Intrinsic and extrinsic regulators of stem cell function in normal and malignant hematopoiesis
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IMAGING HEMATOPOIETIC STEM CELL DIVISION: DETERMINING THE SYMMETRY AND ROLE OF RAC PROTEINS

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ABSTRACT

Hematopoietic stem cells (HSCs) have the potential to both self-renew and give rise to more differentiated progeny that reconstitute all blood lineages. This dual characteristic may originate from the two types of cell division that HSCs undergo: symmetric and asymmetric cell division. In several stem cell models, interactions with the microenvironment dictate the positioning of centrosomes and consequently symmetry of cell division. However, cooperation between cell-extrinsic signals and cell-intrinsic regulators is most likely necessary to determine the type of cell division that an HSC undergoes. Therefore, proteins such as RAC GTPases that translate microenvironmental signals into intracellular responses may be important regulators of HSC cell division. While our insights into the mechanisms that regulate stem cell fate are largely based on experiments performed on heterogeneous populations of stem cells, live cell imaging of single dividing HSCs is necessary to obtain a better understanding of the mechanisms governing the symmetry of HSC division.

Within this study we developed techniques that allow imaging the symmetry of an individual human hematopoietic cell in real time. By generating imaging tools in hematopoietic cell lines, applying them in human hematopoietic stem and progenitor cells (HSPCs) and developing methods of quantitative analysis, we were able to establish a model in which we could observe and quantify the symmetry of HSPC cell divisions. Using these tools we observed specific localization patterns of the two RAC proteins, RAC1 and RAC2 in resting and dividing human hematopoietic cells and noted that RAC activity is necessary in the process of cell division in those cells. Although in this study we focused on RAC proteins, the methods that we developed can be used to assess the effects of various cell-intrinsic and cell-extrinsic factors on the symmetry and progress of HSPC cell division.
INTRODUCTION

Stem cells (SCs), and among them hematopoietic stem cells (HSCs), are characterized by their ability to both self-renew and give rise to more differentiated progeny that sustains tissue regeneration throughout life [1–3]. It has been proposed that this dual characteristic of SCs originates from different types of cell division that they undergo [4]. Asymmetric cell division would give rise to one daughter stem cell and one more differentiated progenitor cell, thus maintaining the stem cell pool and at the same time sustaining production of mature cells [5,6]. Symmetric cell division would result either in generation of two daughter stem cells and therefore SC expansion, or in two progenitor cells leading to SC exhaustion (Fig. 1A). These different types of cell division are most likely not exclusive, but might co-exist within the stem cell pool in a balance that can change depending e.g. on the regenerative requirements of the tissue [4,7–9].

Traditionally, two opposing views on the regulation of the symmetry of stem cell division have been proposed: the cell intrinsic, and the cell extrinsic one [5,10–14] (Fig. 1B). In the cell intrinsic model, determinants of the symmetry of stem cell division are present within the cell and their distribution during cell division determines the type of division and the fate of the daughter cells. Among those factors Brat and Prospero in Drosophila neural stem cells and mammalian Insuteable in developing retina have been shown to influence

Figure 1. Types and regulation of stem cell (SC) cell division. (A) Schematic representation of different types of SC division, in relation to their stroma. (B) Schematic overview of possible determinants of the symmetry of SC division: 1) cell intrinsic that segregate asymmetrically into daughter cells; 2) cell extrinsic influences from the microenvironment; 3) stochastic.
the orientation of the mitotic spindle and therefore the symmetry of SC division [15,16]. However, stem cells reside within a specialized microenvironment and can be accurately described only in relation to their niche [17–22]. Accordingly, the cell extrinsic model proposes that microenvironmental signals can dictate the type of cell division that the stem cell undergoes as well as the fate of daughter cells. Adherent junctions formed by the cell-to-cell contacts between SC and their niche have been shown to influence the symmetry of SC division [14,23,24]. In *Drosophila* male germline stem cells (GSCs), interaction between a stem cell and the hub cell created an anchoring platform for ApC2 proteins that in turn oriented the mitotic spindle perpendicular to the hub. Such a positioning of the mitotic spindle resulted in an asymmetric cell division. Consequently, the daughter cell that remained in contact with the niche retained stem cell properties, while the daughter cell that moved away from the niche underwent differentiation [14]. This suggests that co-operation between cell extrinsic and cell intrinsic factors is required to determine the symmetry of SC cell division [1,25] (Fig. 1B). Interestingly, some of the intrinsic stemness factors are also closely involved in other aspects of SC-niche interactions. Cdc42 is a member of the family of Rho GTPases and acts as a molecular switch transmitting microenvironmental cues to the downstream cellular signaling pathways [26–29]. At the same time, asymmetric distribution of Cdc42 has been associated with a young HSC phenotype [30]. Another member of Rho family, Rac, has been shown to regulate asymmetric cell division in mammalian oocytes. Halet and Carroll have shown that polarized localization of active GTP-bound Rac regulated the stability and anchoring of meiotic spindle to the cortex, which in turn enabled asymmetric cell division [31]. A similar function for Rac-GTP has been described in *Drosophila* female GSCs [32]. Whether Rac proteins have a role in regulating the symmetry of cell division also in other SC types is currently unclear.

Although most of the insight into the mechanisms governing the symmetry of SC cell division comes from studies in the *Drosophila* model, there is evidence that symmetric and asymmetric divisions occur also in hematopoietic system of higher vertebrates [33,34]. In *vitro* studies utilizing the Notch reporter as a marker of stemness showed that the different types of cell division co-exist in murine HSCs and that the balance between symmetric and asymmetric cell divisions can be affected by both microenvironmental signals as well as oncogenes [33]. Nup98-HoxA9 and BMI1 overexpression has been shown to alter the balance in HSC cell division, leading to an increase in symmetric cell divisions and expansion of HSCs [33,35,36]. In the human system, current protocols of HSC purification result in a mixed population of stem and progenitor cells (HSPCs). Measuring an average of such a heterogeneous group, as most experimental approaches do, has the pitfall of omitting small, but relevant subgroups. Methods of live cell imaging at the single cell level developed in recent years can ameliorate this problem and are therefore highly useful in the field of stem cell research [37–40]. Employing these methods for HSCs could lead to a better understanding of the mechanisms governing the symmetry of cell division.

Here, we describe the development of techniques that allow imaging the symmetry of individual hematopoietic cell in real time. Through generation of the imaging
tools in hematopoietic cell lines, applying them in human hematopoietic stem and progenitor cells (HSPCs) and finally through developing methods of quantitative analysis, we were able to establish a model in which we could observe and quantify the symmetry of HSPC cell division.

MATERIALS AND METHODS

PRIMARY CELL ISOLATION AND CULTURE CONDITIONS

Neonatal cord blood (CB) was obtained from healthy full-term pregnancies after informed consent in accordance with the Declaration of Helsinki form the obstetrics departments of the University Medical Centre Groningen (UMCG) and Martini Hospital Groningen, Groningen, The Netherlands. All protocols were approved by the Medical Ethical Committee of the UMCG. After separation of mononuclear cells with lymphocyte separation medium (PAA Laboratories, Coble, Germany), CD34+ cells were isolated using magnetically activated cell sorting (MACS) CD34 progenitor kit (Miltenyi Biotech, Amsterdam, The Netherlands). For the MS5 co-culture experiments cells were grown in Gartner’s medium consisting of α-modified essential medium (α-MEM; Fisher Scientific Europe, Emergo, The Netherlands) supplemented with 12.5% heat-inactivated fetal calf serum (Lonza, Leusden, The Netherlands), 12.5% heat-inactivated horse serum (Invitrogen, Breda, The Netherlands), 1% penicillin and streptomycin, 2 mM glutamine (all from PAA Laboratories), 57.2 μM β-mercaptoethanol (Merck Sharp & Dohme BV, Haarlem, The Netherlands) and 1 μM hydrocortisone (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). For the imaging experiments, Gartner’s medium was supplemented with 100 ng/mL stem cell factor (SCF), FLT3 Ligand (Flt3L; both from Amgen, Thousand Oaks, USA) and thrombopoietin (TPO; Kirin, Tokyo, Japan).

CELL LINES AND CULTURE CONDITIONS

293T embryonic kidney cells (ACC-635 DSMZ), PG13 packaging cells (ATCC CRL-10686) and HeLa cells (ACC-57 DSMZ) were grown in DMEM medium with 200 mM glutamine (BioWhittaker) supplemented with 10% FSC and 1% penicillin and streptomycin. K562 myelogenous leukemia cells (ACC-10, DSMZ) and TF-1 erythroleukemic cells (ACC-334, DSMZ) were grown in RPMI medium with 200 mM glutamine (BioWhittaker) supplemented with 10% FCS, and 1% penicillin and streptomycin, and for TF-1 cells with 5 ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF; Genetics Institute, Cambridge, MA, USA). MS5 murine stromal cells (ACC-441, DSMZ) were grown in αMEM with 200 mM glutamine (BioWhittaker) supplemented with 10% FCS and 1% penicillin and streptomycin.

RETRO- AND LENTIVIRUS GENERATION AND TRANSDUCTION

A C-terminal mCherry in-frame fusion with α-tubulin was cloned into the MSCV vector and stable PG13 producer cell lines were generated and used as published previously [41]. Supernatants from the PG13 cells were harvested after 8-12 hours of incubation in HPGM and passed through
0.45-mm filters before the retroviral transduction rounds (Sigma-Aldrich). Three rounds of transduction were performed on retromere-coated 24-well plates in the presence of 4 µg/mL polybrene (Sigma-Aldrich). With the last round of transduction, lentiviral transduction with the GFP-RAC1 or GFP-RAC2 constructs described below was performed.

N-terminal GFP in-frame fusion constructs of RAC1, RAC2 and C-terminal GFP in-frame fusion construct of γ-tubulin were cloned into the pRRL vector. 293T embryonic kidney cells were transfected using FuGENE6 (Roche, Almere, The Netherlands) with 3 µg pCMV Δ8.91, 0.7 µg VSV-G, and 3 µg of vector constructs (pRRL-GFP-RAC1 (GFP-RAC1), pRRL-GFP-RAC2 (GFP-RAC2), or pRRL-γ-tubulin-GFP (γ-tubulin-GFP)). After 24 hours medium was changed to HPGM and after 12 hours supernatant containing lentiviral particles was harvested and either stored at -80°C or used fresh for transduction of target cells. TF-1 cells, HeLa cells or isolated CD34+ CB cells that were pre-stimulated for 12 hours were subjected to 1 round of transduction with lentiviral particles in the presence of prestimulation cytokines and 4 µg/mL polybrene (Sigma) on retromere-coated 24-well plates (Takara, Tokyo, Japan). After transduction GFP-positive or mCherry-and GFP-double-positive HeLa and TF-1 cells were sorted on a MoFlo sorter (Dako Cytomation). For CB cells, staining for CD34-PE and CD38-APC was performed and GFP+CD34+CD38- cells were sorted.

FLOW CYTOMETRY ANALYSIS AND SORTING

All fluorescence-activated cell sorter (FACS) analyses were performed on a FACScalibur (Becton-Dickinson [BD], Alpen a/d Rijn, the Netherlands) and data were analyzed using WinList 3D (Verity Software House, Topsham, USA). Cells were sorted on a MoFlo sorter. Antibodies: CD34-PE and CD38-APC were obtained from BD.

FLUORESCENT MICROSCOPY AND TIME-LAPSE IMAGING

Transduced TF-1 cells were plated into retromere-coated glass bottom chamber slides (Nagle Nunc International, Naperville, IL, USA) either for time-lapse imaging as described below, or for high-resolution confocal imaging. For the co-localization of γ-tubulin-GFP and RAC, transduced TF-1 cells were cytospun and fixed in 4% paraformaldehyde, and staining with RAC antibody (610650; BD) was performed according to standard protocols. Images were acquired with a Leica SP2 AOBS confocal microscope, 63x objective and 3D reconstructions were generated using Imaris software, version 6.4.2 (Bitplane, Zurich, Switzerland). Transduced HeLa cells were plated on a retromere-coated slides with adhesive square-shaped areas (CYTOO) according to manufacturer's instructions and cell spreading on micropatterns was assessed on a Leica DMIL inverted phase microscope. Subsequently, images were acquired with a Leica SP2 AOBS confocal microscope, 63x objective. GFP+CD34+CD38- CB cells were sorted and plated on MS5 in glass bottom chamber slides in Gartner’s medium supplemented with 100 ng/mL SCF, Flt3L and TPO. Cells were and analyzed every 5 minutes for 16 hours using a Solamere Nipkow Confocal Live Cell Imaging system (based on Leica DM IRE2 Inverted microscope; Leica).
under the control of InVivo software (Media Cybernetics, Silver Springs, USA). For the imaging of GFP-RAC1 and GFP-RAC2, a 63x objective was used and 20 confocal z-stacks were acquired at each timepoint. Images were analyzed with ImageJ software (freeware, developed by NIH). For the imaging of γ-tubulin-GFP, a 40x objective was used and 10 confocal stacks were acquired. Image analysis and 3D reconstructions were performed using Imaris software.

STATISTICAL ANALYSIS
All values are expressed as means ± standard deviation (SD). Student’s t test was used for all other comparisons. Differences were considered statistically significant at p<0.05.

RESULTS
RAC1 AND RAC2 SHOW DISTINCT SUBCELLULAR LOCALIZATION IN RESTING AND DIVIDING HEMATOPOIETIC CELLS

RAC GTPases have been implicated in regulating the symmetry of cell division in Drosophila GSC and mammalian oocytes [31,32]. In the hematopoietic system, two members of that family are expressed (RAC1 and RAC2) and they have been shown to induce cytoskeleton rearrangements in response to microenvironmetal signals [42–44]. We questioned what the distribution pattern of RAC GTPases in human hematopoietic cells during cell division would be and whether their localization could influence the symmetry of HSC division. To allow imaging of living cells, we generated lentiviral constructs containing fluorescent-tagged RAC1 and RAC2, as well as α- and γ-tubulin (Fig. 2A). In the case of GFP-tagged RAC constructs, GFP was fused fused to the N-terminal ends of the proteins to ensure that the tag would not affect their functionality since the C-terminal region contains the polybasic motif that undergoes posttranslational modifications and it responsible for the targeting of RAC proteins to cell membranes. Those lentiviral constructs were then used to stably transduce hematopoietic TF-1 cells that have a propensity to adhere and spread on retronectin-coated plastic or glass surfaces. This ability of TF-1 cells allowed us to culture them in retronectin-coated chamber slides and perform time-lapse imaging of living cells. We observed a distinct localization pattern of the two RAC proteins, with RAC1 enriched at the plasma membrane and RAC2 present in the cytoplasm (Fig. 2B). Importantly, TF-1 cells plated on retronectin were still able to divide normally. During cell division, formerly spread cells were rounding up but remained attached to the surface and relatively immobile. This allowed us to acquire high-resolution confocal stacks of dividing TF-1 cells expressing GFP-RAC1 or -RAC2 and α-tubulin-mCherry, which were then used to create 3D reconstructions. In a 3D representation of a dividing cell it was clearly visible that also during cell division RAC1 was located in the plasma membrane of the cell, while RAC2 was present in the cytoplasm (Fig. 2C and Supplementary movies 1 and 2). However, we did not observe co-localization of RAC proteins with the microtubules of the mitotic spindle, visualized by α-tubulin-mCherry. Subsequently,
**Figure 2.** RAC1 and RAC2 display different subcellular localization patterns in hematopoietic cell lines. (A) Schematic representation of fluorescent-tagged protein constructs used in the imaging experiments. (B) TF-1 cells expressing GFP-RAC1 or GFP-RAC2 were plated in retronectin-coated chamber slides and time-lapse imaging was performed for 16hrs. Stills from the time-lapse movies are shown. (C) TF-1 cells expressing GFP-RAC1 or GFP-RAC2 and α-tubulin-mCherry were plated in retronectin-coated chamber slides and high-resolution confocal imaging was performed. Acquired confocal stacks were used to generate 3D reconstructions of dividing TF1 cells that visualize the localization of GFP-RAC1 or –RAC2 (green channel) and α-tubulin-mCherry (red channel). (D) K562 cells expressing γ-tubulin-GFP were cytospun, fixated in 4% formaldehyde and stained with anti-RAC1/2 antibody. DAPI was used to stain DNA. Blue channel – DAPI, green channel - γ-tubulin-GFP, red channel – RAC1/2.

since both our RAC and γ-tubulin constructs were GFP-tagged, we performed immunostaining using anti-RAC antibody (recognizing both RAC1 and RAC2) on γ-tubulin-GFP-expressing TF-1 cells. Interestingly, co-localization of both signals was seen, indicating possible interaction of RAC proteins with centrosome structures (Fig. 2D) in non-dividing cells.
Figure 3. RAC1 and RAC2 localize differently in resting and dividing human HSPCs. CD34+ CB cells were transduced with GFP-RAC1 or GFP-RAC2 constructs and GFP+ CD34+ CD38- cells were sorted and plated on MS5-coated chamber slides for confocal time-lapse imaging. Stills from time-lapse movies are shown as a composite of 20 confocal z-stacks (experimental setup shown in A). (B) Localization of GFP-RAC1 and GFP-RAC2 in human HSPCs plated on stroma, shown as a still form the time-lapse movie.

TIME-LAPSE IMAGING OF LIVING HSPCs REVEALS SPECIFIC LOCALIZATION PATTERNS OF RAC1 AND RAC2

Next, we wanted to visualize the localization of RAC1 and RAC2 in human HSC-enriched population, defined as lin- CD34+CD38- cells. Since we were interested in both resting and dividing cells, we optimized the experimental protocols to ensure that the imaged cells remained alive and in good condition for the duration of the experiment. To this end, we pre-seeded MS5 cells in an imaging chamber and allowed them to reach confluence to create a feeder layer for hematopoietic cells. CD34+CB cells were then transduced with lentiviral constructs containing GFP-RAC1 or -RAC2 and GFP+C-
D34^+CD38^- cells were sorted and plated on the MS5-coated chamber slides in a cytokine-rich medium. Cells were allowed to recover overnight and imaging was performed on the following day. Exposure time, number of z-stacks acquired and acquisition frequency were set to achieve the highest possible quality of images while minimizing phototoxicity (Fig. 3A). This optimized experimental setup resulted in acquisition of several cell divisions within the HSC-enriched population. We could observe that, similar to TF-1 cells, RAC1 was enriched in the plasma membrane of resting HSCs and remained there also during cell division. In contrast, RAC2 was present in the cytoplasm and its distribution was more dynamic as the cell division progressed. However, no clear asymmetry was observed in the localization of either RAC1 or RAC2 in the dividing cells, or in their distribution between the two daughter cells (Fig. 3B and Supplementary movies 3 and 4).

RETRONECTIN-COATED PATTERNS INDUCE CELL SPREADING AND (A) Asymmetric Organization of Cellular Structures

It has been previously shown that contacts between a cell and the components of the extracellular matrix (ECM) affect the organization of cytoskeleton and can guide the symmetry of cell division [45]. This concept has been used to develop commercially available glass slides on which micrometer-level sized patterns are spotted. Those micropatterns can be coated with components of the ECM, and used to guide the symmetry of cell division. Consequently, depending on the shape of the pattern, cells are forced to divide in either a symmetric or asymmetric manner (Fig. 4A). Therefore, we wondered whether upon imposing different symmetry of cell division by different micropatterns distribution of RAC1 and RAC2 during cell division would also change. To setup the experiment, we first used slides spotted with symmetric micropatterns and coated them with retronectin to enable binding and spreading of TF-1 hematopoietic cells. Although we have previously seen TF-1 cells adhering readily to retronectin, we could not detect any spreading of those cells on the micropatterns, regardless of their size. Therefore, we proceeded to use the adherent HeLa cell line in which we stably expressed either GFP-RAC1 or GFP-RAC2. Those cells were able to adhere and spread on the retronectin-coated patterns, allowing for precise confocal imaging of the localization of RAC1 and RAC2 (Fig. 4B). Similarly to hematopoietic cell lines and primary HSPCs, also in HeLa cells RAC1 was strongly enriched in the plasma membrane. RAC2 was present in the cytoplasm, with clear perinuclear localization (Fig. 4C). However, despite imaging several adherent cells, we did not observe any cell divisions occurring on the micropatterns, deeming this system not suitable for our experiments.

GFP-LABELLED γ-TUBULIN CAN BE USED TO TRACK CENTROSOME LOCALIZATION AND DISTINGUISH DIFFERENT TYPES OF CELL DIVISION

Since we were not able to setup and experimental system in which a certain type
of cell division would be imposed, we focused on determining whether symmetric and asymmetric cell divisions could naturally be observed in human HSC-enriched populations. As described above, we used a co-culture system to ensure optimal culture conditions and register a high number of cell divisions. The GFP-labelled γ-tubulin construct was tested in TF-1 cell line (Supplementary movie 7) and then stably introduced into CD34+ CB cells, after which CD34+38- cells were sorted to enrich for HSCs and plated in stroma-coated chamber slides. For the imaging of the co-cultures a relatively small magnification of 40x was used, since the objective was to observe a high number of cells and acquire images of as many cell divisions as possible, rather than observing details of subcellular organization. We observed that γ-tubulin-GFP was a clear marker of cell division, as the appearance of two centrosomes shortly preceded the beginning of mitosis and their segregation into the daughter cells could be easily followed. Moreover, positioning of the two centrosomes could be used to distinguish two different types of cell division. In the symmetric cell division, two centrosomes were positioned in the same z-plane and both daughter cells remained in contact with stroma. In the asymmetric cell divi-
results

Figure 5. γ-tubulin-GFP can be used to track HSPC cell division and to determine the symmetry of cell division. (A) CD34+ CB cells were transduced with γ-tubulin-GFP construct and GFP+ CD34+ CD38– cells were sorted and plated on MS5-coated chamber slides for confocal time-lapse imaging. Confocal z-stacks were then used for the 3D reconstructions of dividing cells. An example of a symmetric and asymmetric cell division is shown with schematic representations on the left panels. (B) Overall quantification of the symmetric and asymmetric cell divisions registered in 3 independent imaging experiments. (C) Duration time of cell divisions registered in an experiment as described in panel A were analyzed and the two types of cell divisions were compared. Division time is represented as the number of minutes the individual cell spent in mitosis.

Symmetric, centrosomes were located in different z-stacks and while one daughter cell remained attached, the other one was no more in contact with stromal cells after the division (Fig. 5A and Supplementary movie 5 and 6). In the three experiments analyzed, similar number of symmetric and asymmetric cell divisions was observed (Fig. 5B). Moreover, we have analyzed the duration of acquired cell divisions and compared the two types. Symmetric cell division took on average longer
to complete (although not statistically significant), and larger variability in the duration of cell division was observed in this division type (Fig. 5C). Overall, this indicates that imaging GFP-labelled γ-tubulin is useful not only to detect the beginning of cell division, but also to determine its properties such as geometrical symmetry and duration time.

**INHIBITION OF RAC ACTIVITY IN HUMAN HEMATOPOIETIC CELLS RESULTS IN A PROLONGED MITOSIS DURATION TIME**

Having developed the tools to monitor progress of cell division in human hematopoietic cells, we then wondered whether interfering with intracellular signaling pathways would result in changes that could be registered using those tools. Inhibition of RAC activity with a small molecule NSC23766 presented an interesting target, given the role of RAC proteins in mediating the interactions between hematopoietic stem cells and their niche, as well as in regulating cytoskeleton rearrangements [42,44]. TF-1 cell line stably expressing α-tubulin-mCherry was used to enable robust imaging of cell divisions during an overnight time-lapse experiment. Indeed, the first experiment performed yielded acquisition of a large number of cell divisions. Analysis of all the cell divisions obtained for the NSC-treated as well as untreated cells revealed that RAC inhibition resulted in a largely decreased frequency of cell division. Moreover, the duration of mitosis was significantly extended in NSC-treated cells (Fig. 6 and Supplementary movie 8 and 9). The two other experiments performed showed the same trend, although in those cases a much lower number of cell divisions was acquired, highlighting the risk of phototoxicity during prolonged time-lapse imaging. Taken together, this data shows that the imaging setup we developed can be useful to identify and analyze changes in the

**Figure 6.** RAC activity is required for the progress of cell division in hematopoietic cells. TF-1 cells expressing α-tubulin-mCherry were plated on MS5-coated 2 chamber slides for confocal time-lapse imaging. At the beginning of the imaging experiment RAC inhibitor was added to one chamber, while the other chamber remained untreated. Cells were then followed for 16hrs. Duration time of the registered cell division was analyzed and compared between NSC-treated and untreated cells. The results of 3 independent experiments are shown. * $P<0.05$, *** $P<0.001$. 

inhibition of rac activity in human hematopoietic cells results in a prolonged mitosis duration time
frequency and progression of mitosis in primary human HSCs grown on bone marrow stromal cells. Furthermore, we conclude that inhibiting RAC activity results in a decreased number of cell divisions that take longer to complete.

DISCUSSION

RAC PROTEINS AS REGULATORS OF THE SYMMETRY OF HSC CELL DIVISION

RAC GTPases act as molecular switches that in response to microenvironmental stimuli activate various effector proteins, resulting in for instance cytoskeleton re-arrangements in HSCs [42–44]. Since the role of RAC proteins in regulating the symmetry of division of other stem cell types has been described, they presented as potentially interesting candidates for determining the symmetry of HSC cell division. Murine knock-out models have shown that despite the very high amino-acid sequence homology, RAC1 and RAC2 have non-redundant functions in hematopoietic cells [42]. This specificity of function could be explained at least in part by the differences in the C-termini of the two proteins that lead to their distinct subcellular distribution in murine neutrophils [46,47]. Here, we showed that RAC1 and RAC2 displayed different subcellular localization in human hematopoietic cell lines, as well as in HSC-enriched human CD34+38– CB cells and that this distinct localization pattern was also apparent in dividing cells. However, no asymmetry in distribution of RAC1 or RAC2 before or during cell division was observed, and the level of RAC protein that was passed on to the two daughter cells was usually comparable.

Although RAC2 was present in the cytoplasm of dividing cells, no co-localization with the mitotic spindle could be seen. Interestingly, when TF-1 cells expressing γ-tubulin-GFP were immunostained with anti-RAC antibody, a clear co-localization of the two proteins was observed. In proteomic experiments that we have previously performed, several proteins involved in regulation of mitosis were identified in RAC2-associated complexes, including Aurora Kinase B (Capala et al. 2015, in press). The Aurora kinases are highly conserved group of proteins that regulate chromosomal alignment and segregation during cell division. Aurora B specifically controls chromosome condensation, the spindle checkpoint and cytokinesis as a member of the chromosome passenger complex [48]. It is therefore possible that RAC proteins are involved in regulating HSC division in an indirect manner, by activating key players of the mitotic machinery, responsible for the organization of the mitotic spindle and timely progression of cell division. Importantly, when we blocked RAC activity in TF-1 cells, we observed not only fewer cell divisions, but also a significantly prolonged division time. This indicated that the mitotic machinery was disturbed, although the exact mechanisms remain to be determined. Whether blocking RAC activity could also influence the symmetry of HSC division requires further investigation.
USE OF RETRONECTIN-COATED MICROPATTERNS FOR ENFORCING SYMMETRIC OR ASYMMETRIC CELL DIVISION

Since we did not observe asymmetry in the distribution of RAC proteins during or after cell division, we wondered whether by enforcing a particular subcellular organization and consequently cell division type we could affect RAC localization. We coated commercially available CYTOO chips with retronectin that we previously used for inducing spreading of TF-1 cells, yet were not able to stimulate them to attach to the micropatterns. It is possible that TF-1 cells, being naturally a non-adherent cell line, require a larger retronectin-coated surface for adhering. In agreement with that, TF-1 cells were able to spread on the control area of a CYTOO chip, where adherent surface was not limited to the micropatterns. Although adherent HeLa cell lines that we subsequently used were readily attaching and spreading on the retronectin-coated micropatterns, we were not able to observe any cell divisions despite imaging several individual cells. It is possible that the combined stress applied by spreading on a micropattern and by prolonged imaging resulted in a block in cell cycle progression in the observed cells making this system unsuitable for our purpose.

DEFINING THE SYMMETRY OF HSC CELL DIVISION – CHALLENGES AND OPPORTUNITIES

In various types of stem cells, specific positioning of the mitotic spindle in respect to the supporting stromal cells has been shown to result in a defined symmetry of cell division [14,16,25]. Therefore, we analyzed centrosome positioning, visualized by γ-tubulin-GFP, to determine the symmetry of HSC cell divisions and observed both symmetric and asymmetric types of cell division. However, monitoring a sufficient number of cell divisions in these types of experiments poses several technical challenges since cells divide infrequently and prolonged culture periods are therefore necessary. While setting up the time-lapse imaging of RAC1/RAC2 localization in human HSPCs, we were able to find the optimal culture conditions for prolonged imaging experiments. By keeping the HSPCs on stroma and in Gartner’s medium, cells remained in good condition and the addition of cytokines increased the division rate. Moreover, presence of stromal cells allowed modeling the interactions between HSCs and their niche, which we then used for determining the symmetry of HSC division. Despite using optimal culture conditions, phototoxicity remained an issue during long imaging experiments [49]. Our experience highlights the necessity to find balance between obtaining the best images possible and preventing toxic effects from occurring in observed cells. Therefore, in the experiments aiming at determining the symmetry of cell division, we limited the number of confocal z-stacks we acquired for each timepoint to 10. Although the 3D reconstructions obtained in that way were not detailed, they were sufficiently accurate to distinguish position of the two centrosomes, while limiting the exposure time to the minimum. Interestingly, primary HSPCs seemed more resistant to phototoxicity than TF-1 cell lines that suffered significantly in two out of three experi-
ments performed. One reason for that may be the protective effect of stroma, used in the experiments with primary material but not with cell lines. Moreover, we observed more pronounced cytotoxic effects in cells expressing higher levels of GFP. Since transduction efficiency achieved in cell lines is in general higher than in primary cells, higher overexpression of GFP-tagged constructs is obtained, making transduced cells susceptible to phototoxicity. This further stresses the need to select for those cells in which levels of fluorescent-tagged protein are within the range of endogenous protein for all the imaging experiments.

It is postulated that in the normal steady-state hematopoiesis asymmetric cell divisions would be the dominant kind as they allow both maintenance of HSC pool and generation of mature cells [4]. However, in our experiments we observed a comparable numbers of symmetric and asymmetric cell division. This may be explained by the culture conditions in which the HSCs were kept as culturing HSCs in vitro requires using high concentration of cytokines for their survival and proliferation. These conditions may therefore promote symmetric cell division and therefore expansion of HSCs [7,8]. It must be therefore noted that any in vitro imaging experiment provides information on what resembles stress hematopoiesis, rather than on the normal, steady state division of HSCs. Although we were able to visualize two types of HSCs cell division of different geometrical symmetry, it reminds to be verified whether that (a)symmetry corresponds to different fate of the daughter cells. Previous studies on mouse HSCs have used Notch reporter assays to distinguish stem cells from more differentiated progenitors and in that way determine the rate of symmetric versus asymmetric cell divisions in a population [33]. However, determinants of stemness in human HSCs are not as well described as in the murine system and therefore reporter assays are not readily available. In an alternative approach, micromanipulation techniques were used to physically separate daughter cells after division and assess their multilineage potential in a colony-forming assay [35]. Employing single cell assay for monitoring the geometry of stem cell division and combining it with a functional readout of the LTC-IC potential of daughter cell would therefore be the optimal approach for studying the symmetry of HSC division.

In conclusion, although the experimental setup we established still requires functional validation, it provides a new approach to study the symmetry of HSC cell division. Moreover, while giving an insight into the symmetry of division of an individual cell, it is robust enough to provide information on the changes in cell division on a population level, as seen in the experiment using RAC inhibitor. Therefore, this system could be useful for studying the effect of other small molecule inhibitors, e.g. of Aurora Kinase B, growth factors or oncogenes on the symmetry of cell division and ultimately unraveling the mechanism regulating this process in human HSCs.

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