Megakaryocyte endomitosis is a failure of late cytokinesis related to defects in the contractile ring and Rho/Rock signaling

Larissa Lordier\textsuperscript{1,2,3}, Abdelali Jalil\textsuperscript{3}, Frédéric Aurade\textsuperscript{1,2,3,4}, Frédéric Larbret\textsuperscript{1,3}, Jerôme Larghero\textsuperscript{5}, Najet Debili\textsuperscript{1,2,3}, William Vainchenker\textsuperscript{1,2,3}, Yunhua Chang\textsuperscript{1,2,3}

1. Institut National de la Santé et de la Recherche Médicale, U790, 39 rue Camille Desmoulins, 94805 Villejuif, France
2. Université Paris XI, 39 rue Camille Desmoulins, 94805 Villejuif, France
3. Institut Gustave Roussy, IFR54, 39 rue Camille Desmoulins, 94805 Villejuif, France
4. Centre National de la Recherche Scientifique, UPMC University Paris 06, UMRS 787, 75634 Paris, France
5. Institut National de la Santé et de la Recherche Médicale, EMI00-03, Laboratoire de Biologie Cellulaire Hématoïétique, Hôpital Saint-Louis, 75010 Paris, France

Running title: Failure of cytokinesis and endomitosis

This work was supported by grants from the Agence Nationale de la Recherche (contrat blanc, WV) and the Association de la Recherche contre le Cancer (WV).

Corresponding author: William Vainchenker

Telephone number: (33) 1 42 11 42 33

Fax number: (33) 1 42 11 52 40

E-mail: verpre@igr.fr
Abstract:
Megakaryocyte is the naturally polyploid cell that gives rise to platelets. Polyploidization occurs by endomitosis, which was a process considered as an incomplete mitosis aborted in anaphase. Here, we used time-lapse confocal video microscopy to visualize the endomitotic process of primary human megakaryocytes. Our results show that the switch from mitosis to endomitosis corresponds to a late failure of cytokinesis accompanied by a backward movement of the two daughter cells. No abnormality was observed in the central spindle of endomitotic MKs. A furrow formation was present, but the contractile ring was abnormal since accumulation of non muscle myosin IIA was lacking. In addition, a defect in cell elongation was observed in dipolar endomitotic MKs during telophase. RhoA and F-actin were partially concentrated at the site of furrowing. Inhibition of the Rho/Rock pathway caused the disappearing of F-actin at midzone and increased MK ploidy level. This inhibition was associated with a more pronounced defect in furrow formation as well as in spindle elongation. Our results suggest that the late failure of cytokinesis responsible of the endomitotic process is related to a partial defect in the Rho/Rock pathway activation.

Abbreviations: Megakaryocyte (MKs) ; Three dimensions (3D)
Introduction

Megakaryocytes (MKs) are the hematopoietic cells that give rise to platelets by fragmentation of their cytoplasm through pseudopodial formations, called proplatelets. During their differentiation, MKs become polyploid by a unique process called endomitosis. Recent studies have shown that MK endomitosis is an incomplete multipolar mitosis characterized by a failure in both nuclear (karyokinesis) and cytoplasmic division (cytokinesis) producing a cell that contains a unique multilobulated nucleus. Polyploidization (up to 128N) is achieved by successive cycles of endomitosis. Several studies have described the endomitotic stages up to anaphase and the data have led to the assumption that endomitosis corresponded to a late failure of mitosis during anaphase without cleavage furrow formation and spindle elongation \(^1,2\). However, the late endomitotic stages beyond anaphase have not yet been observed in details and the molecular mechanisms controlling the switch from a mitotic to an endomitotic process are still unknown.

During mitosis, the transition from metaphase to anaphase is characterized by the formation of a network of antiparallel microtubules between the separating chromosomes, called the midzone \(^3,4\). The midzone is required for the maintenance of the overall spindle architecture, for spindle elongation and cleavage furrow positioning. Many proteins essential for cytokinesis are localized to the midzone. It was believed that the late mitotic abnormalities in endomitotic MKs were associated with defects in the expression or function of one or more midzone components. However, although controversial, endomitotic MKs appear to form an intact midzone structure with the presence of proteins such as Aurora B, MKLP2 (mitotic kinesin-like protein 2), MgcRacGAP that play an essential role in regulating midzone formation and cytokinesis \(^5\). Thus, these data suggested the failure in mitosis had to take place at stages later than anaphase, as reported in a recent work \(^6\).

Small GTPases from the Rho family (like RhoA, Rac1 and Cdc42) are required for many cellular functions like actin reorganization, transcriptional activation, cell mobility and cytokinesis \(^7\). During mitosis, activated RhoA accumulates in the cleavage furrow promoting the activation of a number of effectors, including Rho kinase (Rock), citron kinase, LIM kinase and mDia/formins. Activation of the RhoA pathway leads to the assembly and constriction of the actomyosin ring, ingestion of the cleavage furrow and completion of cytokinesis \(^4,8,9,11\). In addition, Rho/Rock may also control the cell elongation that occurs in anaphase B \(^10\). Because of the importance of RhoA in late phases of mitosis, a defect in its signaling may be involved in the MK endomitotic process. In favor of this hypothesis, a
previous study has established that there is no accumulation of RhoA or actin at the cleavage furrow in anaphase during endomitosis 5.

Using real-time confocal videomicroscopy, we demonstrate that endomitosis corresponds to a late failure of cytokinesis accompanied by a backward movement of the daughter cells. The phenomenon of reversal of cytokinesis was associated with a partial accumulation of RhoA and F-actin at the midzone. In addition, myosin II was not recruited in the contractile ring in a fraction of cell at the 2N and 4N transition suggesting that it could play an important role in cytokinesis failure. Inhibition of RhoA and Rock accentuated the defects in contractile ring and furrow formation, as well as in spindle elongation. These data indicate that endomitosis is associated with a deficiency in contractile ring formation and spindle elongation that may be associated to a partial defect in Rho/Rock signaling.
Materials and Methods

*In vitro* culture of MKs derived from human CD34\(^+\) cells in liquid serum-free medium

CD34\(^+\) cells were obtained, in agreement with our Institute Ethic Committee (Assistance Publique des Hôpitaux de Paris) and in accordance with the Declaration of Helsinki, from the bone marrow of healthy patients undergoing hip surgery. CD34\(^+\) cells were isolated by using an immunomagnetic cell sorting system (AutoMacs; Miltenyi Biotec, Germany) with "Possel d2" protocol. Purity evaluated by flow cytometry was over 98%. CD34\(^+\) cells were cultured in serum-free medium in the presence of recombinant human thrombopoietin (rhTPO) (10 ng/mL; Kirin Brewery, Japan) for inducing MK differentiation. Ingredients used to prepare the serum-free medium were as previously described 12.

**Lentiviral constructs**

The EF1a-H2BGFP fragment was excised and subcloned into a HIV-derived lentiviral vector (pRRLsin-PGK-eGFP-WPRE) (Généthon, France) in place of the PGK-eGFP sequence. The shRNA cloning and lentiviral construction is described in supplemental Materials and methods 13,14.

**Lentivirus production**

Lentivirus stocks were prepared as previously described 15. The lentivirus stocks containing about 10\(^9\) infectious particules/mL were aliquoted and kept frozen at -80°C.

**Cell transduction by lentivirus**

Isolated CD34\(^+\) cells were cultured 4 days with rhTPO (10 ng/mL). Lentiviral particles were added at a concentration 10\(^7\) infectious particules/1x10\(^5\) cells for 12 h followed by a second transduction. Cells were continuously cultured in the presence of rhTPO.

**Retroviral plasmid construction, retrovirus production and cell infection**

Construction of the Migr-RhoAN19-GFP retroviral plasmid, production of retrovirus were performed as previously described 16. The details of cell infection are described in supplemental Materials and Methods.

**Electroporation**

TatC3 (10\(\mu\)g/ml) and muted TatC3 (10\(\mu\)g/ml) were delivered into MKs by the Amaxa electroporation system (Amaxa Inc MD).

**Measurement of ploidy**

Hoechst 33342 (10\(\mu\)g/mL; Sigma) was added in the medium of cultured MKs for 2 h at 37°C. Cells were centrifuged and then stained with the anti-CD41 APC and anti-CD42 PE MoAbs (Pharmingen, France) for 30 min at 4°C. The ploidy was measured in the CD41\(^+\)/CD42\(^+\) cell population when chemical inhibitors were tested and in the CD41\(^+\)/CD42\(^+\)/GFP\(^+\) population...
after lentiviral infection by means of a LSRII (Becton Dickinson, France) flow cytometer equipped with three lasers (360 nM, 480 nM and 560 nM excitation). The mean ploidy was calculated by the following formula:

\[
2N \times \text{the number of cells at 2N ploidy level} + 4N \times \text{the number of cells at 4N ploidy level} + \ldots + 64N \times \text{the number of cells at 64N ploidy level}
\]

\[
\text{the total number of cells}
\]

**Immunofluorescence**

Fixation and immunofluorescence were performed on CD41+ MKs sorted at day 6 and cultured over night before the experiments as described previously. The following antibodies were used: rabbit anti-PRC1 (Dilution: 1:100, Santa Cruz, CA), rabbit anti-non-muscle Myosin IIA (Dilution: 1:100, Sigma, France), Phalloidin-TRITC (Dilution: 1:800, Dako, Denmark), rabbit anti-α tubulin (Dilution: 1:100, ABR, CO), rabbit anti-MKLP (Dilution: 1:100, Abcam, UK), mouse anti-α tubulin and mouse anti-β tubulin MoAbs (Dilution: 1:200, Sigma, France). For mouse anti-RhoA (Dilution: 1:100, Santa Cruz, CA), the CD41+ MKs were fixed with ice-cold 10% trichloroacetic acid (TCA) for 15 min and then washed with PBS containing 30 mM glycine three times before immunofluorescence. The appropriate secondary antibodies used were conjugated with Alexa 488 or Alexa 546 (Dilution: 1:200, Molecular Probes, Netherlands). TOTO-3 iodide (Dilution: 1:1000, Molecular Probes) was applied for nucleus staining. Cells were examined under a Zeiss laser scanning microscope (LSM 510, Carl Zeiss, Germany) with a 63X1.4NA oil objective.

**Live cell imaging by confocal video-microscopy**

Isolated CD34+ cells were cultured 3 days in serum-free medium in the presence of rhTPO (10 ng/mL) and then infected by the H2B-GFP lentivirus as described above. After infection, cells were cultivated in serum-free medium in the presence of rhTPO for 48-72 hours and then stained with CellTracker Red according to the protocol of product datasheet (Invitrogen, France) and CD41APC moAb. Cells were subsequently seeded in glass bottom culture dish (MatTek, MA).

Cells were imaged under a Zeiss laser scanning microscope (LSM 510, Carl Zeiss, Germany) using a 63X/1.4 NA oil objective at 37°C with 5% CO2. A more detailed description of the process is presented in supplemental Materials and methods.

**Western blot analysis**

Western blot analysis was performed as described previously with the following antibodies: rabbit anti-Rock I (Santa Cruz, CA) and rat anti-Hsc70 (Santa Cruz, CA) and blots were quantified by the program “Image J”.

**Real-time quantitative RT-PCR**
Real time RT-PCR was performed as previously described\textsuperscript{32}. Primers for Rock I mRNA were as follows: sense: 5'-GCTGAACGAAGAGACAGAGGTCAT-3';
antisense: 5'-GCTTCACCTCCTTTGTAAGATGTA-3';
The internal probe: 5'-CTGAGATGATTGGAGACCTTCAAGCTCGA-3'.

Results

Polyplloidization in MKs results from a late reversal of cytokinesis

MKs undergo endomitosis to increase their ploidy during megakaryocytopoiesis. Here, time-lapse microscopy was used to visualize MK endomitosis on living primary human MKs. In order to follow precisely the endomitotic process, MKs were transduced with a lentiviral vector expressing Histone 2B fused to GFP (H2B-GFP) and then images were taken every 3 or 5 min.

In a first set of experiments, we examined MKs defined as CD41APC positive cells at the 2N to 4N transition. As illustrated in Figure 1A and also in Video 1, endomitosis proceeded exactly as a normal mitosis until anaphase. A cell elongation with two separated nuclear masses was accompanied with the formation of a cleavage furrow. The two daughter cells reached a point where they were connected only by a cytoplasm bridge (see 54 - 66 in Figure 1A). However, the two cells failed to separate and stayed almost 30 min at this stage. Thereafter, the two daughter cells moved backwards (Figure 1A, 75-93) and finally re-assembled into one single cell (Figure 1A, 96-99). Thus, the 2N to 4N endomitotic MKs failed to undergo abscission. We could film 11 endomitosis of MKs at the 2N to 4N transition in 8 independent experiments and we observed a late reversal of cytokinesis process in all of them. At the same time, we also filmed 24 diploid MKs undergoing mitosis where completion of cytokinesis and separation of the two daughter cells were always seen (data not shown).

Whereas we observed a failure of cytokinesis in all 2N to 4N endomitotic MKs, nuclear division did not always fail. Among 4N interphase MKs, a fraction of the cells presented a single nucleus (bi-lobulated or mono-lobulated), another fraction two separated nuclei and the third fraction two side by side nuclei but it remained difficult to assume that they were completely separated (Figure S1). The percentage of each fraction varied among experiments. In three independent experiments (more than 300 cells were counted in each experiment), the MKs that presented two separated nuclei could vary from 19.5% to 46.6% and the MKs with two side by side nuclei from 15% to 27%.

The inter-nuclear distance at the end of telophase was evaluated by the length separating the center of each nuclear mass. The average distance in endomitotic dipolar cells was 8.91μm ± 2.13 (n=11), while this distance was 10.54 μm ± 1.73 (n=24) for mitotic cells indicating an about 16% reduction in cell elongation during endomitosis compared to mitosis (p<0.05) (Figure 7C).
Next, we examined the endomitotic sequence in CD41 APC positive cells at the 4N to 8 N transition and at higher ploidy levels. Endomitosis in polyploid MKs proceeded similarly as described above (Figure 1B, see also Video 2), except that during cell elongation from late anaphase to late telophase, four daughter cells were formed which remained linked by a cytoplasm connection, exactly like a flower with four petals (Figure 1B, 30-40). The backward movement occurred asynchronously in the two couples of daughter cells. At first, two incipient daughter cells re-unified leading to a transient presence of three daughter cells (Figure 1B, 45-80). Thereafter, the endomitosis finished by joining up the two polyploid daughter cells together. Sixteen multipolar endomitotic MKs were filmed in 9 independent experiments. A late reversal of cytokinesis, as described above, was observed in 13 polyploid endomitotic MKs. Two polyploid endomitotic MKs showed attenuated furrow ingression compared to dipolar endomitotic MK; a similar phenomenon was observed by Geddis et al. in murine polyploid endomitotic MK (Figure S2A) 6. Another one presented a complete absence of furrowing (Figure S2B).

The defect in furrowing was mostly seen in endomitotic MKs with a high ploidy (> 8N). We could also observe a polyploid endomitotic MK presenting 8 petals at the end of telophase which re-unified together to form one cell (Video S and Figure S3). This reversal of cytokinesis was not due to lentiviral toxicity since a similar phenomenon was observed under bright field examination when H2B-GFP was not transduced (data not shown).

Together, these results indicate that the mechanism responsible for MK polyploidization is not a mitotic abort in anaphase, but a late reversal of cytokinesis. We thus focalized our study on the cytokinetic machinery.

**Core components of the central spindle are normally localized in the midzone during anaphase and in the midbody during telophase in endomitosis**

During anaphase, one of the most remarkable events is the formation of a central spindle, a set of microtubule-based structure 3. Many core components of the central spindle are required for cytokinesis. These include the chromosome passenger proteins, Survivin and Aurora B 17, the microtubule-associated protein, PRC1 (protein regulating cytokinesis 1) 18, a subunit of the centralspindlin complex, MKLP1 (mitotic kinesin-like protein-1) and microtubules etc.. We examined the distribution of PRC1, Survivin, Aurora B, MKLP1 and tubulin in the central spindle during CD41+ MKs endomitosis by immunofluorescence. We observed that these proteins had an appropriate localization and we showed herein the distribution of PRC1.
and tubulin as an example. During anaphase, microtubules congregated midway between the two poles of the central spindle, while PRC1 accumulated in the midzone and co-localized with the central spindle (Figure 2A). During telophase, the central spindle was highly concentrated forming one intercellular bridge. The anti-α and anti-β tubulin antibodies marked the edges of the intercellular bridge, but not the midbody itself. PRC1 was present in the center of the central spindle (Figure 2B). Altogether, these observations indicate that the distribution of PRC1 and microtubules is similar in an endomitosis and a mitosis.

**Myosin II is not recruited in the contractile ring of most mitotic and endomitotic MKs**

During anaphase, the actomyosin contractile ring formation is another principal cytokinetic structure. The contractile ring generally creates a cleavage furrow between the two daughter cells and plays a crucial role in cell abscission. To examine if the contractile ring was correctly formed in endomitotic CD41⁺ MKs, we investigated the accumulation of its two major components, non muscle myosin IIA and actin filaments (F-actin).

Non muscle myosin IIA (thereafter called myosin II) is the prevalent heavy chain of non muscle myosin II in platelets and MKs 19, 20, 21. For mitotic and endomitotic MKs, myosin II was localized ubiquitously throughout the cytoplasm. Surprisingly, among 177 dipolar mitotic/endomitotic MKs examined from the beginning of furrow ingression to the end of telophase, only 13% showed a clear but weak accumulation of myosin II around the furrow cleavage; 13% showed a very weak signal at the threshold of detection (Figure 3A, 4 independent experiments), while the others (~74%) did not show any detectable accumulation of myosin II. When detectable, myosin II accumulation was almost only seen at late telophase. Moreover, in the 24 multipolar endomitotic MKs examined from the beginning of furrow ingression to the end of telophase, we were unable to detect a clear accumulation of myosin II, except for 4 individual MKs where a signal at the threshold of detection was seen in some parts of the cleavage furrow (Figure 3B, 4 independent experiments). In contrast, an obvious accumulation of myosin II in the midzone (anaphase) or in the midbody (telophase) was clearly seen by immunofluorescence in mitosis of the monoblastic U937 cells (Figure 3C) and of primary erythroblasts undergoing differentiation (data non shown). The myosin II fluorescence intensity ratio between the midzone cortex and cytoplasm was quantified by Image J. For dipolar telophase MKs (n=11), the ratio was 1.45 and for U937 (n=13), this ratio increased to 2.41 (P=0.00001). These results point out a main defect in the formation of the contractile ring in MKs endomitosis, even when a furrow ingression is present and show that
MKs with a “normal” bipolar spindle were heterogeneous concerning myosin II accumulation at the cleavage furrow.

**F-actin accumulation is incomplete in the contractile ring of endomitotic MKs**

We also studied another principal component of the contractile ring, F-actin. For dipolar mitotic or endomitotic MKs, an accumulation of actin filaments appearing as a band of fluorescence around the midzone was clearly observed at the site of furrow ingress (Figure 4 A I-II). One hundred and eighty nine dipolar mitotic/endomitotic cells from the beginning of furrowing to the end of cytokinesis were examined in 3 independent experiments. All cells displayed a F-actin ring around the midzone or the midbody cortex. For multipolar endomitotic MKs (4N to 8N or >8N), F-actin accumulation appeared to be incomplete at the cleavage furrow (Figure 4A, III-IV; 4B I-IV). Interestingly, F-actin accumulation on a part of the furrow correlated with its local ingression (Figure 4B, I-VI).

**Rho A is localized to some extent at the cleavage furrow in endomitotic MKs**

The RhoA pathway is a central player in the assembly of the contractile ring during cytokinesis. Activated RhoA could regulate actin polymerization and myosin activation at the midzone through interactions with different effectors. We thus examined RhoA localization during CD41+ MK mitosis and endomitosis. For dipolar MKs, RhoA was concentrated in the midzone cortex during anaphase or on the cleavage furrow during telophase (Figure 5A I-II and 5B I-II). For multipolar endomitotic MKs, no evident accumulation of RhoA in the midzone cortex was seen during anaphase (Figure 5A III-VI). However, during telophase or reversal of cytokinesis, RhoA was usually and incompletely concentrated in the cleavage furrow (Figure 5B III-VI, arrows 1 and 2). Nevertheless, some MKs could be observed with a RhoA accumulation all around the cleavage furrow at the end of telophase (Figure 5C III-VI). In general, the cleavage furrow ingression was more pronounced at sites where RhoA was accumulated (Figure 5C I-II) suggesting that RhoA might be activated at some extent during endomitosis.

**Rho inhibition prevents F-actin accumulation in the midzone and spindle elongation**

A RhoA inhibitor (TatC3) and its control (mutated TatC3) were delivered by electroporation into CD41+ MKs at day 6 of culture. We then checked the accumulation of F-actin in the midzone of dipolar mitotic/endomitotic MK cells 4 hours after electroporation (3 independent experiments). Compared to their controls (Figure 4C I-II and 4D I-II), furrow formation and
cell elongation was strongly inhibited in TatC3 treated dipolar mitotic or endomitotic MKs. Despite the formation of central spindle, there was no accumulation of F-actin in the midzone. Moreover, actin polymerization around the cell cortex was also clearly decreased (Figure 4C III-VI). In addition, accumulation of RhoA in the midzone could not be detected (Figure 4D III-VI). These results suggest that Rho is not only necessary for contractile ring formation but is also implicated in spindle elongation during anaphase B.

Inhibition of Rho and Rock increases MK polyploidization

As Rock is an important effector of RhoA during cytokinesis, we inhibited its action with Y27632. To investigate if Rho/Rock are involved in MK endomitosis, TatC3 and Y27632 were used. TatC3 and its mutated control were delivered into MKs culture by electroporation at day 5 while Y27632 was added directly at day 6. The efficiency of TatC3 delivery was evaluated to exceed 70% by studying stress fiber formation of CD41+ cells (data not shown). The ploidy level of CD41+/CD42+ MKs was analyzed by flow cytometry 72 h after addition of TatC3 and Y27632, respectively (Figure 6A-6B). Addition of Y27632 to MKs cultures led to a significant increase in MK polyploidization because the mean ploidy was increased from 3.68N to 6.02N (n = 3, p < 0.0001). Inhibition of RhoA by TatC3 had very similar effect with an increase in the mean ploidy from 3.79N to 5.69N (n = 3, p<0.005) suggesting that activation of the Rho/Rock pathway may play a negative role on MK polyploidization. In contrast, an inhibition of RhoA activity by TatC3 in CD41- cells has only a minor effect on polyploidization (data not shown).

A RhoA dominant negative (RhoA N19) and a Rock I shRNA increase MK polyploidisation

The above results suggested that Rho/Rock activation was playing a negative role in endomitosis. However, since inhibitors may lack specificity, we used more specific inhibition approaches by employing a RhoA dominant negative (RhoAN19) inserted in a Moloney derived retrovirus (Migr-RhoN19-GFP) and a Rock I shRNA vectorized in a HIV-derived lentivirus. CD34+ cells were infected at day 4 of culture and the ploidy level of GFP+/CD41+/CD42+ cells was analyzed by flow cytometry at day 9 of culture. Expression of both the RhoA dominant negative and the Rock I shRNA moderately, but significantly, increased MK ploidy (Figure 6C and 6E). The mean ploidy level was increased from 2.85N (control) to 3.54N (n = 3, p < 0.03) by RhoN19 and from 3.62N (SCR shRNA) to 4.68N (n = 3, p < 0.04) by Rock I shRNA. In order to confirm that Rock I was efficiently depleted by the
shRNA, the GFP+ cell population was sorted 48 h after infection. Western blot analysis revealed an about 40% reduction in the Rock I protein level in comparison to the scramble (SCR) shRNA used as a control. A similar reduction (35%) was found at the mRNA level by Real time RT-PCR (Figure 6D). This incomplete depletion may explain why the shRNA was less effective than the Rock inhibitor Y27362 which also inhibits Rock II efficiently 23.

**Rock inhibition by Y27632 reduces furrow ingression and spindle elongation**

Our results showed that inhibition of Rock by Y27632 decreased also F-actin accumulation in the midzone and actin polymerization around the cortex of dipolar mitotic or endomitotic MK (data not shown), as observed with RhoA inhibition. To check more precisely if Rock inhibition also inhibits furrowing and spindle elongation, we used real-time video microscopy to monitor endomitosis in H2B-GFP-expressing MKs treated for 12h with Y27632. Thirty-nine endomitotic MKs (25 treated with Y27632 and 17 without Y27632) were filmed in 3 independent experiments. Y27632-treated MKs showed an apparently normal metaphase-to-anaphase transition. However, furrow ingression was less marked than in control MKs (Figure 7, Video 3 and 4). In control cultures, one of the 17 endomitotic MKs examined started an early reversal of cytokinesis, i.e., before the step when the daughter cells remained connected by a thin cytoplasmic bridge. This endomitotic MK had a ploidy over 8N. In contrast, in the presence of Y27632, 30% dipolar endomitosis (4 of 12) exhibited an incomplete furrowing before a reversal of cytokinesis and this ratio reached 77% in multipolar endomitosis (10 of 13) with a mean average of about 56% if all endomitotic MKs were considered (n=25).

Moreover, Rock inhibition also accelerated reversal of cytokinesis. In untreated MKs, the average duration from the beginning of furrow ingression to the end of cytokinesis failure was 46.3 min for dipolar (n=7) and 37.5 min for multipolar (n=10) endomitotic MKs. In contrast, the process took 28.0 min for dipolar (n=12) and 23.3 min for multipolar (n=13) Y27632-treated endomitotic MKs. Interestingly, for dipolar endomitosis, Rock inhibition also decreased the average inter-nuclear distance at the end of telophase (before the beginning of the reversal of cytokinesis) about 20% (n=28 with Y27632, n=35 for control, p<0.001) (Figure 7C).

In conclusion, these data indicate that Rock inhibition markedly impairs furrowing in endomitotic MKs and decreases the cell elongation and duration of the cytokinesis and the reversal of cytokinesis (almost 40%, p<0.05).
Discussion

During differentiation, immature MKs increase their ploidy by endomitosis in order to augment their size. Among mammalian cells, MK is the unique cell type in which polyploidization is an intrinsic part of the differentiation process. Some other cells may become polyploid, but only in response to certain stimuli such as a functional stress or senescence 24. The molecular mechanism of MK endomitosis remains poorly understood. Several studies have suggested that MK endomitosis proceeds like a normal mitosis till anaphase A but aborts at this stage since later phases of the mitosis, including anaphase B, were not observed 1,2. Here, we used H2B-GFP on imaged living human MKs to describe more precisely the process of MK endomitosis. Our results clearly show that anaphase and telophase occur during MK endomitosis. Furthermore, cells begin cytokinesis with a nearly complete furrow completion between the 2N and 4N stages, but the process arrests. Then, the two daughter cells that remain joined by a thin cytoplasm bridge undergo a backward movement to ultimately reunify in one 4N cell with only one or two nuclear masses. This demonstrates that the main defect is the failure of abscission. A similar, but more complex process was observed in MK with higher ploidy undergoing endomitosis because a partial furrow ingression was observed. With the use of a similar approach and primary murine MKs expressing YFP-tubulin, Geddis et al. have recently reported that polyploidization corresponded to a failure of late cytokinesis associated with furrow regression 6. Both results are in overall in agreement except that the timing of endomitotic process from anaphase to reversal of cytokinesis was twice longer in human MKs (around 40 minutes versus 20 minutes in mouse). Thus, video microscopy of living primary MKs used in combination with different GFP fusion proteins provides a powerful approach to understand the precise mechanism of MK polyploidization.

To understand why cytokinesis cannot fully accomplish during MK endomitosis, we examined the central machine for cell division 3,4. The presence of a normal central spindle in endomitosis has been a subject of controversy, especially for the presence and localization of Aurora B and Survivin 25,26. Our results show that the central spindle was almost correctly assembled during endomitosis with the presence of its main components: chromosome passenger proteins such as Survivin and Aurora B (data not shown), the central spindling complex and the microtubule associated protein PRC1. Thus, the cytokinesis failure in MKs
does not seem to be related to a defect in the central spindle. These data concord with the fact that furrow formation localized normally at the equatorial plate in diploid endomitotic MKs. In contrast, we observed a major defect in the contractile ring. In dipolar mitosis or dipolar endomitosis, F-actin accumulated in the midzone, but not myosin II. In multipolar endomitosis, less F-actin and no myosin II were concentrated especially in the midzone. In animal cells, a contractile ring is generally formed to induce the cleavage between the two daughter cells at the end of cytokinesis. However, the contractile ring may be dispensable for both furrow formation and cytokinesis. For example, some adherent animal cells undergo cytokinesis without apparent concentration of F-actin and myosin II in the furrow. Similarly, myosin II-null Dictyostelium cells form a cleavage furrow in the equatorial region and divide efficiently on a substrate. In contrast, non-adherent cells with a disruption of myosin II function cannot undergo cytokinesis. In MKs the defect in myosin II accumulation markedly decreases the constriction forces of the actomyosin ring and may also modify the actin turnover. The polar traction forces are potentially sufficient to induce furrow formation, but the decrease of constricting forces probably causes this failure of cytokinesis. The daughter cells remain thus connected by a cytoplasm bridge for a certain moment under the polar traction forces. However, these forces cannot counterbalance the centripetal forces and the daughter cells reunify by a backward movement. This model is similar to that described for other non-adherent cells without contractile ring formation which form a cleavage furrow but are unable to complete cytokinesis.

The Rho/Rock pathway plays a major role in cytokinesis by regulating actin polymerization and myosin II activity at the cleavage furrow. First, activated RhoA can directly control actin polymerization at the midzone by the formin-profilin machine. Second, RhoA can activate Rock and Citron kinase which in turn regulate myosin II activation by MLC2 (myosin light chain 2) phosphorylation. At the same time, activated Rock can also increase actin polymerization by activating LIMK which subsequently increases cofilin phosphorylation.

Our results also suggest that the endomitotic process is not related to an absence, but to a decrease of RhoA activity. Several evidences support this hypothesis. First, we show that RhoA was localized at the cleavage furrow during a 2N to 4N endomitosis. In higher ploidy endomitotic MKs, Rho A was concentrated together with F-actin at the level of ingression, but not all around the cleavage furrow. A more complete inhibition of RhoA activity by TatC3 led to an absence of RhoA and actin accumulation at the midzone. This suggests that the accumulation of F-actin in the midzone directly correlates with the presence of RhoA and
that RhoA is only partially activated in the midzone or on the cleavage furrow in polyploid MKs. Furthermore, RhoA inhibition induced an absence of contractile ring leading to an increase in MK polyploidization. Second, the spindle elongation was decreased of about 16% in endomitotic 2N-4N MKs in comparison to mitotic MKs. Inhibition of Rho activity by TatC3 inhibited strongly spindle elongation. This further suggests that some Rho activity must remain in endomitotic MKs to allow spindle elongation.

In Drosophila, it has been demonstrated that Rock I activity is required for anaphase cell elongation and that elongation seems essential for complete furrow formation. Inhibition of Rock by Y27632 decreased anaphase elongation of MK. We observed also that inhibition of Rock had an even more pronounced effect than inhibition of RhoA in MK polyploidization. This further suggests that Rock activation plays a crucial role in MK polyploidization.

Altogether, our results suggest that the switch during MK differentiation from mitosis to endomitosis at the 2N stage is related to an incomplete RhoA activation, which leads to a defect in contractile ring and spindle elongation. At higher stages of ploidy, the formation of a complex and short spindle increases the microtubule density which, in association with a more complete defect in RhoA local activation, further inhibits furrow formation. The precise mechanism responsible of the defect in RhoA local activation and the absence of myosin II in MK contractile ring remains to be determined, but this mechanism might be regulated by a lineage specific process. In human cells, the central-spindle-associated RhoGEF (ECT2) and GAP (MgcRacGAP) interact and promote RhoA accumulation and activation in the midzone.

The depletion of ECT2 or MgcRacGAP blocked RhoA activation and both myosin II and F-actin accumulation in the contractile ring. The work of Geddis et al. has shown that MgcRacGAP is located normally in the midzone during anaphase of endomitosis. Overexpression of an active form of RhoA (RhoV14) in MKs did not change MK ploidy level (data not shown). This suggests that the impaired mechanisms in MKs are the localization and concentration of RhoA in the midzone. Thus, it will be important to check if ECT2 and its regulators (Polo like kinase 1 or kinesin-6) accumulate in the midzone of MKs and function to recruit and activate RhoA locally.

In conclusion, our data highlight a new mechanism of cytokinesis failure in mammalian cell, which is related to the defect in contractile ring and in the Rho/Rock pathway. This study offers new avenue for investigation on the precise mechanism of cytokinesis failure and polyploidization in MKs.
Acknowledgments:
We thank J. Bertoglio for kindly providing TatC3 and TatC3mut. We are grateful to F. Wendling for critically reading the manuscript. YC and LL were supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM) et de l’Agence Nationale pour la Recherche (ANR). This research was funded by grants from the INSERM, ANR (contrat blanc 06-10) and the Association pour la Recherche contre le Cancer (ARC).

Author’s contribution statement:
Larissa Lordier: designed and performed experiments, analyzed data and critically reading the paper

Abdelali Jalil: performed experiments and analyzed data

Frédéric Aurade: performed experiments

Jerôme Larghero: performed experiments

Frédéric Larbret: performed sorting experiments

Najet Debili: designed and performed experiments

William Vainchenker: designed the work, supervised the experiments and wrote the paper

Yunhua Chang: designed the work, designed and performed experiments, analyzed data and wrote the paper

The authors declare no competing financial interests
References:

Figures legends

Figure 1: Polyploidization in MK results from a late reversal of cytokinesis
Primary cultured MKs from human bone marrow were transduced with a plasmid encoding H2B-GFP. Time-lapse images were obtained by confocal video microscopy. Time relative to the first image is indicated in min on each photograph. Bars represent 10 μm. (A) a mitosis/endomitosis transition. The cell was stained with CellTracker Orange and serial images were obtained at 3 min interval. Endomitosis proceeded exactly as a mitosis until the late telophase when the 2 nuclear masses are separated and the two daughter cells are connected by a cytoplasm bridge (images 54 and 66). Thereafter, the two daughter cells moved backwards toward each other (images 75 to 96) and reassembled into one single cell with only one nuclear mass (image 114). (B) an endomitosis in a polyploid MK. Serial images were obtained at 5 min interval. Endomitosis proceeded as a mitosis but DNA was scattered at 4 poles until the end of cytokinesis (images 0 to 40). The four daughter cells remained connected (images 30, 35 and 40). Then the incipient daughter cells moved backward and reunified two by two (images 45 to 85). Movies of these time-lapse images are available online as Video 1-2.

Figure 2: PRC1 localized normally to the central spindle during endomitosis.
MKs were stained for tubulin (α and β, green), PRC1 (red) and TOTO (blue). The anti-α and anti-β tubulin antibodies mark the central spindle. (A) During anaphase, microtubules congregated midway between the two poles of the central spindle, while PRC1 accumulated in the midzone and co-localized with the central spindle. (B) During telophase, the central spindle was highly concentrated forming one intercellular bridge. PRC1 was present in the center of the central spindle.

Figure 3: Absence of non muscle Myosin IIA accumulation in the contractile ring during MKs mitosis or endomitosis.
Cells were stained for tubulin (α and β, green) and non muscle Myosin IIA (red). DNA was stained with TOTO (blue). (A) Only 13% dipolar mitotic/endomitotic MKs showed a clear accumulation of Myosin II at the midzone or in the midbody; 13% showed either a signal at the threshold of detection or an incomplete accumulation at one part of the furrow and 74% were negative. (B) Myosin II accumulation was not observed in the midzone or in the midbody of multipolar endomitosis. The arrow indicated the location of midbody. (C) Myosin
II accumulation was clearly detected in the midzone and the midbody of control U937 cells in mitosis.

**Figure 4: Accumulation of F-actin in the contractile ring is incomplete during MK endomitosis**

MKs were stained for F-actin (phalloidin-TRITC, red) or tubulin (green) and TOTO (blue). (A) F-actin accumulation around the midzone at the site of furrow ingress in dipolar mitotic/endomitotic MKs (I-II). Incomplete F-actin accumulation (arrows) at the site of furrow ingress in multipolar endomitotic MKs (III-IV). (B) In multipolar endomitosis, accumulation of F-actin was seen at only some parts of midzone where a local ingress is seen (arrows). (C and D) Rho inhibition by TatC3 prevents F-actin accumulation (C) and RhoA localization (D) in the midzone and spindle elongation.

**Figure 5: Rho A is localized to some extent at the cleavage furrow in endomitotic MK**

Cells were stained for tubulin (α and β, green) and RhoA (red). (A) RhoA localization during anaphase. In dipolar mitotic/endomitotic MK, RhoA was concentrated around the midzone cortex (I-II). In polyploid endomitotic MK, RhoA could not be detected in the midzone (III-VI). (B) RhoA localization during telophase. In dipolar mitotic/endomitotic MK, RhoA was concentrated in the cleavage furrow (I-II). In polyploid endomitotic MK, RhoA was only detected in the deepest ingressions of the cleavage furrow (III-VI, arrows 1 and 2). (C) RhoA localization at the end of telophase. RhoA was localized at all sides of the cleavage furrow in dipolar mitotic/endomitotic MK (III-VI). In multipolar endomitosis, RhoA was accumulated at only one part of the cleavage furrow and at a zone corresponding to a local ingress (indicated by an arrow) (C I-II).

**Figure 6: Effects of RhoA or Rock inhibition on MK polyploidisation**

(A) Effects of RhoA (TatC3) inhibitors on MK polyploidisation. The ploidy level of CD41+/CD42+ MKs was analyzed at day 8 or 9 of culture (72 h after addition of the inhibitors). The mean ploidy was calculated from the number of cells of each ploidy class in three independent experiments. Mean ploidy levels was 3.79N in the control and 5.69N with TatC3 (n=3, p<0.005). The ploidy histograms are illustrated on the right part of the figure. (B) Effects of Rock (Y27632) inhibitor on MK polyploidisation. The mean ploidy levels was 3.67N in the control and 6.02N with Y27632 (n=3, p<0.0001). (C) Effect of a RhoA dominant negative (RhoA N19) on MK polyploidization. The ploidy level of
GFP+/CD41+/CD42+ cells was analyzed by flow cytometry at day 9 of culture. The mean ploidy was calculated from the number of cells in each ploidy class in three independent experiments. The mean ploidy level was 3.72N with RhoN19 and 3.2 N with the empty Migr (n = 3, p < 0.03). (D) The effect of Rock I shRNA was ascertained by Western blotting and real time RT-PCR. Western blot quantification showing that the protein levels of Rock I/Hsc70 (used as a control of protein loading) was about 40% reduced by the specific Rock I shRNA compared to the SCR shRNA (control). Real time RT-PCR showing that the mRNA level of Rock I mRNA was about 35% reduced by the specific Rock I shRNA compared to SCR shRNA (control). (E) Effect of a Rock I shRNA on MK polyploidization. The mean ploidy level was analyzed as described above: SCR shRNA control (3.62N); Rock I shRNA (4.68N) (n = 3, p < 0.04).

**Figure 7: Inhibition of Rock by Y27632 reduces furrow ingression**

MKs transduced with H2B-GFP were treated for 12h with Y27632 and then filmed by real-time video microscopy. Cells were stained with CellTracker Red and serial images were obtained at 3 min interval. Timing (min) relative to the first image is indicated. 

(A) Diploid endomitosis of MK treated with Y27632 proceeded generally as a normal endomitosis, but with a reduced cell elongation (see also Figure 7C) and less evident cleavage furrow formation. (B) Polyploid endomitosis of MK treated with Y27632 showing less cleavage furrow formation (images 21 to 36). The movies of these time-lapse images are available on line as Video 3-4. (C) The distance between two centers of separating nuclear masses in mitosis and dipolar endomitosis was measured at end of telophase of Y27632-treated and untreated MK. In untreated MK (Control), the average distance reduced about 16% during endomitosis compared to mitosis. For Y27632-treated MKs, the average inter-nuclear distance of dipolar endomitosis was continually decreased about 20% compared to untreated MK.
Figure 2

A

Tubulin

PRC1

Tubulin/PRC1/ToTo

Anaphase

B

Tubulin

PRC1

Tubulin/PRC1/ToTo

Telophase
Megakaryocyte endomitosis is a failure of late cytokinesis related to defects in the contractile ring and Rho/Rock signaling

Larissa Lordier, Abdelali Jalil, Frederic Aurade, Frederic Labret, Jerome Larghero, Najet Debili, William Vainchenker and Yunhua Chang