

ACCELERATED COMMUNICATION

Inhibition of Human T-Cell Proliferation by Mammalian Target of Rapamycin (mTOR) Antagonists Requires Noncoding RNA Growth-Arrest-Specific Transcript 5 (GAS5)^[S]

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ABSTRACT

The central importance of the serine/threonine protein kinase mTOR (mammalian Target of Rapamycin) in the control of cell growth and proliferation is well established. However, our knowledge both of the upstream pathways controlling mTOR activity and of the downstream events mediating these effects is still seriously incomplete. We report a previously unsuspected role for the nonprotein-coding RNA GAS5 in the inhibition of T-cell proliferation produced by mTOR antagonists such as rapamycin. GAS5 transcripts are up-regulated during growth arrest and after rapamycin treatment, and GAS5 has recently been shown to be necessary and sufficient for normal T-cell

growth arrest. Down-regulation of GAS5 using RNA interference protects both leukemic and primary human T cells from the inhibition of proliferation produced by mTOR antagonists. The GAS5 transcript is a member of the 5' terminal oligopyrimidine class of RNAs, which is specifically controlled at the level of translation by the mTOR pathway, and the effects of GAS5 on the cell cycle provide a novel and important link to the control of proliferation. These observations point to a significant advance in our understanding of the mechanism of action of mTOR inhibitors, which is likely to lead to improvements in immunosuppressive and cancer therapy.

The serine/threonine protein kinase mTOR occupies a key position in the intracellular pathways that control mammalian cell growth (Hay and Sonenberg, 2004; Wullschleger et al., 2006). Extracellular signals, such as those provided by growth factors and mitogens, and intracellular signals, such as those determined by amino acid concentrations and ATP levels, converge to regulate the state of activation of mTOR (e.g., through dissociation from the endogenous inhibitor FKBP38) (Bai et al., 2007). Subsequently, mTOR regulates both cellular protein synthesis and cell proliferation (Fingar

et al., 2004; Hay and Sonenberg, 2004). The state of activation of mTOR is also important in the regulation of autophagy and apoptosis in many mammalian cells, and all these effects show significant variations among different cell types (e.g., Decker et al., 2003; Chiang and Abraham, 2007).

mTOR, the mammalian homolog of the yeast protein kinase Target of Rapamycin (Sehgal et al., 1975), was identified independently by five groups 15 years ago (for review, see Abraham and Wiederrecht, 1996). Rapamycin binds intracellularly to the immunophilin FKBP12; in turn, this complex binds to mTOR, inhibiting mTOR activity and producing the subsequent effects on cell behavior (Abraham and Wiederrecht, 1996).

Rapamycin (Sirolimus) itself, a lipophilic macrolide isolated from *Streptomyces hygroscopicus*, displays antifungal activity associated with its interaction with fungal TOR ki-

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ABBREVIATIONS: mTOR, mammalian target of rapamycin; GAS5, growth-arrest-specific transcript 5; FKBP38, 38-kDa FK506 binding protein; FKBP12, 12-kDa FK506-binding protein; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; 5'TOP, 5' terminal oligopyrimidine; PHA, phytohemagglutinin; BrdU, 5-bromo 2'-deoxyuridine; siRNA, small interfering RNA; PBS, phosphate-buffered saline; PI, propidium iodide; ELISA, enzyme-linked immunosorbent assay.

nases (Sehgal et al., 1975). Rapamycin also has potent antiproliferative effects on mammalian cells, which accounts for its use as an immunosuppressant (Abraham and Wiederrecht, 1996) and, increasingly, in the therapy of leukemias and other cancers (Abdel-Karim and Giles, 2008; Jiang and Liu, 2008). The specific inhibition of mTOR by rapamycin has enabled many investigators to use it as a powerful pharmacological probe in ongoing investigations aimed at elucidating the critical intracellular signaling pathways upstream and downstream of mTOR (Abraham and Wiederrecht, 1996; Hay and Sonenburg, 2004; Wullschlegel et al., 2006).

In mammalian lymphocytes, the antiproliferative effects of rapamycin depend on the regulation of more than one signaling pathway. Inhibition of mTOR results in inhibition of protein synthesis, in part through reduced phosphorylation of 4E-BP1 (e.g., Brunn et al., 1997; Fingar et al., 2004) and S6 kinase1 (Fingar et al., 2004; Tee and Blenis, 2005). In addition, inhibition of mTOR disproportionately reduces the translation of several groups of RNAs, including 5'TOP transcripts (RNA species containing a 5' terminal oligopyrimidine sequence; for review, see Meyuhas, 2000) and mRNAs encoding several key cell-cycle regulators that have highly structured 5' untranslated regions (Rosenwald et al., 1993). The 5' oligopyrimidine tract of 5'TOP RNAs is found mainly on mRNAs encoding ribosomal and other proteins closely involved with translation, so that mTOR inhibition preferentially inhibits protein synthesis by blocking the production of the protein synthesis machinery itself (Meyuhas, 2000). Inhibition of passage through the cell cycle by inhibition of mTOR has been suggested to result from several effects, first from reduced translation of key cell cycle regulators, including Myc (e.g., Zimmer et al., 2000) and second through effects on signaling pathways controlling crucial cell-cycle regulators such as p21^{Kip1} (Nourse et al., 1994) and p53 (Feng et al., 2005). Therefore, in this second category, inhibition of mTOR acts by mechanisms that are potentially independent of the effects on translation (Soulard and Hall, 2007; Hong et al., 2008).

GAS5 is a 5'TOP RNA; consequently, its translation is controlled by the mTOR pathway. GAS5 encodes snoRNAs (small nucleolar RNAs) in its introns, and its exons contain a small open reading frame that does not appear to encode a functional protein (Muller et al., 1998; Raho et al., 2000). However GAS5 mRNA may still have functional effects through interactions with steroid receptors that inhibit their action (Kino et al., 2010). Several other mammalian snoRNAs are encoded within the introns of genes coding for nonfunctional proteins (e.g., Tycowski et al., 1996; Pelczar and Filipowicz, 1998). Because GAS5 transcript levels increase on growth arrest and after rapamycin treatment (Smith and Steitz, 1998), and GAS5 is both necessary and sufficient for normal growth arrest in human T cells (Mourtada-Maarabouni et al., 2008), we set out to test the working hypothesis that the inhibition of T-cell proliferation produced by mTOR inhibition is mediated in part through GAS5.

Materials and Methods

Materials. Rapamycin was purchased from Calbiochem (San Diego, CA). Temsirolimus and everolimus were purchased from LC

Laboratories (Woburn, MA). Cyclosporine A was purchased from Sigma (St. Louis, MO).

Cell Culture. The human T-leukemic cell lines CEM-C7 CMK1 (Norman and Thompson 1977; Williams et al., 1998) and MOLT-4 (Minowada et al., 1972) were maintained in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and 200 µg/ml gentamicin (Sigma), at 37°C in a 5% CO₂ humidified incubator. All experiments were carried out using cells in logarithmic growth phase.

Primary Lymphocyte Isolation and Culture. Whole blood from healthy volunteers was collected into tubes containing 4% sodium citrate (1:9 ratio). Each experiment was carried out on a different donor. An equal volume of phosphate-buffered saline (PBS) was added to the blood. The blood was pipetted carefully over the Lymphoprep (2:1 volume; Axis-Shield UK, Kimbolton, Cambridgeshire, UK) and centrifuged at 800g for 25 min at room temperature. The mononuclear cells that accumulated at the interface between the plasma and the Lymphoprep were then resuspended in ice-cold PBS and recovered after centrifugation (1500g, 30 min). Cells were resuspended in complete RPMI medium supplemented with 2.5 µg/ml phytohemagglutinin (PHA; Sigma) and incubated at 37°C in a 5% CO₂ humidified incubator. After 24 h, the nonadherent cells (depleted of macrophages) were harvested, resuspended at a density of 10⁶ cells/ml in complete RPMI medium containing 2.5 µg/ml PHA, and incubated for 3 to 4 days at 37°C before being used for the experiments.

Determination of Viable Cell Number. Viable cell number was determined by nigrosin exclusion analysis and the LIVE/DEAD cell viability assay (Invitrogen, Carlsbad, CA). In 96-well plates, 200 µl of cells (2 × 10⁵ cells/ml) were incubated either without or with rapamycin (2.5 µM), temsirolimus (5 nM), or everolimus (10 nM) for 48 h. An aliquot of the control or treated cells was added to the combined LIVE/DEAD assay reagents (as instructed by the manufacturer). Cells were then incubated for 40 min at room temperature. Live cells stained with the green fluorescent dye and dead cells stained with the red fluorescent dye were visualized and counted using a Nikon Eclipse E400 fluorescence microscope.

DNA Synthesis Assay (BrdU Incorporation). Proliferation was assessed by 5-bromo 2'-deoxyuridine (BrdU) incorporation during DNA synthesis using a colorimetric ELISA Kit (Roche Diagnostics, Mannheim, Germany) (Zhang et al., 2009; Gharagozloo and Amirghofran, 2007) according to the manufacturer's instructions. In brief, 200 µl of cells (2 × 10⁵ cells/ml) were cultured in flat-bottomed 96-well plates for 48 h in the presence or the absence of rapamycin or mTOR inhibitors. Subsequent to labeling with 10 µM BrdU (for the final 18 h of the incubation period), DNA was denatured and cells were incubated with anti-BrdU monoclonal antibody before the addition of substrate. The absorbance of the samples was measured using a microplate reader (Wallac 1420 Victor Plate Reader; PerkinElmer Life and Analytical Sciences, Waltham, MA) at 450 nm with the absorbance at 690 nm as reference.

RNA Interference. Transfection of GAS5 and control small interfering RNAs (siRNAs) was as described previously (Mourtada-Maarabouni et al., 2008). Three different GAS5 siRNAs (reference sequence AF141346) were designed by Ambion (Austin, TX). Negative control siRNA was purchased from Ambion. All siRNAs were purified by high-performance liquid chromatography, annealed, and ready to use. Down-regulation of 65 to 80% in all the different cell types and for all three GAS5 siRNAs was confirmed by quantitative reverse transcriptase polymerase chain reaction (Supplemental Tables T1–T5).

Ki-67 Labeling. Expression of the Ki-67 protein is associated with cell proliferation (Scholzen and Gerdes, 2000). Cells (10⁶) were suspended in 70% ethanol/30% PBS while vortexing. They were then incubated at –20°C for 2 h. Fixed cells were then washed twice with staining buffer (PBS with 1% fetal calf serum and 0.09% Na₂S₂O₃), centrifuged for 10 min at 200g, and resuspended in 100 µl in the

staining buffer. Anti-Ki-67 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added at 1:50 dilution. Cells were incubated for 30 min in the dark. Cells were washed twice with the staining buffer, centrifuged at 200g for 10 min, and resuspended in 100 μ l of staining buffer; the secondary antibody (anti-mouse IgG-fluorescein isothiocyanate; Santa Cruz Biotechnology) was added at a dilution of 1:200. Cells were incubated in the dark for 1 h before being washed twice in staining buffer. After centrifugation, the cells were resuspended in 50 μ l of staining buffer, and Ki-67-positive cells were detected using a fluorescence microscope.

Preparation of Cells for Cell Cycle Analysis. Preparation of cells and nuclear propidium iodide (PI) staining procedure for cell-cycle analysis were performed according to standard procedures (White et al., 1990; Kastan et al., 1991). Cells (10^6) were suspended in 200 μ l of PBS, and fixed in 2 ml of ice-cold 70% ethanol/30% PBS. After 30-min incubation on ice, the cells were centrifuged for 5 min at 2000 rpm, and the supernatant was aspirated. Cells were resuspended in 970 μ l of PBS and 3 μ l of DNase-free RNase (Sigma) with 40 μ l of PI (1 mg/ml) and incubated for 30 min at 37°C before

analysis using the MoFlo flow cytometer (Dako UK Ltd. (Ely, Cambridgeshire, UK)).

Statistical Analysis. Data are presented as the mean \pm S.E. Statistical significance was determined by analysis of variance using Origin 6.1 (OriginLab Corp, Northampton, MA). A *p* value of <0.01 was considered statistically significant.

Results and Discussion

GAS5 Down-Regulation Protects Leukemic T Cells from mTOR Antagonists. The initial experimental models we employed to investigate the involvement of GAS5 were the rapamycin-sensitive human cell lines MOLT4 (Minowada et al., 1972) and CEM-C7 CKM1 (Norman and Thompson, 1977; Williams et al., 1998). These leukemic T cells proliferate continuously in culture without stimulation, and this proliferation is inhibited by rapamycin (as described for other T cells by several groups, e.g., Dumont

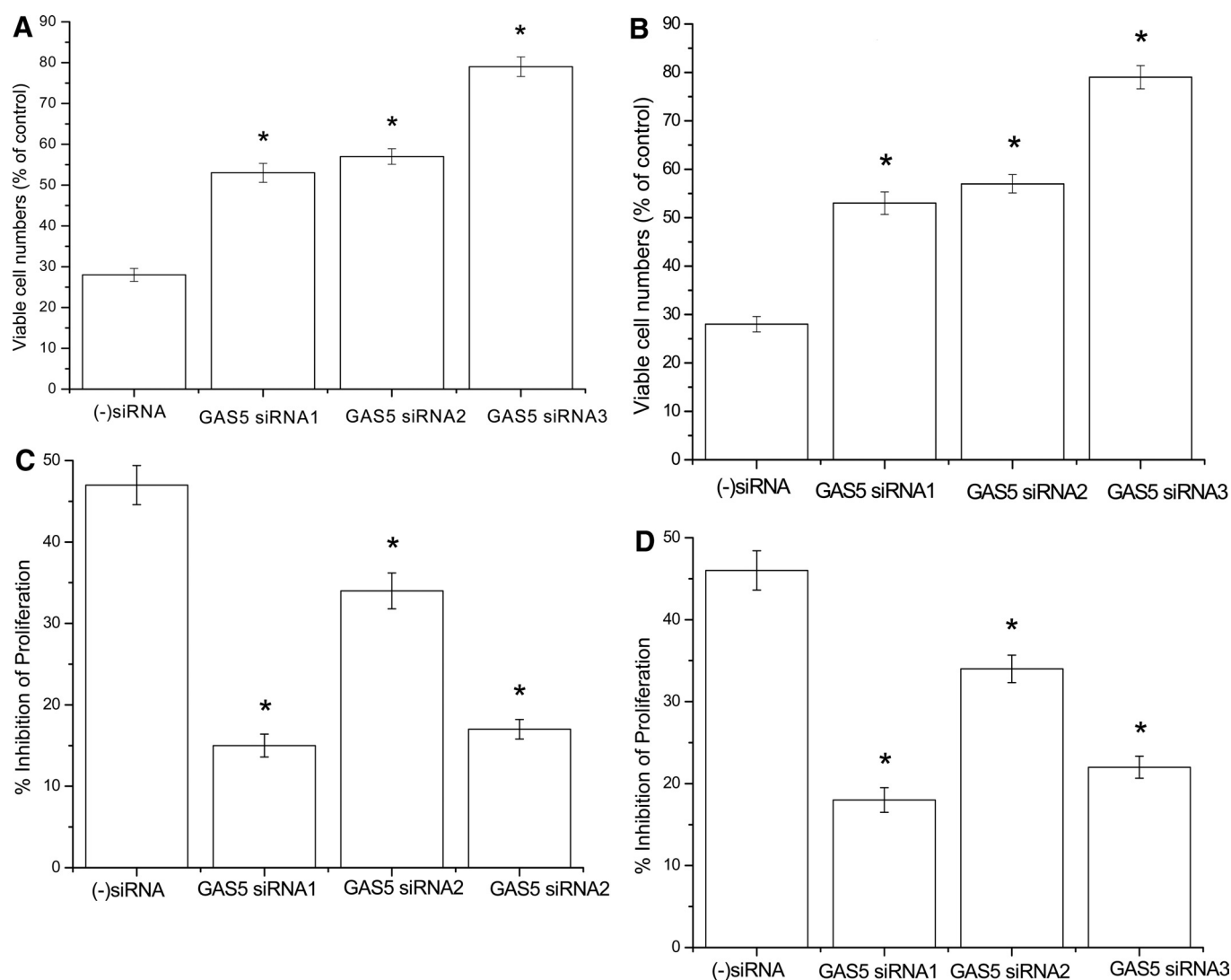


Fig. 1. GAS5-specific siRNAs protect against the inhibition of cell proliferation induced by 2.5 μ M rapamycin in MOLT-4 and CEM-C7 human T cell lines. MOLT-4 and CEM-C7 T cells were transfected with specific GAS5 siRNAs or negative control siRNA [(-)siRNA] and cultured at 37°C. After 48 h, cells were exposed to rapamycin. MOLT-4 (A) and CEM-C7 (B) viable cell numbers were determined after 48 h by vital dye staining and the LIVE/DEAD assay (see *Materials and Methods*). Results are calculated as the percentage of viable cell numbers relative to controls incubated in the absence of rapamycin (mean \pm S.E.M. from five independent experiments). MOLT-4 (C) and CEM-C7 (D) cell proliferation was measured using the BrdU colorimetric ELISA assay. Results are represented as the percentage inhibition of cell proliferation compared with control in the absence of rapamycin. (mean \pm S.E.M. from five independent experiments). *, *P* < 0.01 compared with (-)siRNA. The original data are given as Supplemental Fig. S1.

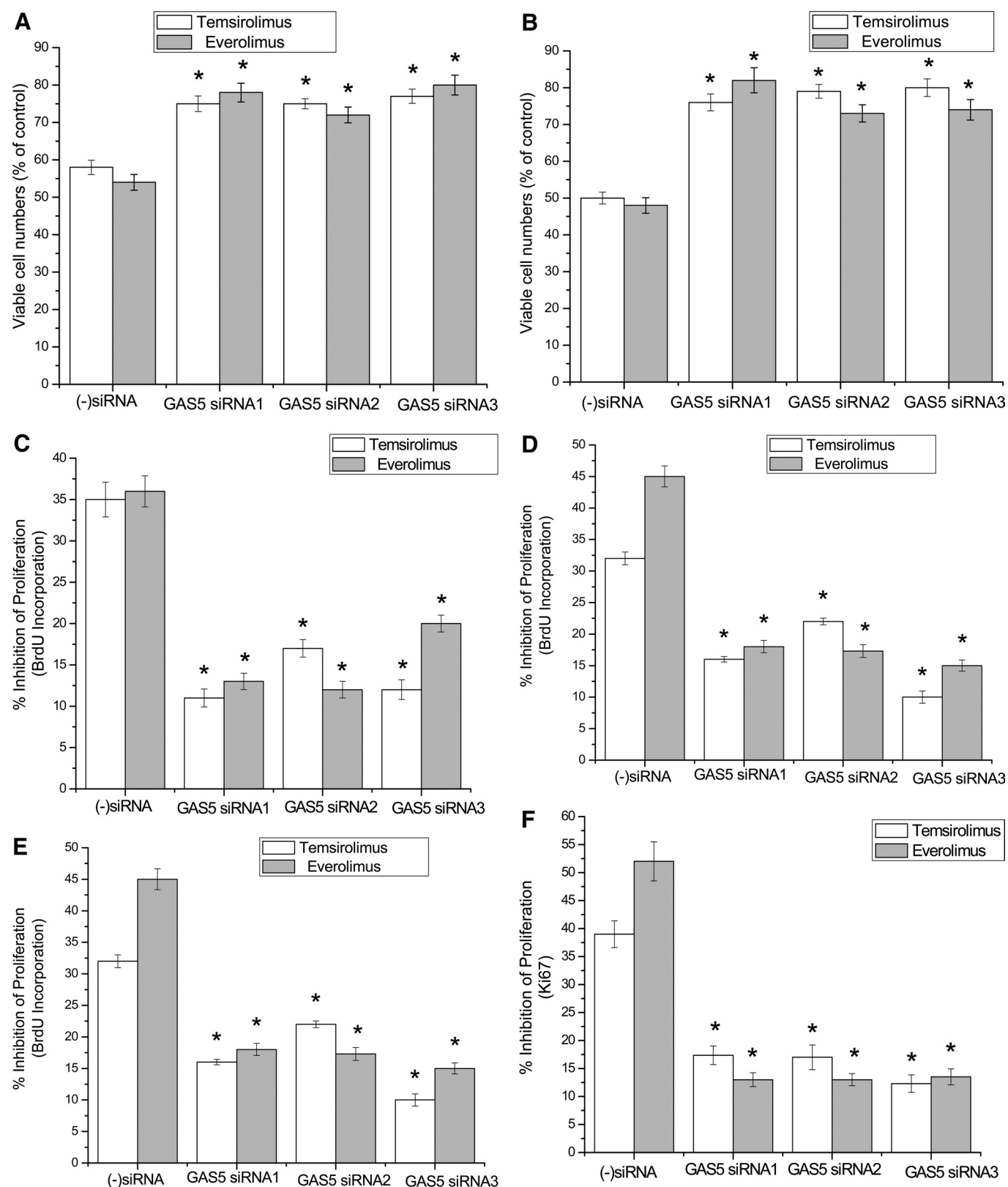


Fig. 2. siRNAs targeting *GAS5* protect the proliferation of MOLT-4 and CEM-C7 T cells from the inhibitory effects of mTOR inhibitors. Forty-eight hours after transfection, siRNA-transfected cells were incubated with temsirolimus (5 nM) or everolimus (10 nM) for 48 h. Viable cell numbers for MOLT-4 (A) and CEM-C7 (B) were determined by vital-dye staining and LIVE/DEAD assay. Results are calculated as the percentage of viable cell numbers relative to controls incubated in the absence of inhibitors (mean \pm S.E.M. from five independent experiments). MOLT-4 (C) and CEM-C7 (D) cell proliferation was assessed using the BrdU colorimetric ELISA assay. Results are represented as the percentage inhibition of cell proliferation compared with controls in the absence of rapamycin. (mean \pm S.E.M. from five independent experiments). E and F, percentage of cells stained by anti-Ki67 antibody. Data are represented as percentage inhibition of cell proliferation and represent means \pm S.E.M. from five independent experiments. *, $P < 0.01$ compared with (-)siRNA. Original data are provided in Supplemental Fig. S2.

et al., 1990; Powell et al., 1999), as determined by counting viable cells and by incorporation of bromodeoxyuridine. The reduction in accumulated viable cell number of cell populations treated with rapamycin for 48 h was substantially alleviated by down-regulating GAS5 using any one of three different siRNAs (Fig. 1, A and B). The inhibition of T-cell DNA synthesis induced by rapamycin treatment, as measured by the incorporation of BrdU, was also clearly alleviated by all three GAS5 siRNAs (Fig. 1, C and D). Although the exact alleviation of inhibition produced and the relative order of effectiveness of the different GAS5 siRNAs showed some differences when different parameters were monitored, the effects produced approached or exceeded 50% in all cases. Such siRNA-mediated down-regulation of GAS5 has no effect on untreated T cells over the 48-h period examined (Mourtada-Maarabouni et al., 2008).

These observations strongly suggested that a substantial proportion of the effect produced by inhibition of mTOR on these leukemic T-cell lines is mediated through GAS5. However, although there is strong evidence that the effects of rapamycin are indeed due to inhibition of mTOR (Abraham and Wiederrecht, 1996), it is always useful with pharmacological agents to examine the effects of other inhibitors to exclude other potential mechanisms of action. The effects of

two other mTOR inhibitors, temsirolimus (Ma and Jimeno, 2007) and everolimus (Majewski et al., 2003), on the T-cell lines were therefore examined. The effects of both these mTOR inhibitors on MOLT4 and CEM-C7 proliferation (as measured by monitoring viable cell numbers, by bromodeoxyuridine incorporation, and by Ki-67 staining) were affected by GAS5 down-regulation in a manner very similar to the effects of rapamycin; at least 50% of the effects produced by these reagents was abolished by each of the GAS5 siRNAs (Fig. 2). These experiments with the three different mTOR inhibitors and three different GAS5 siRNAs are therefore in agreement, indicating that a substantial part of the reduction in MOLT4 and CEM-C7 cell proliferation induced by mTOR inhibition is mediated through GAS5. These effects are not seen for the calcineurin inhibitor cyclosporine (Supplemental Fig. S4).

Analysis of the cell cycle by propidium iodide staining and fluorescence-activated cell sorting revealed that the accumulation of cells in G_1 caused by rapamycin treatment was clearly inhibited by GAS5 down-regulation in both MOLT4 and CEM-C7 T-cell lines (Fig. 3). The corresponding reductions in the proportions of cells in S and G_2/M phases were also inhibited. Rapamycin also caused a small increase in the proportion of cells with sub- G_1 DNA content (i.e., cells un-

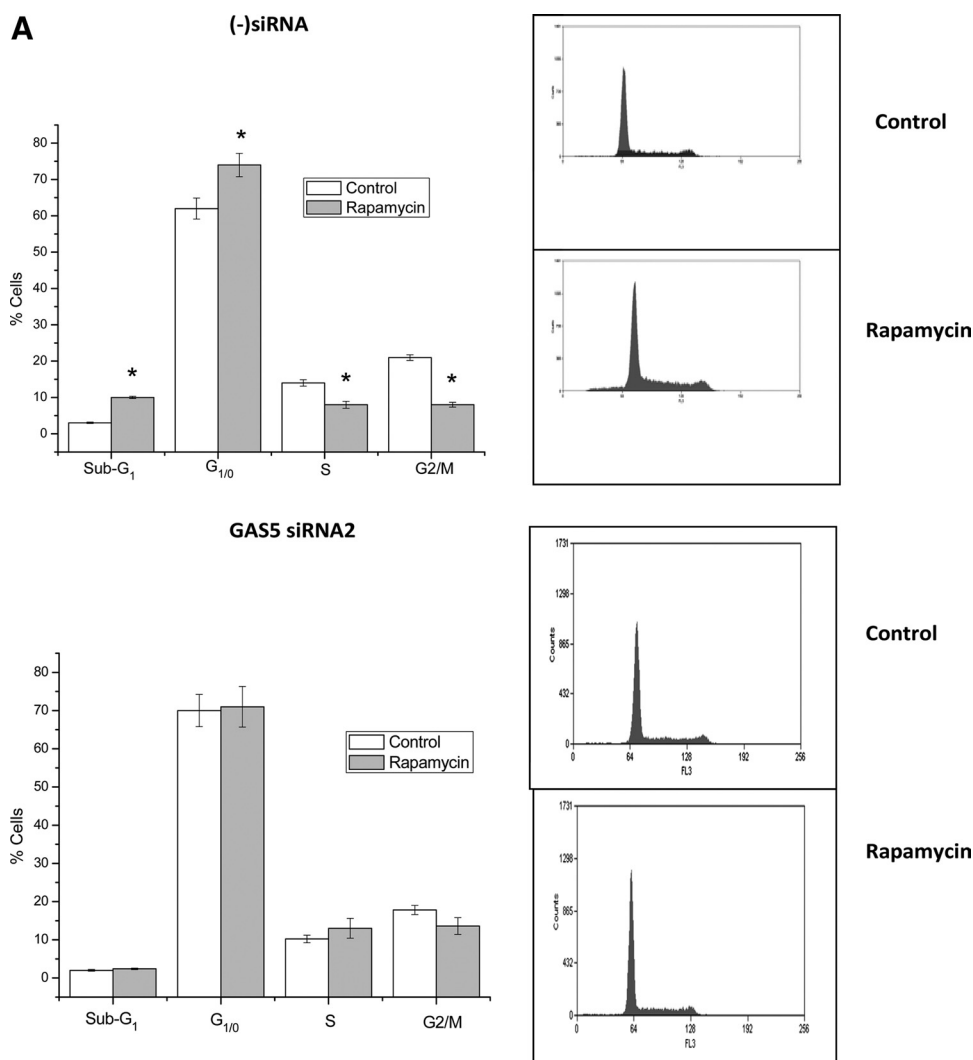


Fig. 3. GAS5-specific siRNAs protect MOLT-4 and CEM-C7 T cells from cell cycle perturbations caused by rapamycin. A, MOLT-4; B, CEM-C7.

dergoing apoptosis). This increase as well was inhibited by down-regulation of GAS5 (Fig. 3).

GAS5 Down-Regulation Protects Untransformed T Cells from mTOR Antagonists. It is clearly important to examine the role of GAS5 in untransformed cells, because all of the above observations were made with transformed leukemic T cells, which may not accurately reflect the physiological situation. Primary cultures of human peripheral blood leukocytes were therefore monitored after stimulation with the mitogen PHA, which selectively stimulates the proliferation of T cells (Greaves and Bauminger, 1972), as described previously (Mourtada-Maarabouni et al., 2008). Rapamycin produced a clear reduction in proliferation over 24 h of incubation, and this effect was substantially reduced by each of the GAS5 siRNAs (Fig. 4, A and B). The rapamycin-induced accumulation of T cells in G₁ and the increase in cells with sub-G₁ DNA were again inhibited by down-regulation of GAS5 (Fig. 4C). Although all three GAS5 siRNAs again alleviated the effects of rapamycin, there were noticeable differences in the response profiles, possibly because of the expression of different GAS5 splice variants in transformed versus untransformed T cells.

It was important to test the effect of GAS5 down-regulation on the action of temsirolimus and everolimus on these nor-

mal human T cells. Once again, the reduction in T-cell proliferation produced by the two mTOR inhibitors was reduced by at least 50% by each of the GAS5 siRNAs (Fig. 4, D–F).

The consistent observations made in the above experiments, using both T-cell lines and untransformed T cells, with three mTOR inhibitors and three GAS5 siRNAs, strongly suggest that a substantial proportion of the effects of mTOR inhibition on these cells involves GAS5. This conclusion is entirely consistent with the established effect of mTOR in regulating GAS5 transcript levels (Smith and Steitz, 1998) together with the recently established critical role of GAS5 in the regulation of T-cell growth arrest (Mourtada-Maarabouni et al., 2008).

When mTOR is active (i.e., when conditions are conducive to cell growth), 5'TOP RNAs are actively translated (for review, see Meyuhas, 2000). For most of these RNAs, this results in the synthesis of proteins required for increased protein synthesis. However, in the case of GAS5, the active translation of the short open reading frame in exon 3, which is normally followed by several further exons, results in the rapid degradation of the GAS5 transcript, most likely by nonsense-mediated decay (Smith and Steitz, 1998; Isken and Maquat, 2007). When mTOR activity is reduced (e.g., by the pharmacological agents used

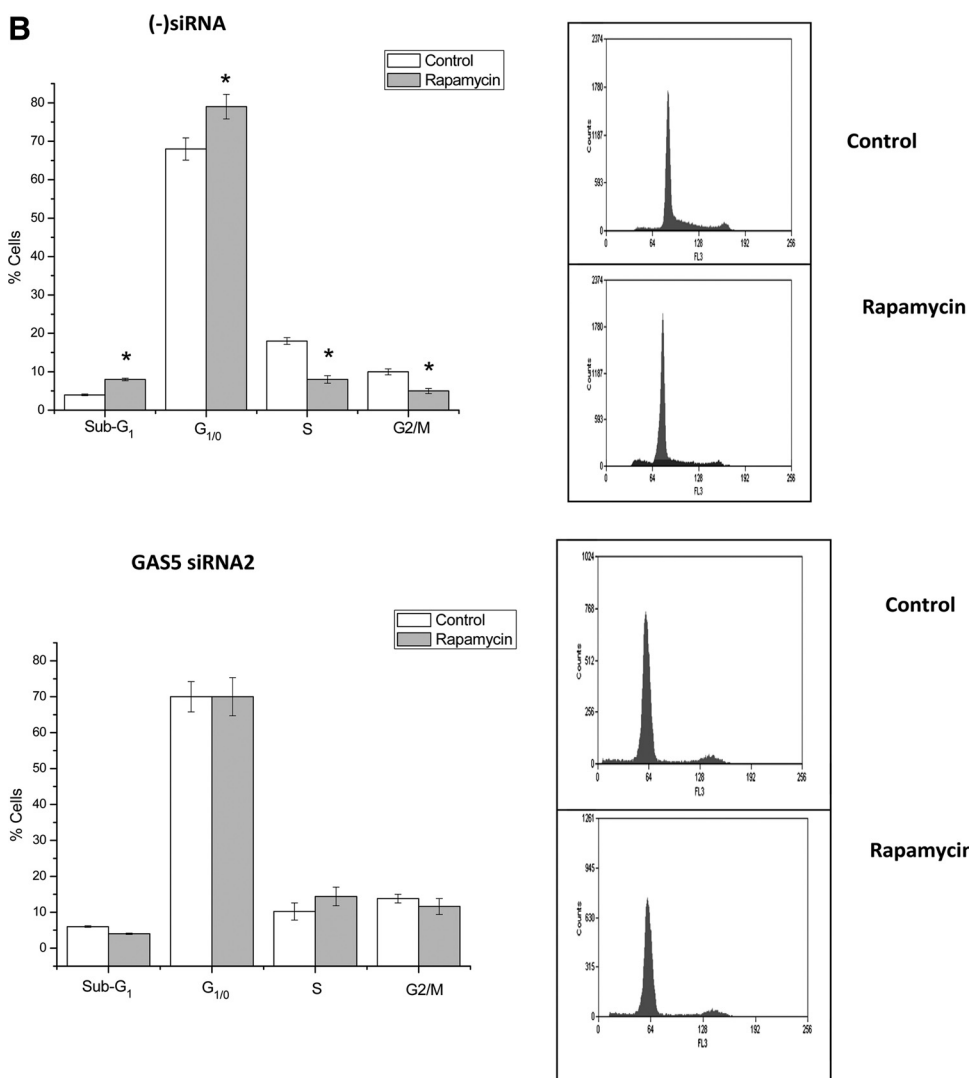


Fig. 3. Continued. B, T cells were transfected with specific GAS5 siRNAs or negative control siRNA [(–)siRNA] and cultured at 37°C. After 48 h, cells were exposed to 2.5 μ M rapamycin. Cell-cycle analysis was carried out after a further 24 h. DNA content was quantified by propidium iodide staining of fixed cells and fluorescence flow cytometry. Results are represented as the means \pm S.E.M. ($n = 5$). *, $P < 0.01$ compared with control in the absence of rapamycin. Representative histograms are also shown, and full results for all three GAS3 siRNAs are given in Supplemental Tables T6 and T7.

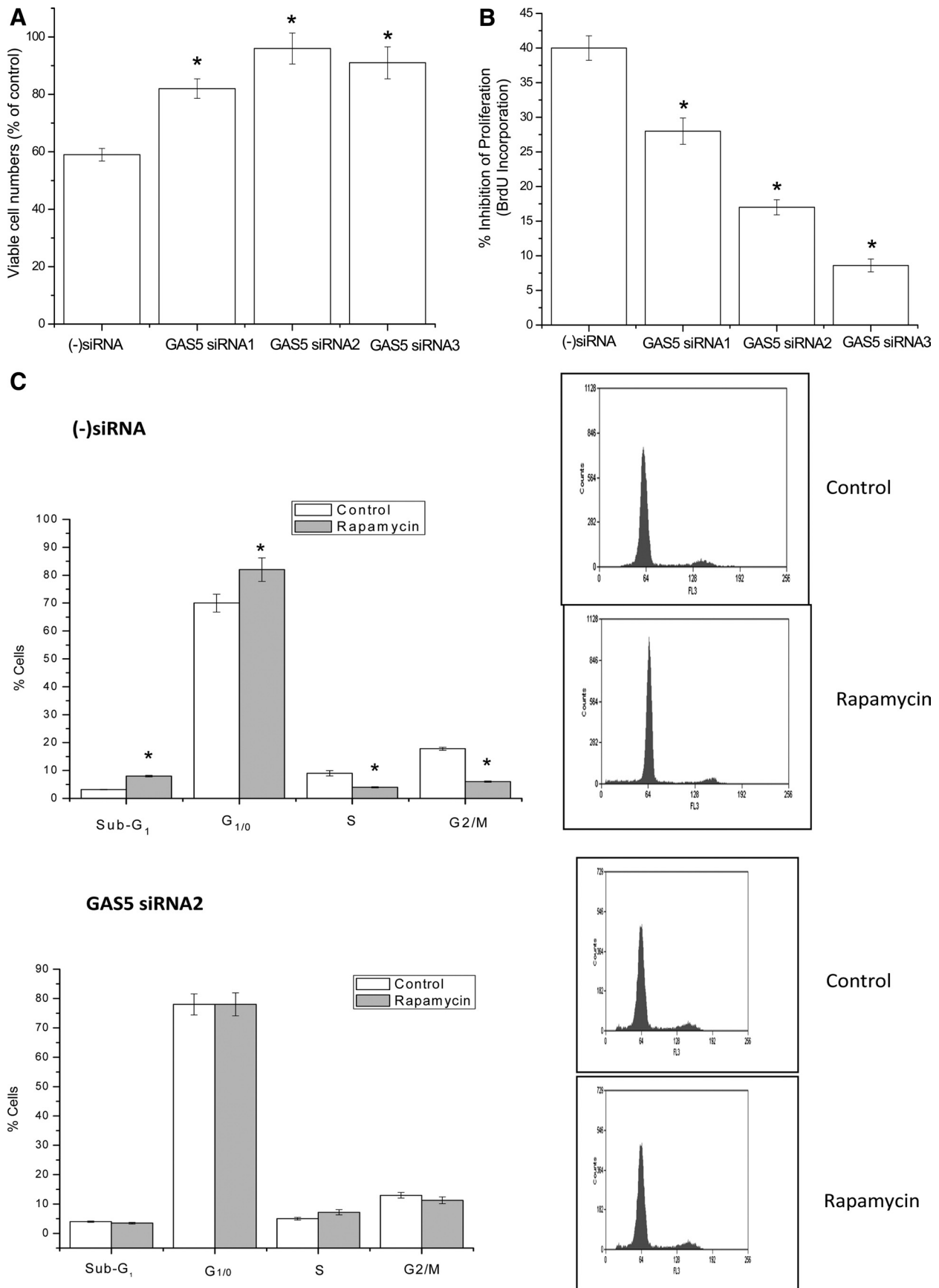


Fig. 4. Changes in *GAS5* expression modulate the response of human peripheral blood lymphocytes to mTOR inhibitors. Peripheral blood lymphocytes from a single healthy volunteer were stimulated with 2.5 μ g/ml PHA and transfected with siRNAs targeting *GAS5* or with negative control (–)siRNA. Forty-eight hours after transfection, siRNA-transfected cells were incubated with either rapamycin 2.5 μ M (A–C) or 5 nM temsirolimus or 10 nM everolimus (D–F) for 48 h.

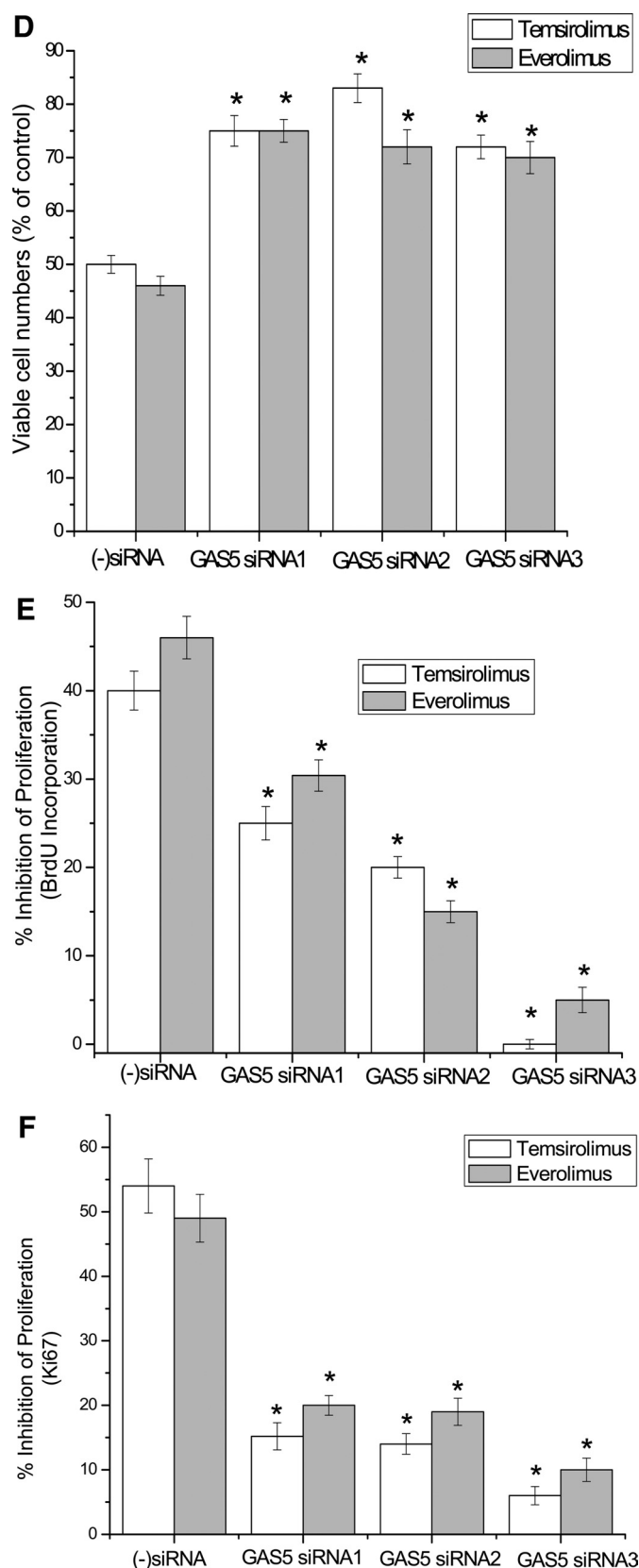


Fig. 4. Continued. A and D, viable cell numbers were determined by vital-dye staining and LIVE/DEAD assay. Results are represented as the percentage viable cell number relative to controls incubated in the absence of mTOR inhibitors (mean \pm S.E.M. from six independent experiments). Changes in GAS5 expression modulate the response of human peripheral blood lymphocytes to mTOR inhibitors. Peripheral blood

lymphocytes from a single healthy volunteer were stimulated with 2.5 μ g/ml PHA and transfected with siRNAs targeting GAS5 or with negative control (-)siRNA. Forty-eight hours after transfection, siRNA-transfected cells were incubated with either rapamycin 2.5 μ M (A-C) or 5 nM temsirolimus or 10 nM everolimus (D-F) for 48 h. A and D, viable cell numbers were determined by vital-dye staining and LIVE/DEAD assay. Results are represented as the percentage viable cell number relative to controls incubated in the absence of mTOR inhibitors (mean \pm S.E.M. from six independent experiments), C, DNA content of transfected lymphocytes 24 h after exposure to rapamycin was monitored by PI staining and flow cytometry. The results obtained from one of the GAS5 siRNAs are presented (full results for all three GAS5 siRNAs are given in Supplemental Table T8). Data represent the mean \pm S.E.M. from four independent experiments, each carried out with cells from a different donor). B and E, cell proliferation was measured using the BrdU colorimetric ELISA assay. F, percentage inhibition of proliferation as monitored by staining by anti-Ki67 antibody. Results are represented as inhibition of cell proliferation compared with controls in the absence of mTOR inhibitors (mean \pm S.E.M. from six independent experiments). *, $P < 0.01$ compared with controls.

here), translation is inhibited, and nonsense-mediated decay, which depends on active translation (Isken and Maquat, 2007), no longer occurs. GAS5 transcript levels therefore increase (Smith and Steitz, 1998). Because up-regulation of GAS5 results in T-cell growth arrest (Mourtada-Maarabouni et al., 2008), cell proliferation and viability would be expected to decrease in these cell cultures, and this prediction is amply confirmed by the experimental observations presented here. GAS5 mRNA has recently been shown to produce effects on epithelial cell growth and survival by antagonizing the action of steroid hormones (Kino et al., 2010) through competing with steroid response elements in DNA for steroid hormone/receptor complexes. Although these effects may account in part for some of our observations, the best known effects of glucocorticoid hormones on T cells are proapoptotic rather than antiapoptotic (e.g., Ashwell et al., 2000). Indeed, we have already shown that the well established proapoptotic effects of glucocorticoids on T cells are alleviated by down-regulation of GAS5 (Mourtada-Maarabouni et al., 2008), indicating that GAS5 carries out its effects by mechanisms other than by antagonizing the glucocorticoid receptor in these circumstances.

Although our observations show that GAS5 plays a major role, the effects of GAS5 siRNAs are incomplete. Because siRNA transfection efficiency is itself below 100% (65–85%; see Supplemental Tables T1–T5), this probably accounts for much but not all of the incompleteness of these effects. The observation that GAS5 down-regulation does not entirely alleviate the effects of mTOR inhibitors, however, is consistent with previous reports, which indicate that the regulation of cell cycle control points, such as the down-regulation of p27^{Kip1} (Nourse et al., 1994; Hong et al., 2008) and the inhibition of translation of critical cell cycle regulators (e.g., Rosenwald et al., 1993), can play important roles in the effects of mTOR inhibitors. Given the significant cell-type dependence of the effects of mTOR inhibitors (e.g., Decker et al., 2003; Chiang and Abraham, 2007), it is likely that the relative contributions of GAS5 and the other factors vary among different cell types.

GAS5 was originally identified from a subtraction cDNA library enriched for sequences overexpressed at growth arrest (Schneider et al., 1988), and its level of expression

lymphocytes from a single healthy volunteer were stimulated with 2.5 μ g/ml PHA and transfected with siRNAs targeting GAS5 or with negative control (-)siRNA. Forty-eight hours after transfection, siRNA-transfected cells were incubated with either rapamycin 2.5 μ M (A-C) or 5 nM temsirolimus or 10 nM everolimus (D-F) for 48 h. A and D, viable cell numbers were determined by vital-dye staining and LIVE/DEAD assay. Results are represented as the percentage viable cell number relative to controls incubated in the absence of mTOR inhibitors (mean \pm S.E.M. from six independent experiments), C, DNA content of transfected lymphocytes 24 h after exposure to rapamycin was monitored by PI staining and flow cytometry. The results obtained from one of the GAS5 siRNAs are presented (full results for all three GAS5 siRNAs are given in Supplemental Table T8). Data represent the mean \pm S.E.M. from four independent experiments, each carried out with cells from a different donor). B and E, cell proliferation was measured using the BrdU colorimetric ELISA assay. F, percentage inhibition of proliferation as monitored by staining by anti-Ki67 antibody. Results are represented as inhibition of cell proliferation compared with controls in the absence of mTOR inhibitors (mean \pm S.E.M. from six independent experiments). *, $P < 0.01$ compared with controls.

has been shown to increase in several situations in which cell growth is inhibited [e.g., in saturation density-arrested cells (Coccia et al., 1992) and under conditions of amino acid insufficiency (Fleming et al., 1998)]. The functional importance of GAS5 was first indicated by its isolation by functional expression cloning as a gene regulating T-cell survival (Williams et al., 2006) and was confirmed by the demonstration that GAS5 is both necessary and sufficient for normal growth arrest in both normal and leukemic human T cells (Mourtada-Maarabouni et al., 2008).

Regulation of GAS5 transcript levels by mTOR therefore provides a clear link between the control of translation and the control of cell division and survival. Because other 5'TOP RNAs encode proteins required for translation (Meyuhas, 2000), it may be that the GAS5 open reading frame also encoded such a functional protein at some time in its evolutionary history. This open reading frame subsequently would become redundant through gene duplication and would be down-regulated during active cell growth through nonsense-mediated decay after the appearance of a stop codon in an early exon (Isken and Maquat, 2007). The subsequent acquisition of growth- and survival-suppressing properties by GAS5 transcripts, potentially mediated in part by the inhibition of steroid hormone action by GAS5 RNA reported recently (Kino et al., 2010), therefore allowed inhibition of translation to be linked both to inhibition of cell cycle progression and to induction of cell death (Mourtada-Maarabouni et al., 2008).

The importance of GAS5 and related genes in the control of the proliferation and survival of normal cells had not been anticipated before its identification by functional cloning (Williams et al., 2006; Mourtada-Maarabouni et al., 2008), illustrating the power of such forward genetics strategies (Stark and Gudkov, 1999; Williams and Farzaneh, 2004). The demonstration of the key role played by GAS5 in the mTOR pathway represents an unexpected twist in the control of mammalian cell proliferation and survival. It is likely that this advance will lead to further improvements in the therapy of diseases involving the mTOR pathway, particularly leukemia, other cancers, and autoimmune disease.

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