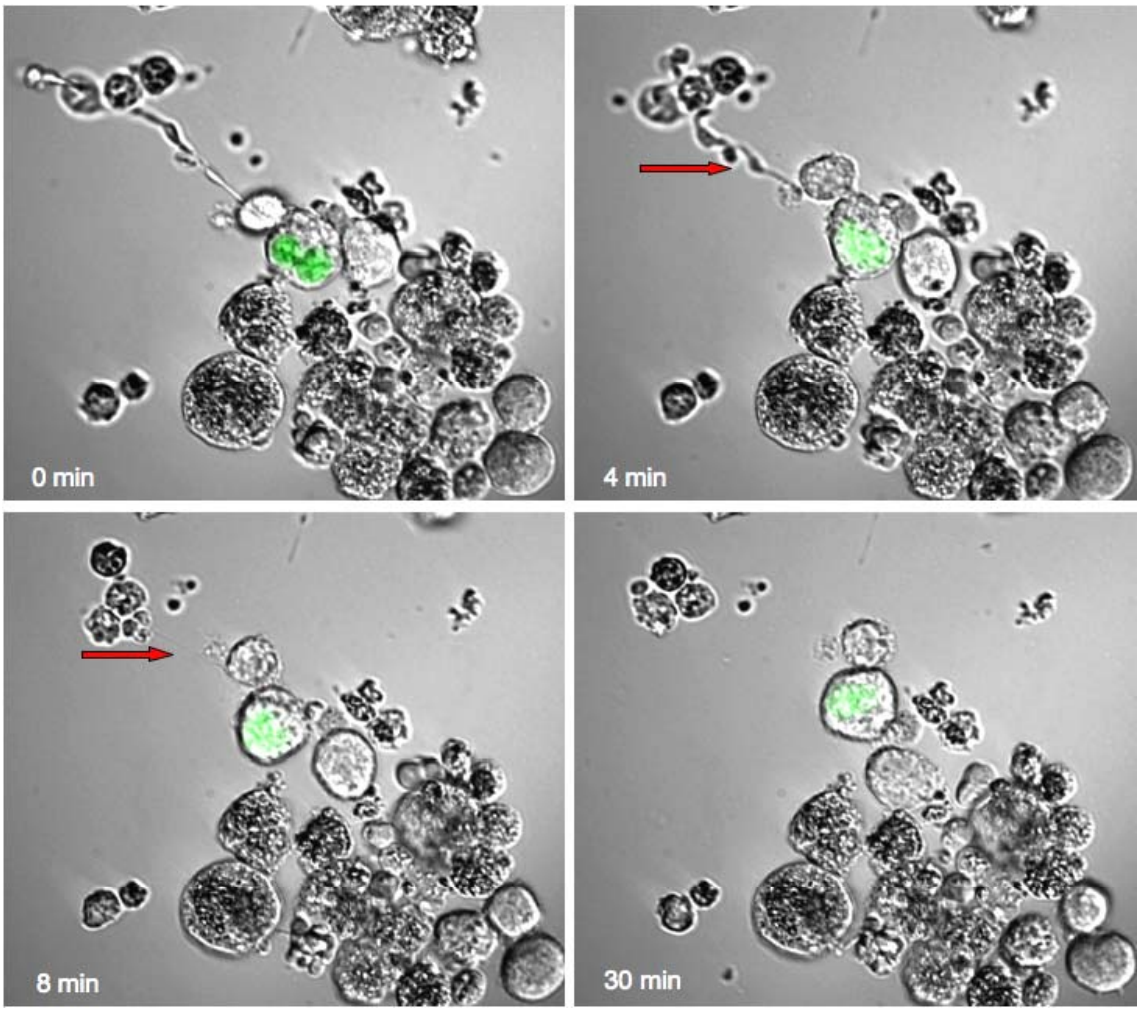


C

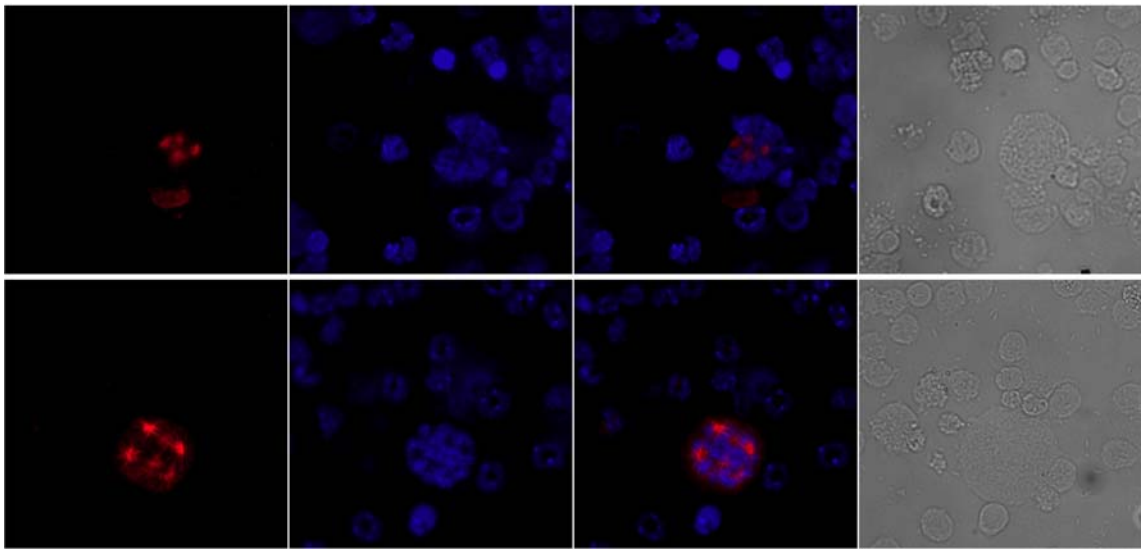
Figure 6. Proplatelet formation during Ex-vivo imaging.

Representative composite captions of time lapse imaging of PF4-H2B-GFP MKs exhibiting proplatelet formations. (**A**) An MK with cytoplasmatic protrusions consisted with proplatelet formations, was documented to release a

cytoplasmatic fraction (depicted with arrow) near the distal end of proplatelet. The released fraction assumed a spherical conformation after its release. **(B)** An MK exhibiting a single long proplatelet formation. The proplatelet was gradually reduced in size but at the distal end of proplatelet, spherical formations were generated. The spherical fragments were connected with a cytoplasmatic bridge to the MK cytoplasm. However, the cytoplasmatic bridge progressively diminished. **(C)** An MK with multiple proplatelet formations exhibiting gradual shortening of its main proplatelet formation. The length of the dominant proplatelet formation was considerable and its end bifurcated into two nascent bulbs. Composite captions where generated by merging time lapse images acquired at bright field and fluorescent phase. Original magnification 600X. Depicted time is relative to the first caption of each panel.

Immunofluorescence analysis of fixed MKs

Immunofluorescence studies of PF4-H2B-GFP and wild type (control) mice were performed (Figure 7). The cells were stained with DAPI to view DNA, as well as with anti-tubulin antibody to view the microtubules. In Diploid cells, a midzone was formed between segregating chromosomes. Furthermore, formation of territories consistent with Midzone were observed between segregating chromosomes in low ploidy MKs (four spindle poles) derived from fetal liver and bone marrow of PF4-H2B-GFP mice. By contract, in high ploidy MKs, the DNA content assumed a ring type alignment with no clear space typical of midzone formation. The same pattern was also observed in bone marrow of wild type mice.



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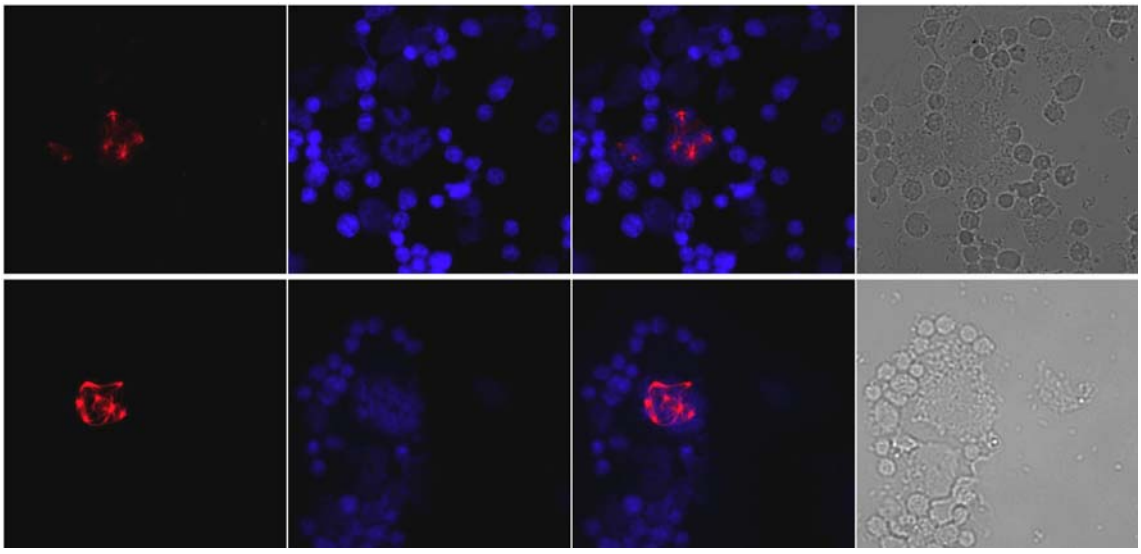

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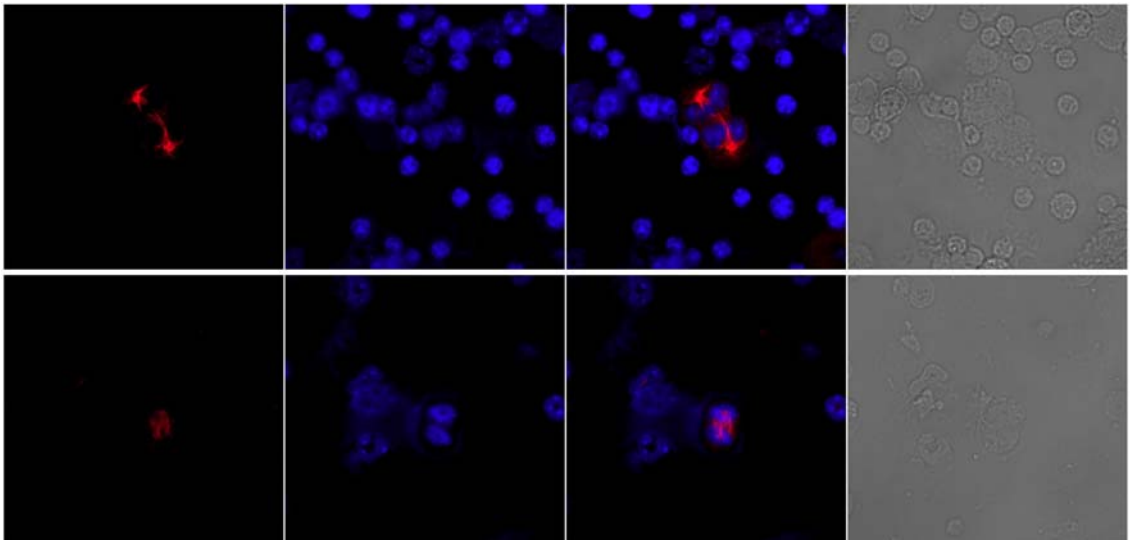

C

Figure 7 . Immunofluorescence imaging of high and low ploidy MKs derived from wt mouse and PF4-H2B-GFP transgenic mouse model.

(A) Wild type (WT) mouse bone marrow immunofluorescence imaging exhibiting low (upper row) and high (lower row) ploidy MKs during anaphase. DNA was stained with DAPI (blue) and microtubules are stained red with anti-tubulin (see Materials and Methods). During anaphase of the low ploidy MK the chromosomes assumed a mirror shaped conformation with the presence of a space between them consistent with midzone. By contrast, a clear midzone is absent in high ploidy MK. **(B)** Immunofluorescence images of low (upper row) and high (lower row) MKs derived from PF4-H2B-GFP mouse model fetal liver cultures. In the low ploidy MK, midzone is again formed during anaphase (depicted by arrow) but is absent in the high ploidy MKs.

(**C**) Diploid cells of wt bone marrow (upper row) and PF4-H2B-GFP fetal liver cultures (lower row) exhibit formation of midzone during anaphase. Original magnification 600X.

Chapter IV. DISCUSSION

Here, we report the first chromosomal tracking study of living MKs during endomitosis, utilizing a transgenic mouse model that allows visualization of MK chromosomes through the fusion of Histone 2B with GFP. Studies regarding aspects of MK physiology are inherently difficult because MKs comprise only a small fraction of the total adult bone marrow or fetal liver cell populations. Furthermore, the study of endomitosis is even more challenging, because most MKs are cell cycle-arrested and the phenomena related to the last steps of endomitosis occur rapidly.

The PF4-H2B-GFP transgenic model offers specific visualization of MK DNA and has several advantages over potential approaches involving MK DNA tagging based on viral vectors gene transfer. Namely, stable tagging of MK DNA with GFP circumvents the issue of transient expression hampering adenoviral-mediated gene transfer methods, as well as the potential toxicity that is a major concern in gene transfer methods based on retroviral vectors.

We utilized MKs of a PF4-H2B-GFP transgenic mouse model to visualize DNA content in a subnuclear scale and to study endomitosis ex-vivo. The in vivo cell imaging approach has key advantages compared to data obtained by immunofluorescence microscopy. Studies based on fixed MKs are hampered by low yield of isolated MKs in anaphase as well as with fixation artifacts. In addition, reagents utilized for immunofluorescence may exhibit specificity and sensitivity problems and hence, produce conflicting results. These issues were clearly manifested in the detection of Survivin and Aurora-B proteins during endomitosis[49]. Another drawback is that immunocytochemistry studies can not discern between an MK with 4N content of chromosomes that are going to divide, from 4N cells that will undergo endomitosis.

Most importantly, static images obtained by immunocytochemistry studies can not capture the details of chromosome/microtubule dynamics during endomitosis.

By contrast to previous studies[32], we performed ex-vivo imaging at high magnifications focusing on a single MK cell at a time. Higher magnifications have the disadvantage that only very few, or most commonly, a single MK is visualized per optical field, and image clarity can be affected by subtle movement of cells. However, it has the key advantage of visualizing in greater detail the phases of endomitosis. In addition, the use of high magnification is crucial for the demonstration that in high ploidy MKs territories resembling midzone are formed.

This novel ex-vivo cell imaging model permitted tracking of chromosomal segregation during all phases of endomitosis, including anaphase of both low and high ploidy MKs. This approach has the key advantage that the chromosomal dynamics were directly visualized in contrast to previous reports[32] where microtubule network was visualized instead.

Both, in high and low ploidy MKs, the chromosomes assumed a tight lobulated form that was positioned in the equator of the MK cell. The DNA content was compacted into multi-branched form in agreement with previous reports[31]. However, in low ploidy MKs chromosomes were segregated into two groups and a territory consistent with midzone was formed between departing chromosomal groups. These phenomena are consistent with anaphase A. However, the two chromosomal groups were subsequently merged in the last steps of endomitosis without the phenomena observed during cell division. The whole process occurred rapidly and is on agreement with previous estimations[66] based on data from fixed cells.

On the other hand, images obtained from high ploidy MKs did not follow the above pattern. The first steps of mitosis were also retained intact but the condensed chromosomes formed

ring type alignments followed by separation of uneven amounts of DNA to form territories resembling midzones. The subsequent steps included chromosomal pulling to one side without the events associated with cytokinesis.

The formation of midzone is an important milestone of anaphase because it is the place where a battery of proteins with significant role in the last step of mitosis are assembled. The chromosomal passenger protein complex has been implicated as an important mediator of endomitosis. Recent studies detected Aurora B protein[49,52] during anaphase indicating that formation of midzone during endomitosis of low ploidy MKs is preserved. Midzone formation also heralds the site of cleavage furrow an important step for the assembly of factors regulating cytokinesis and cytoskeleton rearrangements. Although, we did not document furrowing in the extent of the previous reports, low ploidy MKs exhibited cell morphology changes and assumed a pinched shape before reverting back to spherical. The pinched cell shape correlated with anaphase A and the spherical conformation was assumed during re-gathering of chromosomal groups. By contrast, in high ploidy MKs furrowing was attenuated even though the size of the cell increased considerably.

Imaging of the last steps of MK maturation indicated that the nucleus is retained in the cytoplasm of the MK cell. The MKs were documented to form proplatelets that consumed most of the cytoplasm. However, we also observed release of proplatelet fractions that assumed spherical shape. On other cases, the Proplatelet formed were documented to be separated into two parts that remained connected with a thin cytoplasmatic bridge. We detected MKs that had numerous cytoplasmatic protrusions closely resembling proplatelet formations and had assumed an apoptotic morphology. Intriguingly, the MK chromosomal DNA was shown to be

packed into vesicles that decorated the periphery of the MK. Our model may be useful to decipher how MK DNA fragmentation occurs and if release of vesicles that contain chromosomal DNA is an active process. This objective may be the focus of future experiments. In addition, our approach of ex-vivo imaging utilizing histone tagging of MK DNA can serve as a platform for the future study in vivo of endomitotic aspects that remain enigmatic. For example, fluorescently tagged proteins with a key role in endomitosis could be utilized in conjunction with PF4-H2B-GFP model and offer important information their pattern of localization and interaction. A similar approach can be applied to study the localization of proteins with key role in MK apoptosis and terminal steps of maturation. This will provide useful information about the dynamics of apoptosis and elucidate how viable platelets are released from a dying cell.

The finding that high and low ploidy MKs have differences in the formation of midzones opens new vistas in the our understanding of MK endomitosis. The fact that chromosomal groups are not pulled apart completely, but rather they assume an annular formation, indicate that the spindle checkpoint may be bypassed. However, the mechanisms underlying this potential bypass of the spindle checkpoint remain speculative. For example, the high content of DNA could reduce the space available for the endomitotic machinery to execute anaphase. On the other hand, a key mediator of the spindle checkpoint specifically due to lack of microtubule tension is Aurora B. A hypothesis could be that, in high ploidy MKs Aurora B can not perform it's function properly. Elucidation of this hypothesis would require time lapse microscopy to detect the presence and localization of Aurora B during MK transition from low to high ploidy state. However, a more intriguing scenario involves the Polo Like Kinase (PLK) family of

proteins[67]. PLK proteins have a crucial role for the formation of mitotic spindle and in their absence mitotic arrest occurs followed by apoptosis[68]. However, if in cells lacking PLK the spindle checkpoint is bypassed, then mitotis is completed without anaphase, resulting in polyploidy. A recent study[69] indicated that PLK-1 was absent in polyploid MKs and forced expression of PLK-1 was correlated with attenuated polyploidization. Clearly, further studies are required to elucidate the role of PLK family of proteins in endomitosis.

However, central role in the spindle checkpoint have the microtubule network. In order to study their role during endomitosis, construction of transgenic mouse models that offer visualization of microtubule network must be engineered. The information that will be gained from these models can be further expanded by utilizing the PF4-H2B-GFP mouse and generating a double transgenic mouse that will offer simultaneous visualization of DNA and microtubules.

Bibliography

1. Szalai G LA, Watson DK. Molecular mechanisms of megakaryopoiesis. Cell Mol Life Sci. 2006;63:2460-2476.
2. Deutsch VR, Tomer A. Megakaryocyte development and platelet production. British Journal of Haematology. 2006;134:453-466.
3. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov. 2007;6:273-286.
4. Healy AM, Pickard MD, Pradhan AD, et al. Platelet expression profiling and clinical validation of myeloid-related protein-14 as a novel determinant of cardiovascular events. Circulation. 2006;113:2278-2284.
5. Rey Vazquez G, Guerrero GA. Characterization of blood cells and hematological parameters in Cichlasoma dimerus (Teleostei, Perciformes). Tissue Cell. 2007;39:151-160.
6. Meseguer J, Esteban MA, Rodriguez A. Are thrombocytes and platelets true phagocytes? Microsc Res Tech. 2002;57:491-497.
7. de Jong JL, Zon LI. Use of the zebrafish system to study primitive and definitive hematopoiesis. Annu Rev Genet. 2005;39:481-501.
8. Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. Development. 2007.
9. Kunicki TJ, Newman PJ. Synthesis of analogs of human platelet membrane glycoprotein IIb-IIIa complex by chicken peripheral blood thrombocytes. Proc Natl Acad Sci U S A. 1985;82:7319-7323.
10. Scott T, Owens MD. Thrombocytes respond to lipopolysaccharide through Toll-like receptor-4, and MAP kinase and NF-kappaB pathways leading to expression of interleukin-6 and cyclooxygenase-2 with production of prostaglandin E2. Mol Immunol. 2008;45:1001-1008.
11. Junt T, Schulze H, Chen Z, et al. Dynamic visualization of thrombopoiesis within bone marrow. Science. 2007;317:1767-1770.
12. Kopp HG, Avecilla ST, Hooper AT, Rafii S. The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda). 2005;20:349-356.
13. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell. 2005;121:1109-1121.
14. Tavassoli M, Aoki M. Migration of entire megakaryocytes through the marrow--blood barrier. Br J Haematol. 1981;48:25-29.
15. Mandal RV, Mark EJ, Kradin RL. Megakaryocytes and platelet homeostasis in diffuse alveolar damage. Exp Mol Pathol. 2007.
16. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 1997;91:661-672.
17. Szalai G, LaRue A, Watson D. Molecular mechanisms of megakaryopoiesis. Cellular and Molecular Life Sciences (CMLS). 2006;63:2460-2476.

18. Tomer A. Human marrow megakaryocyte differentiation: multiparameter correlative analysis identifies von Willebrand factor as a sensitive and distinctive marker for early (2N and 4N) megakaryocytes. Blood. 2004;104:2722-2727.
19. Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest. 2005;115:3339-3347.
20. Avecilla ST, Hattori K, Heissig B, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med. 2004;10:64-71.
21. Kaushansky K, Drachman JG. The molecular and cellular biology of thrombopoietin: the primary regulator of platelet production. Oncogene. 2002;21:3359-3367.
22. Kaushansky K. Thrombopoietin: the primary regulator of megakaryocyte and platelet production. Thrombosis & Haemostasis. 1995;74:521-525.
23. Kaushansky K. Thrombopoietin: basic biology, clinical promise. International Journal of Hematology. 1995;62:7-15.
24. Pang L, Weiss MJ, Poncz M. Megakaryocyte biology and related disorders. J Clin Invest. 2005;115:3332-3338.
25. Patel SR, Hartwig JH, Italiano JE, Jr. The biogenesis of platelets from megakaryocyte proplatelets. J Clin Invest. 2005;115:3348-3354.
26. Zimmet J, Ravid K. Polyploidy: Occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system. Experimental Hematology. 2000;28:3-16.
27. Ravid K, Lu J, Zimmet JM, Jones MR. Roads to polyploidy: the megakaryocyte example. J Cell Physiol. 2002;190:7-20.
28. Raslova H, Kauffmann A, Sekkai D, et al. Interrelation between polyploidization and megakaryocyte differentiation: a gene profiling approach. Blood. 2007;109:3225-3234.
29. Chang Y, Bluteau D, Debili N, Vainchenker W. From hematopoietic stem cells to platelets. J Thromb Haemost. 2007;5 Suppl 1:318-327.
30. Yang D, McCrann DJ, Nguyen H, et al. Increased polyploidy in aortic vascular smooth muscle cells during aging is marked by cellular senescence. Aging Cell. 2007;6:257-260.
31. Roy L, Coullin P, Vitrat N, et al. Asymmetrical segregation of chromosomes with a normal metaphase/anaphase checkpoint in polyploid megakaryocytes. Blood. 2001;97:2238-2247.
32. Geddis AE, Fox NE, Tkachenko E, Kaushansky K. Endomitotic megakaryocytes that form a bipolar spindle exhibit cleavage furrow ingression followed by furrow regression. Cell Cycle. 2007;6:455-460.
33. Geddis AE, Kaushansky K. Endomitotic megakaryocytes form a midzone in anaphase but have a deficiency in cleavage furrow formation. Cell Cycle. 2006;5:538-545.
34. Margolis RL, Lohez OD, Andreassen PR. G1 tetraploidy checkpoint and the suppression of tumorigenesis. J Cell Biochem. 2003;88:673-683.

35. Malumbres M, Barbacid M. TO CYCLE OR NOT TO CYCLE: A CRITICAL DECISION IN CANCER. Nature Reviews Cancer. 2001;1:222-231.
36. Nagata Y, Muro Y, Todokoro K. Thrombopoietin-induced Polyploidization of Bone Marrow Megakaryocytes Is Due to a Unique Regulatory Mechanism in Late Mitosis. J Cell Biol. 1997;139:449-457.
37. Bloom J, Cross FR. Multiple levels of cyclin specificity in cell-cycle control. Nat Rev Mol Cell Biol. 2007;8:149-160.
38. Muntean AG, Pang L, Poncz M, Dowdy SF, Blobel GA, Crispino JD. Cyclin D-Cdk4 is regulated by GATA-1 and required for megakaryocyte growth and polyploidization. Blood. 2007:blood-2006-2011-059378.
39. Zimmet JM LD, Jackson CW,Stenberg PE,Ravid K. A role for cyclin D3 in the endomitotic cell cycle.
. Mol Cell Biol. 1997;17:7248-7259.
40. Geng Y, Yu Q, Sicinska E, et al. Cyclin E Ablation in the Mouse. Cell. 2003;114:431-443.
41. Musacchio A, Salmon ED. The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol. 2007;advanced online publication.
42. Lens SMA, Vader G, Medema RH. The case for Survivin as mitotic regulator. Current Opinion in Cell Biology. 2006;18:616-622.
43. Nguyen HG, Ravid K. Tetraploidy/aneuploidy and stem cells in cancer promotion: The role of chromosome passenger proteins. J Cell Physiol. 2006;208:12-22.
44. Altieri DC. The case for survivin as a regulator of microtubule dynamics and cell-death decisions. Current Opinion in Cell Biology. 2006;18:609-615.
45. Fukuda S, Pelus LM. Survivin, a cancer target with an emerging role in normal adult tissues. Mol Cancer Ther. 2006;5:1087-1098.
46. Anthony G. Uren LW, Miha Pakusch, Kerry J. Fowler, Francis J. Burrows, David L. Vaux and K. H. Andy Choo. Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype. Current Biology. 2000;10:1319 -1328.
47. Gurbuxani S, Xu Y, Keerthivasan G, Wickrema A, Crispino JD. Differential requirements for survivin in hematopoietic cell development. PNAS. 2005;102:11480-11485.
48. Zhang Y, Nagata Y, Yu G, et al. Aberrant quantity and localization of Aurora-B/AIM-1 and survivin during megakaryocyte polyploidization and the consequences of Aurora-B/AIM-1-deregulated expression. Blood. 2004;103:3717-3726.
49. Geddis AE, Kaushansky K. Megakaryocytes express functional Aurora-B kinase in endomitosis. Blood. 2004;104:1017-1024.
50. C. Kotwaliwale SB. Microtubule Capture: A Concerted Effort. . Cell. 2006;127:1105-1108.
51. Murata-Hori M, Tatsuka M, Wang Y-L. Probing the Dynamics and Functions of Aurora B Kinase in Living Cells during Mitosis and Cytokinesis. Mol Biol Cell. 2002;13:1099-1108.
52. Geddis AE KK. Endomitotic megakaryocytes form a midzone in anaphase but have a deficiency in cleavage furrow formation. Cell Cycle. 2006;5:538-545.

53. Rubin CI, French DL, Atweh GF. Stathmin expression and megakaryocyte differentiation: a potential role in polyploidy. Exp Hematol. 2003;31:389-397.
54. Iancu-Rubin C NC, Atweh GF. Stathmin prevents the transition from a normal to an endomitotic cell cycle during megakaryocytic differentiation. Cell Cycle. 2005;4:1774-1782.
55. Fichelson S, Dreyfus F, Berger R, et al. Evi-1 expression in leukemic patients with rearrangements of the 3q25-q28 chromosomal region. Leukemia. 1992;6:93-99.
56. Testoni N, Borsaru G, Martinelli G, et al. 3q21 and 3q26 cytogenetic abnormalities in acute myeloblastic leukemia: biological and clinical features. Haematologica. 1999;84:690-694.
57. Kilbey A, Alzuherri H, McColl J, Cales C, Frampton J, Bartholomew C. The Evi1 proto-oncoprotein blocks endomitosis in megakaryocytes by inhibiting sustained cyclin-dependent kinase 2 catalytic activity. Br J Haematol. 2005;130:902-911.
58. Patel SR, Richardson JL, Schulze H, et al. Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. Blood. 2005;106:4076-4085.
59. Marino-Ramirez L, Kann MG, Shoemaker BA, Landsman D. Histone structure and nucleosome stability. Expert Review of Proteomics. 2005;2:719-729.
60. Nguyen HG, Yu G, Makitalo M, et al. Conditional overexpression of transgenes in megakaryocytes and platelets in vivo. Blood. 2005;106:1559-1564.
61. Ravid K, Beeler DL, Rabin MS, Ruley HE, Rosenberg RD. Selective Targeting of Gene Products with the Megakaryocyte Platelet Factor 4 Promoter. PNAS. 1991;88:1521-1525.
62. Kanda T SK, Wahl GM. Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. Curr Biol. 1998;8:377-385.
63. Fraser ST, Hadjantonakis AK, Sahr KE, et al. Using a histone yellow fluorescent protein fusion for tagging and tracking endothelial cells in ES cells and mice. Genesis. 2005;42:162-171.
64. Lecine P, Blank V, Shivdasani R. Characterization of the Hematopoietic Transcription Factor NF-E2 in Primary Murine Megakaryocytes. J Biol Chem. 1998;273:7572-7578.
65. Italiano JE, Jr., Lecine P, Shivdasani RA, Hartwig JH. Blood Platelets Are Assembled Principally at the Ends of Proplatelet Processes Produced by Differentiated Megakaryocytes. J Cell Biol. 1999;147:1299-1312.
66. Vitrat N, Cohen-Solal K, Pique C, et al. Endomitosis of Human Megakaryocytes Are Due to Abortive Mitosis. Blood. 1998;91:3711-3723.
67. Glover DM, Ohkura H, Tavares A. Polo kinase: the choreographer of the mitotic stage? J Cell Biol. 1996;135:1681-1684.
68. van Vugt MA, van de Weerdt BC, Vader G, et al. Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. J Biol Chem. 2004;279:36841-36854.
69. Yagi M, Roth GJ. Megakaryocyte polyploidization is associated with decreased expression of polo-like kinase (PLK). J Thromb Haemost. 2006;4:2028-2034.