Biochemical Pharmacology, Vol. 51, pp. 1181–1185, 1996.


ELSEVIER

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00044-5

# Inhibition of Proliferation but not Erythroid Differentiation of J2E Cells by Rapamycin

*Robert Jaster,*‡ Thomas Bittorf,* S. Peter Klinken† and Josef Brock**

*INSTITUTE OF MEDICAL BIOCHEMISTRY, MEDICAL FACULTY OF THE UNIVERSITY OF ROSTOCK, SCHILLINGALLEE 70, 18057 ROSTOCK, GERMANY, AND †ROYAL PERTH HOSPITAL, GPO BOX X2213, PERTH WESTERN AUSTRALIA 6001

**ABSTRACT.** During erythropoiesis, replication and maturation are tightly coupled processes. Here, we show that the immunosuppressant rapamycin inhibited basal- as well as erythropoietin-stimulated proliferation of the erythroid cell line J2E. In addition, it enhanced the antiproliferative effect of sodium butyrate. Although rapamycin suppressed erythroid cell division, it did not affect terminal differentiation induced by erythropoietin or sodium butyrate. The proliferative status of J2E cells correlated well with the activity of the ribosomal S6 kinase $p70^{S6k}$, an enzyme effectively blocked by rapamycin. It was concluded from this study that erythroid maturation proceeded normally despite the rapamycin-induced inhibition of mitosis and of $p70^{S6k}$ activity. These data provide further evidence that separate signalling pathways for proliferation and differentiation exist in erythroid cells. BIOCHEM PHARMACOL 51;9:1181–1185, 1996.

**KEY WORDS.** rapamycin; erythropoietin; proliferation; differentiation; 70 kDa S6 kinase

Production of red blood cells is mainly regulated by EPO,§ a hematopoietic growth factor promoting survival, proliferation, and terminal differentiation of immature erythroid progenitor cells [1, 2]. EPO action is mediated through binding of the hormone to a specific cell surface receptor, a member of the cytokine receptor superfamily [3]. The signalling cascades triggered by the stimulated receptor still require complete elucidation (reviewed in [4]; in particular, the relationships between putative signalling pathways involved in the induction of proliferation and differentiation remain to be deciphered.

The erythroid cell line J2E, generated by the transformation of immature progenitors with the *raf/myc*-containing J2 virus and immortalized at the basophilic erythroblast stage [5], provides a useful model for studying terminal differentiation. In response to EPO stimulation, the cells undergo enhanced proliferation and maturation, whereas the chemical inducer sodium butyrate uncouples these processes by stimulating terminal differentiation while inhibiting replication [6]. In the past, studies on the EPO-dependent regulation of erythropoiesis have been impaired by the lack of suitable EPO-responsive cell lines; primary cultures of bone marrow, spleen, or murine fetal liver cells consist of heterogeneous cell populations and provide limited numbers of cells. The major advantage of the erythroid cell line J2E, used in this study, is that the cells show virtually the same biological responses to EPO stimulation as normal erythroid progenitors (i.e. elevated cell division, hemoglobin synthesis, and morphological maturation).

Rapamycin is an immunosuppressive drug blocking T-cell activation and growth by interfering with IL-2 or IL-4 induced signals [7, 8]. Rapamycin-treated T lymphocytes are arrested in the G1 phase of the cell cycle [9, 10]. However, the drug has been shown to suppress the proliferation of other mammalian cells, including endothelial cells, fibroblasts following FGF-beta-stimulation [11], and primary bone marrow cells after exposure to hematopoietic growth factors such as GM-CSF and IL-3 [12]. The mode of action of rapamycin after binding to its intracellular receptor FKBP12 [13], an immunophilin that also interacts with the structurally related immunosuppressant FK506 [14], is only partially understood so far. FKBP12-FK506 complexes associate with the $Ca^{2+}$/calmodulin-dependent phosphatase calcineurin and induce an inhibition of the enzyme [15], whereas FKBP12-rapamycin binds to a distinct intracellular target, the FRAP/RAFT1 protein [16, 17].

Rapamycin was shown to inhibit the activation of the ribosomal S6 kinase $p70^{S6k}$ [18, 19] and cyclin-dependent kinases (Cdk's) $p34^{cdc2}$ and $p33^{cdk2}$ [9, 20]. Furthermore, it was demonstrated that the drug blocks both the IL-2-mediated elimination of the $p27^{kip1}$- and the IL-2-dependent induction of the p21 Cdk inhibitors [21].

Here, we show that rapamycin suppressed the proliferation of the erythroid cell line J2E, in the presence and absence of EPO, and augmented the antiproliferative effect of sodium butyrate without affecting terminal differentiation of the cells. These results indicated that the rapamy-

‡ Corresponding author. Tel. 49-381-4945756; FAX 49-381-4945752.

§ *Abbreviations:* $p70^{S6k}$, 70 kDa S6 protein kinase; EPO, erythropoietin; IL, interleukin; FGB-beta, fibroblast growth factor-beta; GM-CSF, granulocyte-macrophage colony-stimulating factor; FKBP12, FK506- and rapamycin-binding protein; ECL, enhanced chemoluminescence.

Received 4 September 1995; accepted 18 December 1995.

cin-sensitive pathway regulates replication, but not maturation. Experiments designed to investigate the role of $p70^{S6k}$ in the proliferation and differentiation of J2E cells revealed that cell growth correlated with enzyme activity, whereas maturation proceeded despite the rapamycin-induced inhibition of p70 S6 kinase.

## MATERIAL AND METHODS

### *Reagents*

Recombinant human EPO was obtained from Boehringer Mannheim (Germany). Rapamycin, a product of Calbiochem (Bad Soden, Germany), was stored as a stock solution at −20°C and diluted appropriately in ethanol prior to use. The peptide RRRLSSRA derived from the S6 sequence (referred to as S6 peptide) as well as the polyclonal $p70^{S6k}$ antibody (from rabbit) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). ECL-reagents were purchased from Amersham (Bucks, U.K.).

### *Cell Culture*

J2E cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% fetal calf serum, penicillin 100 U/mL and streptomycin 100 μg/mL. Logarithmic phase growth was maintained by resuspending the cells each day at $1.5 \times 10^5$ cells/mL. Terminal differentiation induced by EPO or sodium butyrate was assayed by benzidine staining of hemoglobin [22] 48 hr later. If rapamycin was applied in combination with EPO or sodium butyrate, it was added 30 min before the induction of maturation.

### *Cell Proliferation*

J2E cells were washed free of serum and cultivated for 4 hr in serum-free DMEM. Without this pretreatment, the effects described were less pronounced, but still detectable (data not shown). Thirty minutes prior to the readdition of serum, cells were plated into 24-well plates and exposed to rapamycin as indicated. EPO, or sodium butyrate was added together with the serum, with each well containing $7.5 \times 10^4$ cells in a total volume of 0.5 mL. After an incubation period of 20 hr, DNA synthesis was measured; three 90-μL aliquots were transferred from each well to a 96-well plate and 0.6 μCi [$^3$H] thymidine (83 Ci/mmol [methyl $^3$H] thymidine; Amersham) in 10 μL culture medium was added to each sample. Incubation was continued for an additional 4 hr before [$^3$H] thymidine incorporation into DNA was quantified by liquid scintillation counting. Simultaneously, the Trypan Blue exclusion test was performed to check cell viability.

### *$P70^{S6k}$ Assay*

J2E cells were exposed to EPO, sodium butyrate, or rapamycin, or left untreated for the indicated periods of time. Aliquots of $2 \times 10^6$ cells were lysed in 500 μL ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 0.15 U/mL aprotinin, 1 mM sodium orthovanadate and 25 mM β-glycerophosphate), frozen immediately in liquid nitrogen and stored at −70°C until assayed. The samples were thawed on ice, centrifuged for 10 min at 10,000 *g*, and the supernatant incubated with the $p70^{S6k}$ antibody prebound to protein A-sepharose beads (0.5 μg antibody/5 μL beads per sample) for 2 hr at 4°C. After washing the immunoprecipitates 3 times with lysis buffer and once with kinase buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM dithiothreitol, 10 mM $MgCl_2$ and 25 mM β-glycerophosphate), the $p70^{S6k}$ assay was started by resuspending the beads in a total volume of 30 μL of kinase buffer supplemented with 100 μM ATP (Boehringer Mannheim), 5 μCi [$\gamma$-$^{32}$P] ATP (>5000 Ci/mmol, Amersham) and 250 μM S6 peptide. The samples were incubated for 30 min at 37°C before reactions were terminated by a short centrifugation at 10,000 *g* and by spotting 2 15-μL aliquots of the supernatant onto phosphocellulose discs. Thereafter, the filters were washed once in 1% acetic acid and 3 times in $H_2O$, and the S6 peptide-associated $\gamma$-$^{32}$P radioactivity bound to the phosphocellulose was quantified by Cerenkov counting.

### *Immunoblotting*

Cell extracts were prepared by boiling $1 \times 10^6$ cells of each sample in 100 μL hot SDS sample buffer (50 mM Tris pH 6.8, 10% glycerol, 1.5% SDS, 4.2% mercaptoethanol, 0.01% bromophenolblue). Lysates were resolved by 8% SDS-PAGE and transferred to nitrocellulose filters (Hybond-ECL, Amersham, U.K.). Nonspecific binding sites on the filter were blocked by incubation in PBS pH 7.5 + 0.1% Tween 20 (PBS-T) + 1% bovine serum albumin. Then 200 ng of the $p70^{S6k}$ antibody was added to 10 mL of the solution and the incubation continued overnight at 4°C. After washing with PBS-T and incubation with a horseradish peroxidase-labelled antirabbit Ig antibody, blots were washed again and developed with ECL.

## RESULTS

To investigate the effect of rapamycin on proliferation, J2E cells were exposed to various concentrations of the drug alone or in combination with EPO or sodium butyrate for 24 hr before [$^3$H]-thymidine uptake was measured (Fig. 1a). Rapamycin inhibited the normal growth of J2E cells in a dose-dependent manner. The maximum effect, a decrease in DNA-synthesis by more than 50%, was observed for concentrations of 1 ng/mL or above. Significantly, rapamycin displayed no cytotoxic effect even at high concentrations (cell viability > 95%).

Although EPO was able to stimulate DNA synthesis by approximately 40% (Fig. 1a), rapamycin was still capable of inhibiting hormone-initiated proliferation. Given that the

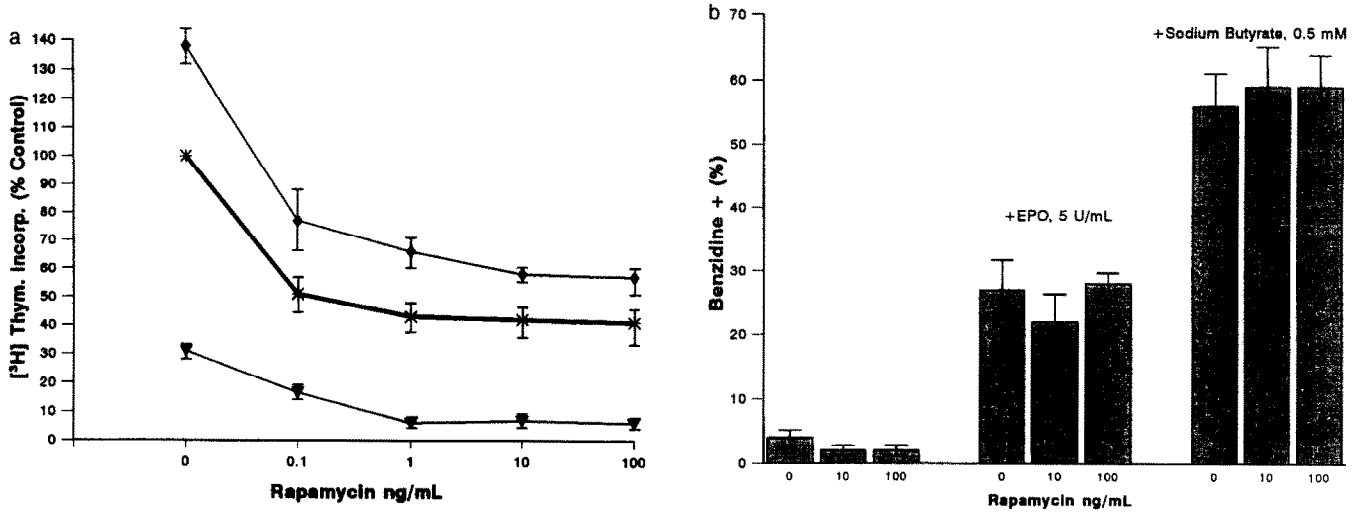


**FIG. 1. Rapamycin inhibits proliferation without affecting terminal differentiation of J2E cells. (a) Effects of rapamycin on the proliferation of J2E cells in the presence of EPO (5 U/mL, ♦), sodium butyrate (0.5 mM, ▼) and in the absence of inducers (*) were determined by measuring [$^3$H] thymidine uptake; untreated control = 100%. (b) J2E cells were cultured for 48 hr in the presence of rapamycin, EPO, sodium butyrate, or in combination as indicated, and terminally differentiated cells were detected by benzidine staining of hemoglobin. Data are the mean ± SEM of 3 independent experiments.**

cells were dividing more rapidly with EPO, the degree of inhibition was similar to that of control cells, and the two curves changed in parallel. In contrast to EPO, sodium butyrate restricted the replication of J2E cells (Fig. 1a), but rapamycin impeded cell division even further. Thus, under these experimental regimes, rapamycin effectively suppressed the proliferation of J2E cells.

Next, we examined the effect of rapamycin on erythroid maturation of J2E cells by determining the percentage of benzidine-positive cells (Fig. 1b). Rapamycin itself, at 10 and 100 ng/mL, did not affect hemoglobin production by the cells. Although EPO and sodium butyrate induced almost 30 and 60% differentiation, respectively, rapamycin did not interfere with this process. Therefore, rapamycin was shown to reduce mitogenic activity of J2E cells (Fig. 1a), but had no impact on hemoglobin synthesis (Fig. 1b).

Among the biochemical effects of rapamycin described, inhibition of the ribosomal S6 kinase p70$^{S6k}$ is well established [18, 19]. In J2E cells, rapamycin (10 ng/mL) induced a rapid and strong inhibition of p70$^{S6k}$ activity, which was maintained for at least 24 hr (Fig. 2). The presence of EPO or sodium butyrate on these cultures did not alter the inhibitory effect (data not shown), indicating that erythroid differentiation of J2E cells (Fig. 1b) may occur in the absence of active p70$^{S6k}$.

Interestingly, the activity of p70$^{S6k}$ correlated well with the proliferative status of the cells (Fig. 2). When cells underwent enhanced proliferation with EPO, p70$^{S6k}$ activity rose. On the other hand, the enzyme's activity fell as sodium butyrate reduced cell division. Moreover, the level of p70$^{S6k}$ protein decreased continuously when cells were cultured in the presence of sodium butyrate, to become almost undetectable after 72 hr (Fig. 3a), but did not change significantly during EPO-induced differentiation (Fig. 3b).

## DISCUSSION

The immunosuppressive drug rapamycin has been shown to inhibit T-cell growth and activation [7, 8], as well as to block the cytokine-driven proliferation of other cell types [11, 12, 23]. The suppression of cell growth by inducing a G1-arrest [9, 10] is the best characterized, but not the only biological effect of the drug, because rapamycin also stimulated differentiation in a myogenic cell line [24].

Investigating the effect of rapamycin on the proliferation of J2E cells, we found a dose-dependent inhibitory effect of the drug both in the presence and absence of EPO. Additionally, a marked increase was found in the antiprolifera-

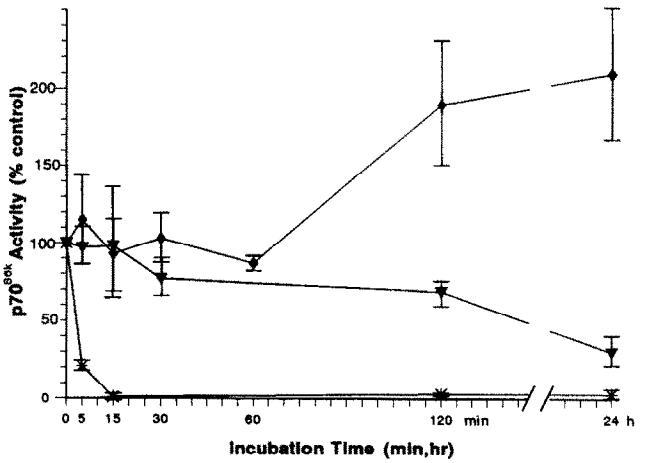


**FIG. 2. Effects of EPO, sodium butyrate, and rapamycin on p70$^{S6k}$ activity. J2E cells were exposed to EPO (5 U/mL, ♦), sodium butyrate (0.5 mM, ▼), or rapamycin (10 ng/mL, *) for the indicated periods of time followed by cell lysis, immunoprecipitation of p70$^{S6k}$, and assay of S6 peptide phosphotransferase activity. Values represent the mean ± SEM of at least 3 independent experiments.**

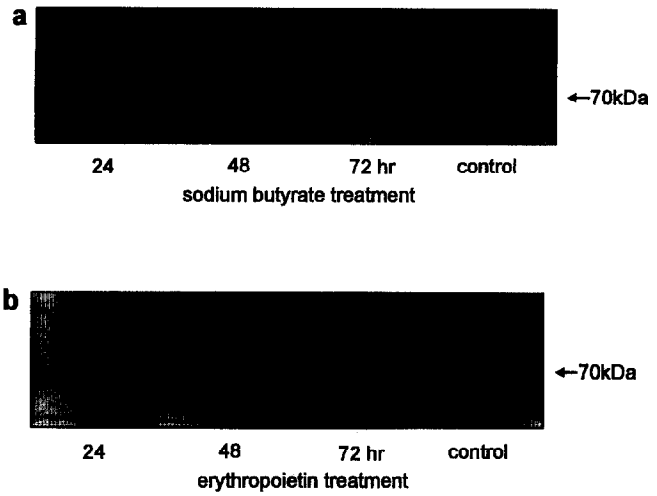


**FIG. 3. Sodium butyrate (a) but not erythropoietin (b) induces a downregulation of the $p70^{S6k}$ protein level. J2E cells were incubated with sodium butyrate (0.5 mM) or erythropoietin (5 U/mL) and lysates from an equal number of cells taken after 0, 24, 48, and 72 hr were resolved by SDS-PAGE and $p70^{S6k}$ detected by Western blotting using a specific antibody. The experiments are typical of 3 performed.**

tive effect of sodium butyrate, a chemical inducer of terminal differentiation [6]. Although replication of J2E cells was severely restricted by rapamycin, the drug had no effect on the capacity of the cells to undergo terminal differentiation. Thus, rapamycin blocked cell division, but maturation was not impeded. This observation indicates that proliferation and differentiation in erythroid cells may be activated by distinct intracellular pathways.

During the process of EPO-induced differentiation of J2E cells, the initial period of enhanced replication is followed by a cessation of cell division as maturation proceeds [25, 26]. Sodium butyrate blocks proliferation, and terminal differentiation occurs more rapidly than in EPO-stimulated cultures [6]. These data suggest that reducing the replication rate might facilitate the maturational process, a hypothesis that is further supported by the observation that the $Na^+/H^+$ antiport-blocker amiloride suppresses proliferation but potentiates the EPO-initiated differentiation of J2E cells [27]. Therefore, it is striking that rapamycin also inhibited replication without affecting the EPO-induced erythroid differentiation of J2E cells. Although the mechanisms by which the EPO receptor induces different signals leading to proliferation and differentiation have yet to be fully identified, several studies have shown that distinct regions in the cytoplasmic domain are essential for the induction of mitogenesis [28, 29]. It is still unknown, however, whether or not a specific differentiation domain exists in the EPO receptor.

Despite progress, in the characterization of the FRAP/RAFT1 protein as the intracellular target of the FKBP12-rapamycin complex [16, 17], many details concerning the mode of action of the drug are poorly understood. Nevertheless, two main effects have been established. First, the immunosuppresant was shown to interfere with the activation of cyclin-dependent kinases involved in the regulation of the G1-phase of the cell cycle. For example, rapamycin suppressed the IL-2 induced activation of $p34^{cdc2}$ and $p33^{cdk2}$ in T lymphocytes [9, 20]. Second, rapamycin blocks the mitogen-induced activation of the ribosomal S6 kinase $p70^{S6k}$ [18, 19], an enzyme shown to be essential for G1 progression [30]. A recent analysis, in mutant clones of a T-cell lymphoma with altered sensitivities to rapamycin, revealed a strong correlation between the growth-inhibitory effect of rapamycin and $p70^{S6k}$ activity [31]. In this study with J2E cells, rapamycin effectively suppressed both $p70^{S6k}$ activity and cell division. Furthermore, inhibition of cell proliferation by sodium butyrate was accompanied by reduced $p70^{S6k}$ activity and downregulation of the $p70^{S6k}$ protein content. In contrast, EPO stimulated cell growth and induced an increase in enzyme activity. We have shown, elsewhere, that EPO-induced activation of $p70^{S6k}$ is much more pronounced in a hematopoietic cell line, which strictly depends on the presence of the growth factor for its proliferation [32]. Together, these data demonstrate a strong correlation between proliferation of erythroid cell lines and $p70^{S6k}$ activity. In addition, the observation that J2E cells differentiate despite almost completely suppressed $p70^{S6k}$ activity indicates that an active $p70^{S6k}$ pathway is not essential for the process of erythroid maturation. Furthermore, our data suggests that the recently identified target of the FKBP12-rapamycin complex, the FRAP/RAFT1 protein [16, 17], might be involved in the regulation of replication, but not differentiation, of erythroid cells.

*This work was supported by grants from the Deutsche Forschungsgemeinschaft and the SANDOZ Foundation.*

## References

1. Youssouffian H, Longmore G, Neumann D, Yoshimura A and Lodish HF, Structure, function and activation of the erythropoietin receptor. *Blood* **81:** 2223–2236, 1993.
2. Krantz SB, Erythropoietin. *Blood* **77:** 419–434, 1991.
3. Bazan JF, Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA* **87:** 6934–6938, 1990.
4. Wojchowski DM and He T-Ch, Signal transduction in the erythropoietin receptor-system. *Stem Cells* **11:** 381–392, 1993.
5. Klinken SP, Nicola NA and Johnson GR, *In vitro*-derived leukemic erythroid cell lines induced by a *raf*- and *myc*-containing retrovirus differentiate in response to erythropoietin. *Proc Natl Acad Sci USA* **85:** 8506–8510, 1988.
6. Busfield SJ and Klinken SP, Erythropoietin-induced stimulation of differentiation and proliferation in J2E cells is not mimicked by chemical induction. *Blood* **80:** 412–419, 1992.
7. Bierer BE, Mattila PS, Standaert RF, Herzenberg LA, Burakoff SJ, Crabtree G and Schreiber SL, Two distinct signal transmission pathways in T lymphocytes are inhibited by alternative complexes formed between an immunophilin and either FK506 or rapamycin. *Proc Natl Acad Sci USA* **87:** 9231–9235, 1990.
8. Dumont FJ, Staruch MJ, Koprak SL, Melino MR and Sigal NH, Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J Immunol* **144:** 251–258, 1990.
9. Morice WG, Brunn GJ, Wiederrecht G, Siekierka JJ and Abraham RT, Rapamycin-induced inhibition of $p34^{cdc2}$ ki-

nase activation is associated with G1/S-phase growth arrest in T lymphocytes. *J Biol Chem* **268:** 3734–3738, 1993.
10. Terada N, Lucas JJ, Szepesi A, Franklin RA, Domenico J and Gelfand EW, Rapamycin blocks cell cycle progression of activated T cells prior to events characteristic of the middle to late G1 phase of the cycle. *J Cell Physiol* **154:** 7–15, 1993.
11. Akselband Y, Harding MW and Nelson PA, Rapamycin inhibits spontaneous and fibroblast growth factor beta-stimulated proliferation of endothelial cells and fibroblasts. *Transplant Proc* **23:** 2833–2836, 1991.
12. Quesniaux FJ, Wehrli S, Steiner C, Joergensen J, Schuurman H-J, Hermann P, Schreier MH and Schuler W, The immunosuppressant rapamycin blocks in vitro responses to hematopoietic cytokines and inhibits recovering but not steady-state hematopoiesis in vivo. *Blood* **84:** 1543–1552, 1994.
13. Schreiber SL and Crabtree BR, The mechanism of action of cyclosporin A and FK506. *Immunol Today* **13:** 136–142, 1992.
14. Harding MW, Galat A, Uehling DE and Schreiber SL, A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature* **341:** 758–760, 1989.
15. Liu J, Farmer JD, Jr., Lane WS, Friedman I and Schreiber SL, Calcineurin is a common target of the cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66:** 807–815, 1991.
16. Brown EJ, Albers MW, Bum Shin T, Ichikawa K, Keith CT, Lane WS and Schreiber SL, A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* **369:** 756–758, 1994.
17. Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P and Snyder SM, A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78:** 35–43, 1994.
18. Chung J, Kuo CJ, Crabtree GR and Blenis J, Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* **69:** 1227–1236, 1992.
19. Kuo CJ, Chung J, Fiorentino DF, Flanagan DF, Blenis J and Crabtree GR, Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature* **358:** 70–73, 1992.
20. Morice WG, Wiederrecht G, Brunn GJ, Siekierka JJ and Abraham RT, Rapamycin inhibition of interleukin-2-dependent $p33^{cdk2}$ and $p34^{cdc2}$ kinase activation in T lymphocytes. *J Biol Chem* **268:** 22737–22745, 1993.
21. Nourse J, Firpo E, Flanagan WM, Coata S, Polyak K, Lee M-H, Massague J, Crabtree GR and Roberts JM, Interleukin-2-mediated elimination of the $p27^{kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* **372:** 570–573, 1994.
22. Cooper MC, Levy J, Cantor LN, Marks PA and Rifkind RA, The effect of erythropoietin on colonial growth of erythroid precursor cells *in vitro*. *Proc Natl Acad Sci USA* **71:** 1677–1681, 1974.
23. Hatfield SM, Mynderse JS and Roehm NW, Rapamycin and FK506 differentially inhibit mast cell cytokine production and cytokine-induced proliferation and act as reciprocal antagonists. *J Pharmacol Exp Ther* **261:** 970–976, 1992.
24. Jayaraman T and Marks AR, Rapamycin-FKBP12 blocks proliferation, induces differentiation and inhibits cdc2 kinase activity in a myogenic cell line. *J Biol Chem* **268:** 25385–25388, 1993.
25. Busfield SJ, Farr TJ, Singh T, Sainsbury AJ and Klinken SP, Clonal analysis of erythropoietin stimulated J2E cells reveals asynchrony during terminal differentiation. *Growth Factors* **9:** 307–315, 1993.
26. Busfield SJ, Meyer GT and Klinken SP, Erythropoietin induced ultrastructural alteration and loss of proliferative capacity with terminal differentiation. *Growth Factors* **9:** 317–328, 1993.
27. Callus B, Tilbrook PA, Busfield SJ, Cull VS, Bittorf Th and Klinken SP, Amiloride suppresses erythropoietin-induced proliferation and MAP kinase, but potentiates differentiation of J2E cells. *Exp Cell Res* **219:** 39–46, 1995.
28. He T-C, Jiang N, Zhuang H, Quelle DE and Wojchowski DM, The extended box 2 subdomain of erythropoietin receptor is nonessential for JAK2 activation yet critical for efficient mitogenesis in FDC-ER cells. *J Biol Chem* **269:** 18291–18294, 1994.
29. D'Andrea A, Yoshimura A, Youssoufian H, Zon LI, Koo JW and Lodish HF, The cytoplasmic region of the erythropoietin receptor contains nonoverlapping positive and negative growth regulatory domains. *Mol Cell Biol* **11:** 1980–1987, 1991.
30. Lane HA, Fernandez A, Lamb NJC and Thomas G, $P70^{S6k}$ function is essential for G1 progression. *Nature* **363:** 170–172, 1993.
31. Dumont FJ, Altmeyer A, Kastner C, Fischer PA, Lemon KP, Chung J, Blenis J and Strauch MJ, Relationship between multiple biologic effects of rapamycin and the inhibition of pp70S6 protein kinase activity. *J Immunol* **152:** 992–1003, 1994.
32. Jaster R, Bittorf Th, Markewitz M, Selig G and Brock J, Erythropoietin induces biphasic activation of $p70^{S6k}$: evidence for a different regulation of early and late phase of activation. *Cell Signal* **7:** 325–330, 1995.