Mammalian target of rapamycin activity is required for expansion of CD34+ hematopoietic progenitor cells

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ABSTRACT

Background

The mammalian target of rapamycin is a conserved protein kinase known to regulate protein synthesis, cell size and proliferation. Aberrant regulation of mammalian target of rapamycin activity has been observed in hematopoietic malignancies, including acute leukemias and myelodysplastic syndromes, suggesting that correct regulation of mammalian target of rapamycin is critical for normal hematopoiesis.

Design and Methods

An *ex vivo* granulocyte differentiation system was utilized to investigate the role of mammalian target of rapamycin in the regulation of myelopoiesis.

Results

Inhibition of mammalian target of rapamycin activity, with the pharmacological inhibitor rapamycin, dramatically reduced hematopoietic progenitor expansion, without altering levels of apoptosis or maturation. Moreover, analysis of distinct hematopoietic progenitor populations revealed that rapamycin treatment inhibited the expansion potential of committed CD34+ lineage-positive progenitors, but did not affect early hematopoietic progenitors. Further examinations showed that these effects of rapamycin on progenitor expansion might involve differential regulation of protein kinase B and mammalian target of rapamycin signaling.

Conclusions

Together, these results indicate that mammalian target of rapamycin activity is essential for expansion of CD34+ hematopoietic progenitor cells during myelopoiesis. Modulation of the mammalian target of rapamycin pathway may be of benefit in the design of new therapies to control hematologic malignancies.

Key words: mammalian target of rapamycin, mTOR, rapamycin, myeloid, hematopoiesis, CD34+.

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Introduction

Hematopoiesis is a highly regulated process resulting in the formation of blood cells. It occurs predominantly in the bone marrow, and is regulated at the level of proliferation, survival and differentiation.[1] Although it is evident that these complex processes are regulated by cytokines and depend on the correct function of the bone marrow microenvironment, the cytokine-mediated intracellular signal transduction pathways regulating hematopoietic stem cell function are thus far incompletely understood. The phosphatidylinositol 3-kinase (PI3K) signal transduction pathway has been demonstrated to play an important role in survival and proliferation of a plethora of cell types.[2] It has, for example, been demonstrated that PI3K plays an essential role in regulation of hematopoietic progenitor survival and expansion during myelopoiesis.[3] Furthermore, impaired regulation of PI3K and its downstream effector protein kinase B (PKB/c-akt) has been implicated in carcinogenesis. In particular, activation of the PI3K/PKB signaling module is observed in a variety of hematopoietic malignancies, including acute leukemias and high-risk myelodysplastic syndromes.[4-6] One of the downstream targets of PI3K/PKB is the mammalian target of rapamycin (mTOR), which is a conserved serine/threonine kinase that has been demonstrated to regulate cell size and cell cycle progression in various cell types.[7] Activation of mTOR is mediated by PKB through direct phosphorylation.[8] In addition, PKB inhibitor phosphorylates the GTPase activating protein tuberous sclerosis protein 2 (TSC2), which results in accumulation of GTP-bound Rheb and subsequent activation of mTOR.[9] mTOR exists in two distinct multiprotein complexes: mTORC1 and mTORC2. mTORC1 consists of the regulatory-associated protein of mTOR (Raptor) adaptor protein, mLST8 and mTOR.

The mammalian translational initiation machinery governs the recruitment of ribosomes to mRNA to commence the production of protein synthesis. This machinery consists of various eukaryotic initiation factors (eIF) that tightly regulate protein synthesis. Importantly, activation of mTORC1 positively stimulates mRNA translation via its downstream substrates p70S6 kinase and 4E-BP1/eIF4E.[10,11] Phosphorylation of 4E-BP1 by mTORC1 results in its dissociation from eIF4E, promoting assembly of the eIF4F complex, a protein complex that mediates recruitment of ribosomes to mRNA.[12] The mTORC2 complex consists of mTOR, rapamycin-insensitive companion of mTOR (Rictor) and mLST8,[13] and is involved in the activation of PKB. In contrast to mTORC1, mTORC2 activity is not abrogated by treatment with rapamycin, a potent pharmacological inhibitor of mTOR activity.[14]

Analysis of mTOR-deficient mice has revealed that mTOR is essential for embryonic development. mTOR-deficient mice die *in utero* due to impaired cell proliferation.[15] Moreover, exposure of mouse embryos to rapamycin results in a block in cell proliferation, indicating that the rapamycin-sensitive mTORC1 complex plays an essential role in the regulation of developmental processes.[16] Rapamycin has also been demonstrated to regulate the differentiation and proliferation of various adult cell types, including human endothelial progenitors, epithelial cells, chondrocytes, osteoblasts and myoblasts.[17-24] Although these data demonstrate the importance of mTOR signaling in the regulation of multiple cellular processes, a role for mTOR in the regulation of myelopoiesis remains to be investigated. In this study, we investigated the role of the mTOR signal transduction pathway in the regulation of myelopoiesis utilizing a human *ex vivo* granulocyte differentiation system.

Design and Methods

Isolation and culture of human CD34$^+$ cells

Mononuclear cells were isolated from umbilical cord blood by density centrifugation over a Ficoll-paque solution (density 1.077 g/mL). MACS immunomagnetic cell separation (Miltenyi Biotech, Auburn, CA, USA) using a hapten-conjugated antibody against CD34, which was coupled to beads, was used to isolate CD34$^+$ cells. CD34$^+$ cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Paisley, UK) supplemented with 9% fetal calf serum (FCS) (Hyclone, Logan, UT, USA), 50 μM β-mercaptoethanol, 10 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM glutamine at a density of 0.3×10^6 cells/mL. Cells were differentiated towards eosinophils upon addition of stem cell factor (SCF) (50 ng/mL), FLT-3 ligand (FTL-3L) (50 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 nmol/L), interleukin (IL)-3 (0.1 nmol/L), and IL-5 (0.1 nmol/L). Every 3 days, cells were counted and fresh medium was added to a density of 0.5×10^6 cells/mL. After 3 days of differentiation, only IL-3 and IL-5 were added to the cells. Neutrophil differentiation was induced upon addition of SCF (50 ng/mL), FLT-3L (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L) and granulocyte colony-stimulating factor (G-CSF) (30 ng/mL). After 6 days of culture only G-CSF was added to the cells. Twenty ng/mL of rapamycin (Biomol International LP, Hamburg, Germany) used to inhibit mTOR activity, was added freshly to the cells every 3 or 4 days. Cord blood samples were collected from healthy donors after informed consent had been given according to the Declaration of Helsinki. Protocols were approved by the local ethics committee of the University Medical Center in Utrecht.

Flow cytometric analysis of myeloid progenitors

Hematopoietic progenitors were isolated as described by Manz *et al.*[25] In short, CD34$^+$ cells were isolated as described above and cultured for 2 days in the presence of SCF, FLT-3L, GM-CSF, IL-3 and G-CSF in the absence or presence of rapamycin. Cells were subsequently washed and resuspended in PBS/5% FCS and incubated for 30 min on ice with a mixture of antibodies (Becton Dickinson, Alphen a/d Rijn, The Netherlands). Lineage markers included CD2, CD3, CD4, CD7, CD8, CD14, CD19, CD20 and CD235a. Myeloid progenitors are negative for these lineage markers. The lineage negative (Lin$^-$), CD34$^+$, and CD38$^-$ populations consist of hematopoietic stem cells. Lin$^-$, CD34$^+$, CD38$^+$, CD123$^+$, and CD45RA$^-$ cells are common myeloid progenitors, whereas Lin$^-$, CD34$^+$, CD38$^+$, CD123$^+$, and CD45RA$^+$ cells are granulocyte-macrophage progenitors and Lin$^-$, CD34$^+$, CD38$^+$, CD123$^-$, and

CD45RA$^-$ cells are megakaryocyte-erythrocyte progenitors. Different hematopoietic stem cells, common myeloid progenitor and granulocyte-macrophage progenitor populations were analyzed using a FACS ARIA (from Becton Dickinson). Appropriate isotype-matched, control antibody staining was used to determine the level of background staining.

Measurement of apoptosis

Apoptotic cells were measured by staining with annexin V (Alexis, Leiden, The Netherlands) according to the manufacturer's protocol. Necrotic cells were visualized in the same assay by staining with propidium iodide.

Colony-forming unit assay

Freshly isolated CD34$^+$ cells were used in colony-forming unit (CFU) assays. Cells were plated in IMDM supplemented with 35.3% FCS, 44.4% methylcellulose-based medium called Methocult (StemCell Technologies, Vancouver, Canada), 11.1 μmol/L of β-mercaptoethanol, 2.2 units/mL of penicillin, 2.2 μg/mL of streptomycin, and 0.44 mmol/L of glutamine at a density of 500 cells/well. CFU assays were done in the presence of SCF (50 ng/mL), FLT-3L (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L), and G-CSF (0.2 nmol/L). Colonies were scored after 7 days of culture.

Histochemical staining of hematopoietic cells

May-Grünwald Giemsa staining was used to analyze myeloid differentiation. Cytospins were prepared from 5×10^4 differentiating granulocytes and were fixed in methanol for 3 min. After fixation, cytospins were stained in a 50% eosin methylene blue solution according to May-Grünwald (Sigma-Aldrich GmbH, Seelze, Germany) for 20 min, rinsed in water for 5 seconds, and the nuclei were counterstained with 10% Giemsa solution (Merck kGaA, Darmstadt, Germany) for 15 min. During eosinophil differentiation, cells could be characterized as differentiating from myeloblasts towards pro-myelocyte type I, pro-myelocyte type II, myelocyte, meta-myelocyte, and finally mature eosinophils with segmented nuclei. These stages can be distinguished by the size of the cells, ratio of cytoplasm versus nucleus present, presence of azurophilic granules, appearance of eosinophilic granules and the shape of the nuclei. Differentiated eosinophils were characterized as cells belonging to the stages of myelocyte, metamyelocyte and mature eosinophils. Neutrophil differentiation can also be characterized by distinct stages from myeloblast, promyelocyte I, promyelocyte II, myelocyte, and metamyelocytes towards neutrophils with banded or segmented nuclei. Differentiated neutrophils were characterized as cells containing either banded or segmented nuclei. Micrographs were acquired with an Axiostar plus microscope (Carl Zeiss, Sliedrecht, The Netherlands) fitted with a 100x/1.3 NA EC Plan Neofluor oil objective using Immersol 518F oil (Carl Zeiss), a Canon Powershot G5 camera (Canon Nederland, Hoofddorp, The Netherlands), and Canon Zoombrowser EX image acquisition software. Photoshop CS2 was used for image processing (Adobe Systems Benelux, Amsterdam, The Netherlands). A minimum of 100 cells per cytospin were counted in two to three randomly selected microscopy fields.

Western blot analysis

Western blot analysis was performed using standard techniques. In brief, differentiating granulocytes were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 μg/μL bromophenol blue, and 35 mM β-mercaptoethanol) and boiled for 5 min. Equal amounts of total lysate were analyzed by 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (Tris buffered saline/Tween-20) containing 5% low-fat milk for 1 h before being incubating with antibodies against PKB (Cell Signaling Technology, Beverly, MA, USA) or tubulin (Sigma) overnight at 4 °C in the same buffer. Before incubation with an antibody against phosphorylated PKB, phosphorylated S6, phosphorylated p70S6kinase or phosphorylated eIF4B (all obtained from Cell Signaling Technology, Beverly, MA, USA) for 16 h at 4°C, blots were incubated for 1 h in blocking buffer containing 5% bovine serum albumin (BSA). Blots were subsequently incubated with peroxidase-conjugated secondary antibodies for 1 h. Enhanced chemical luminescence was used as a detection method according to the manufacturer's protocol (Amersham Pharmacia, Amersham, UK).

^{3}H-thymidine incorporation assays

Eighty thousand CD34$^+$ cells, resuspended in normal culture medium (see *Isolation and culture of human CD34$^+$ cells*), were incubated with 1 μCi/mL ^{3}H-thymidine for 72 h at days 0, 7 and 10. The amount of ^{3}H-thymidine incorporated was analyzed after 3 days of culture.

Single-cell proliferation assay

For single-cell proliferation assays, freshly isolated CD34$^+$ cells were seeded in 60-well plates (Nunc, Kamstrup, Denmark) by limiting dilution at a density of 1 cell per well in 20 μL of normal culture medium containing SCF, FLT-3L, GM-CSF, IL-3, and G-CSF in the presence or absence of 20 ng/mL rapamycin. Per condition, 120 wells were scored for both colony size and number of colonies after 7 days of incubation.

Proliferation assays

For proliferation assays, freshly isolated CD34$^+$ cells were first resuspended in normal culture medium containing 2 μM of LavaCell™ (Active Motif, Rixensart, Belgium), a commercially available non-toxic fluorescent cell stain, and stained for 15 min at 37°C. Subsequently cells were washed twice and resuspended in normal culture medium containing SCF, FLT-3L, GM-CSF, IL-3 and G-CSF in the absence or presence of rapamycin. Proliferation was evaluated as the decrease of fluorescent LavaCell™-probe per cell, as measured by flow cytometric analysis.

Statistics

An independent sample *t* test was performed to compare the differences in proliferation, differentiation, and annexin-positive cells between the control cells and rapamycin-treated cells. A p value of 0.05 or less was considered statistically significant.

Results

Inhibition of rapamycin-sensitive mTOR signaling decreases cellular expansion of granulocyte progenitors during myelopoiesis

In order to determine whether mTOR activity plays a critical role in the regulation of myelopoiesis, an *ex vivo* differentiation system was utilized. Human CD34$^+$ hematopoietic progenitor cells, isolated from umbilical cord blood, were cultured in the presence of either G-CSF or IL-3 and IL-5 to induce neutrophil and eosinophil differentiation, respectively. Cells were cultured in either the absence or presence of rapamycin, a specific pharmacological inhibitor of mTOR, and differences in expansion were determined both by analysis of the number trypan blue-negative cells as well as by performing ^{3}H-thymidine incorporation assays. Rapamycin reduced expansion during neutrophil differentiation (Figure 1A, 1B). To determine whether rapamycin-mediated inhibition of expansion was due to enhanced levels of apoptosis, the percentage of annexin-V positive cells was analyzed. Inhibition of mTOR activity did not significantly affect the levels of annexin-V positive cells during neutrophil differentiation (Figure 1C). As for neutrophil differentiation, inhibition of mTOR reduced progenitor expansion during eosinophil development (Figure 1D), without altering progenitor survival (Figure 1E). These results demonstrate that inhibition of mTOR decreases cellular expansion of neutrophil and eosinophil progenitors during differentiation.

mTOR signaling is not essential for myeloid differentiation of hematopoietic progenitors

In order to investigate the clonogenicity of distinct hematopoietic progenitor populations, CFU assays were performed, in either the absence or presence of rapamycin, and colony formation was analyzed after 7 days. Inhibition of mTOR did not affect the granulocyte-macrophage colony formation capacity of hematopoietic progenitor cells (Figure 2A). To determine whether mTOR plays a role in regulating terminal maturation, CD34$^+$ progenitor cells were differentiated towards neutrophils or eosinophils for 17 days in either the presence or absence of rapamycin. After 10, 14 and 17 days of culture, cytospins were prepared and the morphology of the differentiating granulocytes was analyzed as described in the Design and Methods section. Treatment of hematopoietic progenitor cells with rapamycin did not significantly alter the percentages of mature neutrophils or eosinophils (Figure 2B, 2D). However, since rapamycin inhibited progenitor expansion during both neutrophil and eosinophil development, the absolute numbers of mature neutrophils and eosinophils were also reduced (Figure 2C, 2E). Together these results show that rapamycin-sensitive mTOR signaling is not involved in the regulation of early lineage development or terminal maturation.

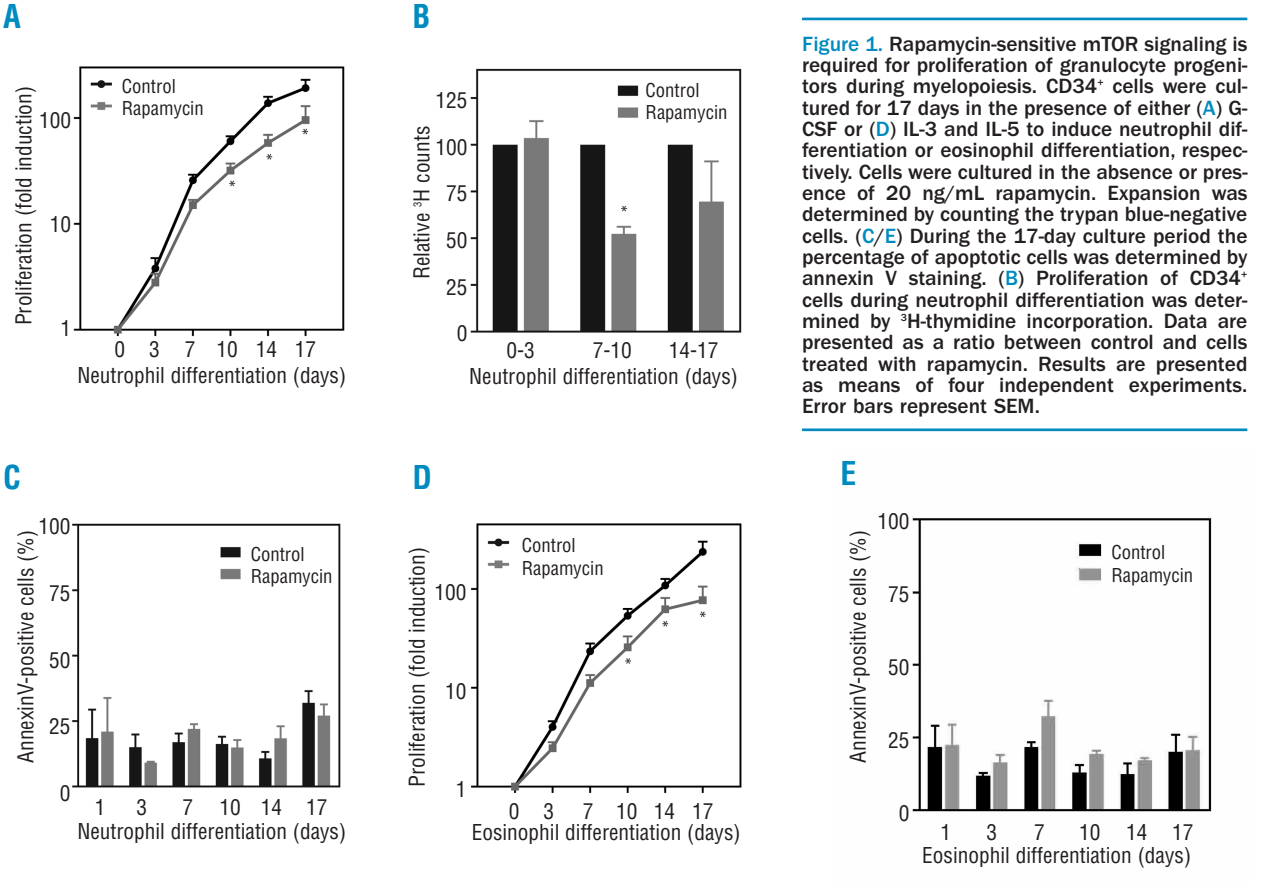
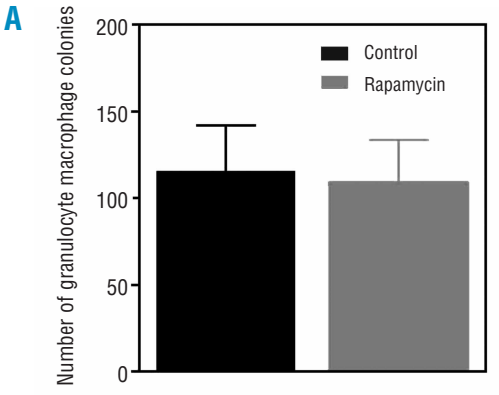
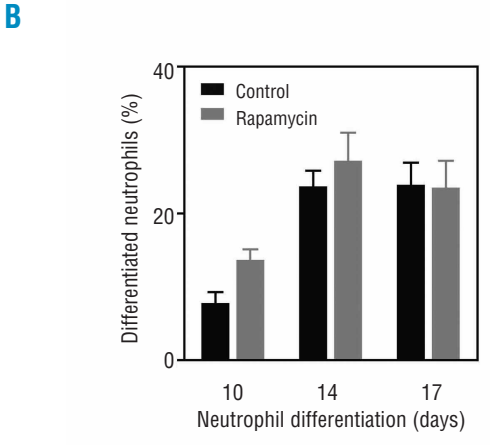
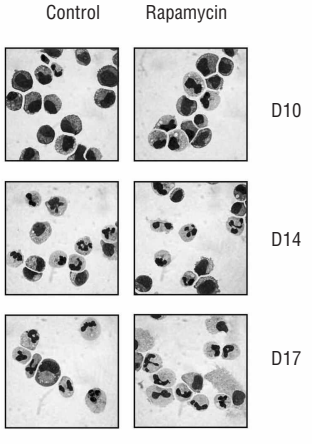
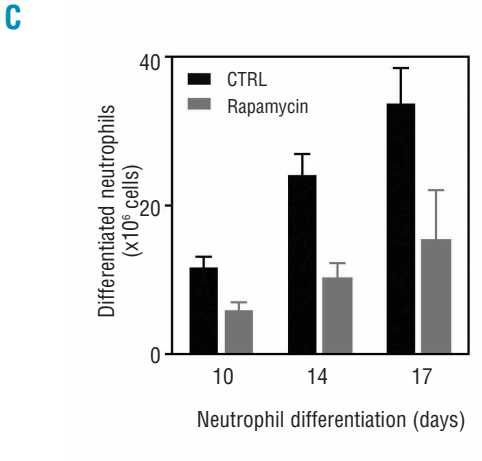


Figure 1. Rapamycin-sensitive mTOR signaling is required for proliferation of granulocyte progenitors during myelopoiesis. CD34$^+$ cells were cultured for 17 days in the presence of either (A) G-CSF or (D) IL-3 and IL-5 to induce neutrophil differentiation or eosinophil differentiation, respectively. Cells were cultured in the absence or presence of 20 ng/mL rapamycin. Expansion was determined by counting the trypan blue-negative cells. (C/E) During the 17-day culture period the percentage of apoptotic cells was determined by annexin V staining. (B) Proliferation of CD34$^+$ cells during neutrophil differentiation was determined by ^{3}H-thymidine incorporation. Data are presented as a ratio between control and cells treated with rapamycin. Results are presented as means of four independent experiments. Error bars represent SEM.



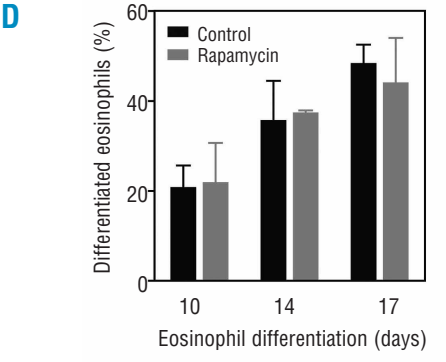


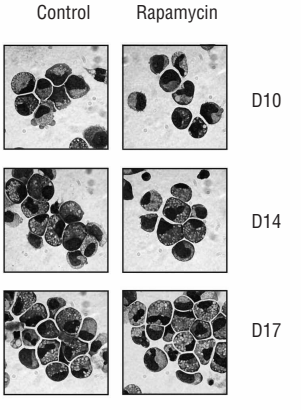




Rapamycin-sensitive mTOR is not implicated in the regulation of cell-size of myeloid progenitors

mTOR activity has been implicated in the regulation of cell size in a variety of cell lineages.[26] In order to investigate whether mTOR is also involved in regulation of the cell size of hematopoietic progenitors, human CD34+ progenitors were cultured for 2 days in the presence of SCF, FLT-





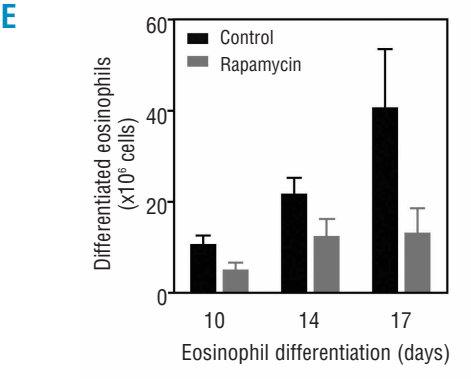


Figure 2. mTOR signaling is not essential for myeloid differentiation of hematopoietic progenitors. (A) Freshly isolated CD34+ progenitor cells were plated in CFU assays, in the presence or absence of 20 ng/mL rapamycin, and colony formation was analyzed after 7 days. CD34+ cells were cultured for 17 days in the presence of (B/C) G-CSF or (D/E) IL-5 and IL-3 to induce neutrophil differentiation or eosinophil differentiation, respectively. After 10, 14 and 17 days of differentiation, cytospins were prepared to analyze the morphology of the differentiating granulocytes. Data are expressed as either (B/D) the percentage of differentiated cells or (C/E) as absolute cell numbers. Results are presented as means of four independent experiments. Error bars represent SEM.

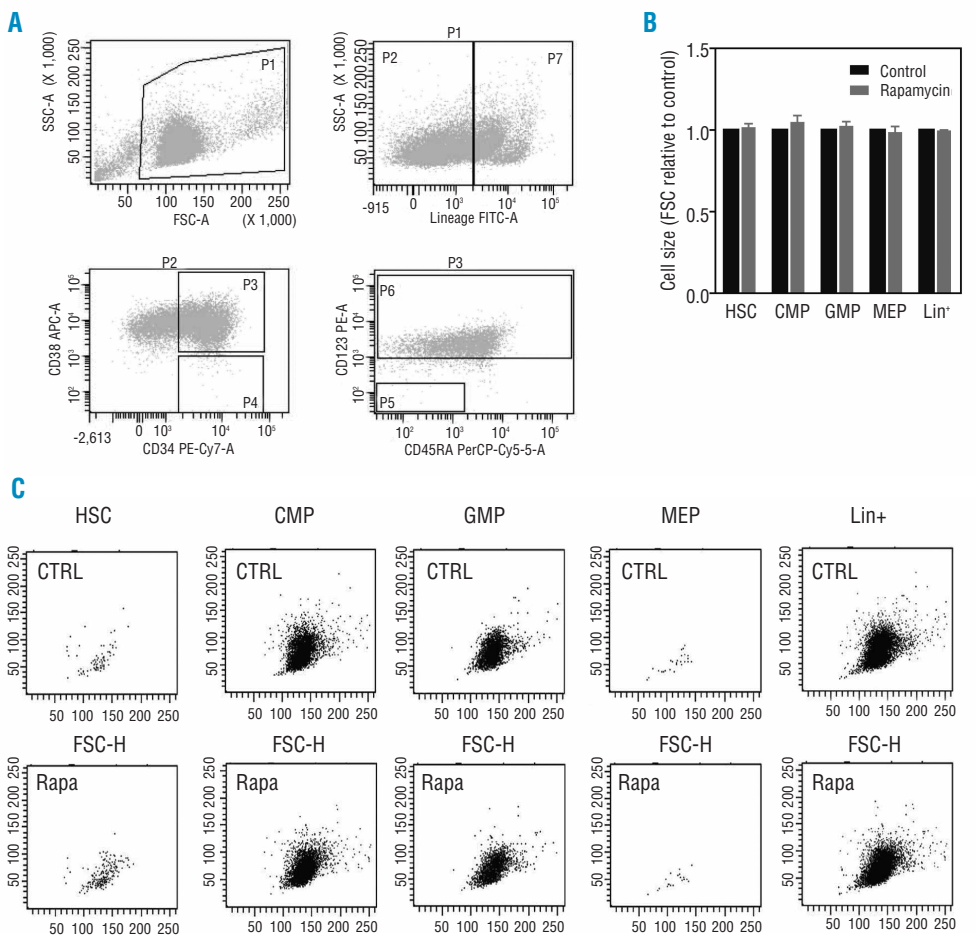


Figure 3. Rapamycin-sensitive mTOR is not implicated in the regulation of cell-size of myeloid progenitors. (A) CD34+ cells were cultured for 2 days in the presence of SCF, FLT-3 ligand, GM-CSF, IL-3 and G-CSF, in either the absence or presence of rapamycin and different hematopoietic progenitor populations, including hematopoietic stem cells (HSC), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), megakaryocyte-erythroid progenitors (MEP), and Lin+ cells were analyzed by multilineage flow cytometry. Analysis of the distinct progenitor populations was performed as depicted in panel A. (B) Differences in cell size were determined by analysis of the value of the FSC-H in all individual progenitor populations. Data are expressed as a ratio between controls and cells treated with rapamycin. Results are presented as means of four independent experiments. Error bars represent SEM. (C) The FSC-H plots of all individual hematopoietic progenitor populations are depicted. One representative experiment is shown.

3L, GM-CSF, IL-3 and G-CSF in either the absence or presence of rapamycin and myeloid progenitors were analyzed by multilineage flow cytometry. Different hematopoietic progenitor populations, including hematopoietic stem cells and common myeloid, granulocyte-macrophage, megakaryocyte-erythrocyte progenitors (CMP, GMP, MEP) and Lineage positive (Lin+) cells can be distinguished by a combination of extracellular lineage markers as described in the Design and Methods section (Figure 3A). Differences in cell size were determined by analysis of the forward scatter (FSC-H). Analysis of distinct myeloid progenitor populations revealed that inhibition of mTOR by rapamycin did not result in a significant change in overall cell-size (Figure 3B, 3C). These results suggest that rapamycin-sensitive mTOR is not implicated in the regulation of cell-size of myeloid progenitors.

mTOR activity regulates expansion of hematopoietic progenitor cells during myeloid differentiation in a stage-specific manner

To further investigate the role of mTOR in regulating expansion during myelopoiesis, single-cell proliferation assays were performed to evaluate the expansion capacity of individual hematopoietic progenitors. Freshly isolated CD34+ cells were plated singly in culture medium containing G-CSF in the presence or absence of rapamycin. After 7 days, wells with colonies were scored (Figure 4). Treatment with rapamycin resulted in a decreased number of colonies, which were also reduced in size, showing that mTOR is involved in the regulation of expansion of myeloid progenitors.

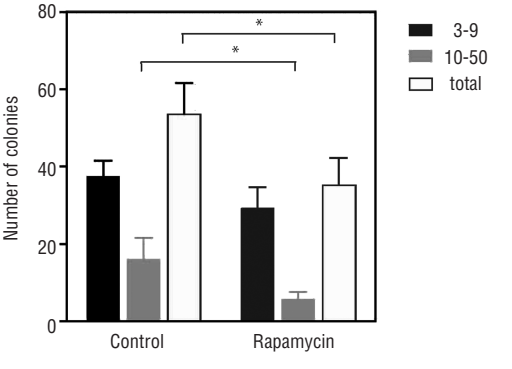


Figure 4. mTOR activity regulates expansion of myeloid progenitor cells. Freshly isolated CD34+ cells were seeded in 60-well plates at a density of 1 cell per well (120 wells in total) in normal culture medium containing G-CSF in the presence or absence of 20 ng/mL rapamycin. After 7 days, wells with colonies were scored. Results are presented as means of four independent experiments. Error bars represent SEM.

To further dissect how the mTOR pathway regulates progenitor expansion, CD34+ progenitor cells were differentiated towards neutrophils in the presence or absence of rapamycin. After 3 and 7 days of culture, cells were plated in CFU assays, and colony formation was analyzed after 7 days of culture. Interestingly, granulocyte-macrophage colony formation of CD34+ cells that had been cultured in the presence of rapamycin for 7 days was significantly increased compared to that of control CD34+ cells (Figure 5A). These data suggest that rapamycin selectively inhibits expansion and thereby preserves the clonogenic capacity of a specific population of progenitors.

To define what cell population is the target of rapamycin, freshly isolated CD34+ cells were stained with LavaCell™, a fluorescent dye, and cultured in either the presence or absence of rapamycin in the presence of SCF, FLT-3L, GM-CSF, IL-3 and G-CSF. Subsequently, after 3 days of culture, myeloid progenitor populations were analyzed by flow cytometry and the level of proliferation was visualized by the decrease in the mean fluorescent intensity of LavaCell™. Inhibition of mTOR activity significantly decreased proliferation of Lin+ CD34+ hematopoietic cells (Figure 5B), while expansion of hematopoietic stem cells and common myeloid and granulocyte-macropage progenitor populations (Figure 5D, 5E) and the more differentiated Lin+ progeny that no longer expresses the CD34 (Figure 5C) was not altered upon treatment with rapamycin. Together, these observations show that rapamycin selectively inhibits expansion of the most committed CD34+ progenitors, indicating that rapamycin-sensitive mTOR activity regulates proliferation of hematopoietic progenitor cells during myeloid differentiation in a stage-specific manner.

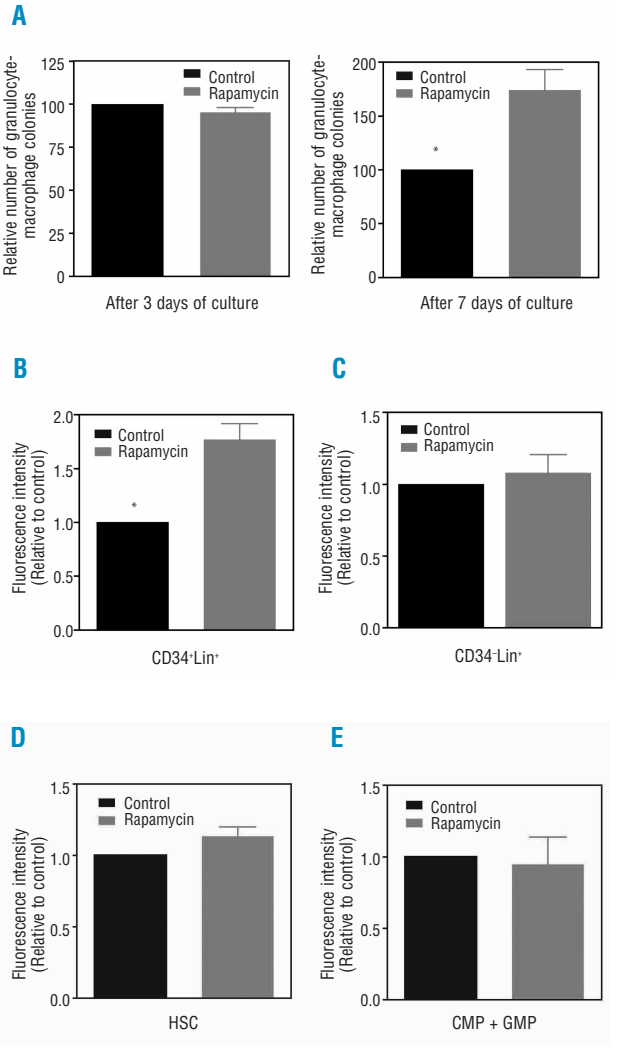


Figure 5. mTOR activity regulates proliferation of hematopoietic progenitor cells during myeloid differentiation in a stage-specific manner. (A) CD34+ progenitor cells were differentiated towards neutrophils in the presence or absence of rapamycin. After 3 and 7 days of culture, cells were plated in CFU assays, and colony formation was analyzed after another 7 days of culture. (B-E) Freshly isolated CD34+ cells were stained with LavaCell™, a fluorescent dye, and cultured in either the presence or absence of rapamycin in the presence of SCF, FLT-3 ligand, GM-CSF, IL-3 and G-CSF for 3 days. Different myeloid progenitor populations, including (B) CD34+Lin+, (C) CD34-Lin+, (D) HSC, and (E) CMP/GMP cells were analyzed by flow cytometry. Proliferation was visualized by the decrease in the mean fluorescent intensity per cell of LavaCell™. Results are presented as means of four independent experiments. Error bars represent SEM.

Inhibition of mTOR activity by rapamycin differentially regulates protein kinase B and mTOR signaling during granulopoiesis

To investigate the effect of rapamycin on mTOR signal-

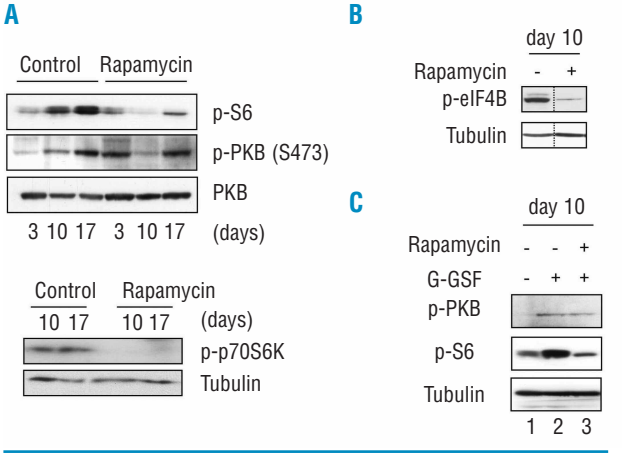


Figure 6. Inhibition of mTOR activity by rapamycin differentially regulates PKB and mTOR signaling during granulopoiesis. (A) CD34+ cells were cultured in the presence of G-CSF to induce neutrophil differentiation, in either the presence or absence of rapamycin. After 3, 10 and 17 days of culture, protein lysates were made and western blot analysis was performed using an antibody against phosphorylated p70S6K, phosphorylated S6, phosphorylated PKB or total PKB as a control for equal loading. (B) CD34+ cells were cultured in the presence of G-CSF to induce neutrophil differentiation, in either the presence or absence of rapamycin. After 10 days of culture, protein lysates were made and western blot analysis was performed using an antibody against phosphorylated eIF4B or tubulin as a control for equal loading. (C) CD34+ cells were cultured in the presence of G-CSF to induce neutrophil differentiation. After 10 days of culture, cells were left untreated (lanes 1, 2) or treated with rapamycin (lane 3) for 3 h before stimulation with G-CSF (lanes 2, 3) for 15 min. Protein lysates were prepared and western blot analysis was performed with an antibody against phosphorylated PKB, phosphorylated S6, and as a control for equal loading an antibody against tubulin. Similar results were obtained in three independent experiments.

ing during granulopoiesis, CD34$^+$ cells were cultured in the presence of G-CSF, to induce neutrophil differentiation, in the presence or absence of rapamycin. After 3, 10 and 17 days of culture, protein lysates were made and western blot analysis was performed using an antibody against phosphorylated p70S6K and phosphorylated S6, a direct substrate of the mTOR substrate p70S6K (Figure 6A). S6 phosphorylation levels were low early during neutrophil differentiation, but increased after 10 to 17 days of differentiation. However, phosporylation of both p70SK and S6 was inhibited or completely blocked in cells cultured in the presence of rapamycin for 10 or 17 days, respectively. However, no effect of rapamycin on S6 phosphorylation could be detected after 3 days of culture. In addition, rapamycin treatment resulted in a block in PKB phosphorylation at day 10, while phosphorylation of PKB at day 3 of culture in the presence of rapamycin was induced compared to that in control cells and was unaffected at day 17. In addition, rapamycin treatment resulted in a decrease in phosphorylation of eIF4B, a downstream target of PKB,[27] at day 10 (Figure 6B). In contrast, G-CSF-induced PKB phosphorylation was not inhibited upon short exposure to rapamycin at the same time point (Figure 6C). These observations suggest that the effects of rapamycin might be due to differential regulation of PKB and mTOR signaling.

Discussion

Although mTOR signaling has been demonstrated to play an important role in a plethora of cellular processes, a role in the regulation of myelopoiesis remains relatively unexplored. In this study, we investigated the role of mTOR in the regulation of myelopoiesis utilizing a human *ex vivo* granulocyte differentiation system. Our results show that rapamycin-sensitive mTOR signaling plays an important role in the regulation of expansion of hematopoietic progenitors during myelopoiesis in a stage-specific manner.

mTOR is known to be a regulator of cell cycle progression and proliferation.[28] In B and T lymphocytes, rapamycin induces G_1-phase arrest and can, therefore, be used as a potent anti-proliferative drug.[29] In most other cell types, however, rapamycin reduces the proliferation rate by delaying cell cycle progression. For example, proliferation of human endothelial progenitors, epithelial cells, osteoblasts and myoblasts is inhibited by rapamycin.[19-22] Our data demonstrate that mTOR activity is also required for the proliferation of hematopoietic progenitors during myelopoiesis. Progenitor expansion in the presence of rapamycin was significantly reduced, as measured by ^{3}H-thymidine incorporation experiments (Figure 1B) and single cell proliferation assays (Figure 4). Cell cycle analysis of myeloid progenitors showed that rapamycin did not significantly alter cell cycle distribution (*data not shown*), suggesting that the observed inhibition of proliferation was due to a delay in cell cycle progression rather than an arrest in G_0/G_1 phase. mTOR mediates cell growth at the translational level through phosphorylation of p70S6K and 4E-BP1, two essential regulators of ribosome biogenesis and translation initiation. Ectopic expression of p70S6K and eIF4E in quiescent U2OS osteosarcoma cells, which are stimulated with serum to enter the G_1 phase, accelerates entry into the S phase, whereas reduction of p70S6K expression with RNAi or expression of a dominant-negative 4E-BP1 mutant inhibits the rate of S phase entry.[30] In addition, ectopic expression of eIF4E or rapamycin-resistant mutants of p70S6K partially rescues the rapamycin-induced delay in cell cycle progression, indicating that p70S6K and eIF4E are important mediators of mTOR-dependent cell division.[30] It has been demonstrated that mTOR regulates the translation of proteins involved in G_1/S transition during cell cycle progression, including retinoblastoma protein, cell-cycle inhibitors of the Cip/Kip family p21, p27, and cyclin D or E.[31-36] Thus, it is likely that mTOR also regulates hematopoietic progenitor expansion at the translational level by regulating translation of cell-cycle modulating proteins.

Recently, Fingar *et al*. demonstrated that rapamycin not only delays proliferation, but is also involved in regulation of cell size. Although rapamycin-treated osteosarcoma cells are significantly smaller in size, proliferation is not completely blocked,[37] indicating that cell division does not require a fixed size. It has been suggested that both cell size and proliferation can be regulated by the same mTOR-dependent downstream effectors.[38] A reduction of, for example, S6K1 expression in osteosarcoma cells has been demonstrated both to delay proliferation and to reduce cell size.[30] However, a reduction in energy sources results in an inhibition of cell size in yeast, but does not block cell division.[39] In addition, the cell size of rat neuronal cells varies depending on the level of extracellular growth factors, whereas proliferation is unaltered.[40] Moreover, deletion of p70S6K in mouse myoblasts mimics the inhibitory effect of rapamycin on cell size but not on proliferation.[22] Our data demonstrate that mTOR activity is essential for proliferation of myeloid progenitors, whereas cell-size appears not to be regulated by mTOR (Figure 3), indicating that in certain cell lineages cell cycle and cell size are controlled by multiple independent signal transduction pathways.

Analysis of different progenitor cells revealed that rapamycin differentially affects the diverse populations. Inhibition of mTOR activity significantly decreased proliferation of CD34$^+$ Lin$^+$ hematopoietic cells, whereas proliferation of CD34$^-$ Lin$^+$, hematopoietic stem cells, common myeloid progenitor and granulocyte-macrophage progenitor populations was unaffected (Figure 5B-E). These observations suggest that the response of hematopoietic progenitors to rapamycin is dependent on the stage of differentiation. It is likely that the observed resistance to rapamycin of the early hematopoietic progenitors is due to these cells being less dependent on the mTOR pathway compared to the more committed CD34$^+$Lin$^+$ hematopoietic cells. Correspondingly, a recent analysis of TSC1-deficient mice revealed that constitutive mTORC1 activation induces severe multilineage defects including anemia and progenitor expansion.[41] These data, combined with our results, suggest that correct regulation of mTOR activity is critical for optimal progenitor expansion. Zeiser *et al*. recently showed that regulatory T cells and conventional T cells also display differential expansion kinetics upon exposure to rapamycin. Expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative regulator of the PI3K/PKB/mTOR pathway, was found to remain high

in regulatory T cells but not in conventional T cells during stimulation, which may explain the difference in susceptibility to rapamycin between these T-cell populations.[42] Furthermore, our results suggest that the effect of rapamycin on progenitor expansion during granulopoiesis might be due to differential regulation of PKB and mTOR activity (Figure 6). It has, for example, been demonstrated that modulation of PKB activity regulates the sensitivity of glioblastoma cells to the mTOR inhibitors rapamycin and CCI-779 by expression of cyclin D1 and c-myc, two proteins found to be essential for cell cycle transit.[43] Furthermore, we have previously demonstrated that PKB activity is indeed critical for expansion of hematopoietic progenitors during myelopoiesis.[3] Although regulation of PKB activity is believed to be mediated by mTORC2, which is insensitive to rapamycin, Sarbassov *et al*. demonstrated that treatment with rapamycin can result in either increased or decreased levels of PKB phosphorylation, depending on the cell type.[44] A possible explanation for this could be that, although rapamycin cannot inhibit mTORC2, it can associate with free mTOR, thereby preventing the association with Rictor and assembly of the mTORC2 complex.[45] Alternatively, differential expression of PTEN or other components of the PI3K/PKB/mTOR pathway, such as Raptor, may explain the difference in susceptibility of distinct hematopoietic progenitors to rapamycin.

Constitutive activation of PI3K and its downstream effectors PKB and mTOR has also been implicated in the pathogenesis of a variety of hematopoietic malignancies, including acute myeloid leukemia.[46,47] It has been demonstrated that tumors displaying enhanced expression or activation of the PI3K signaling module are highly sensitive to rapamycin and its analogs CCI-779 and RAD001.[48,49] Low doses of rapamycin were found to inhibit colony formation of acute myeloid leukemia progenitors, suggesting that inhibition of the mTOR pathway could be of clinical interest in this leukemia.[50,51] However, recent studies also demonstrated that in acute myeloid leukemia samples with constitutive PI3K-PKB activation, rapamycin treatment resulted in increased PKB phosphorylation, suggesting the need for dual inhibition of both the mTORC and the PI3K-PKB pathway in treatment of this disease.[52,53]

Our results demonstrate that mTOR selectively regulates proliferation of $CD34^+Lin^+$ hematopoietic progenitors, while expansion of hematopoietic stem cells and common myeloid/granulocyte macrophage progenitor populations is not affected. In addition, mTOR signaling appears not to be essential for differentiation of myeloid progenitors. Taken together, our data suggest that modulation of the mTOR pathway could play an important role in future therapies to control hematologic malignancies.

Authorship and Disclosures

CRG performed experiments, made the figures, analyzed results, designed the research and wrote the paper; MB performed experiments, made the figures, analyzed results, designed the research and wrote the paper; FJZ designed the research; EV analyzed results and designed the research; PJC analyzed results, designed the research and wrote the paper.

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