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Rapamycin induces the fusion-type independent downregulation of the EWS/FLI-1 proteins and inhibits Ewing's sarcoma cell proliferation

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Ewing's sarcoma (ES) is the prototype of a family of tumors (ESFT) of neuroectodermal origin formed by small, round cells with limited neural differentiation, which arise most frequently within bones in children or adolescents. The proliferation of ESFT cells is highly dependent on the establishment of, and signaling through several growth factor-mediated autocrine loops. The mammalian target of rapamycin (mTOR) is a central regulator of translation and cell proliferation, involved in the cellular response to various nutritional, stress and mitogenic effectors. As mTOR has recently been associated with certain human cancers, we investigated the possibility that mTOR played a role in the regulation of ES cell proliferation. Results showed that ES cell lines carrying *EWS/FLI-1* alleles of different types expressed different levels of total and phosphorylated mTOR protein. We demonstrate that rapamycin, an mTOR inhibitor, efficiently blocked the proliferation of all cell lines by promoting cell cycle arrest at the G_1 phase. This was paralleled by the downregulation of the levels of the EWS/FLI-1 proteins, regardless of their fusion type, and the concomitant restoration of the expression of the TGF-β type 2 receptor (TGFβ RII), which is known to be repressed by several EWS-ETS fusion proteins. The expression of a rapamycin-resistant mTOR construct prevented both the proliferation blockade and the EWS/FLI-1 downregulation. These data demonstrate that mTOR signaling plays a central role in ES cell pathobiology and strongly suggest that the use of rapamycin as a cytostatic agent may be an efficient tool for the treatment of ES patients.
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Ewing's sarcoma (ES) and primitive neuroectodermal tumors (PNET) are characterized by the presence of a specific chromosomal translocation leading to the fusion of the 5′ end of the *EWS* gene and the 3′ end of various genes encoding transcription factors of the ETS family, usually *FLI-1* and *ERG*. The most frequent fusion, resulting from a t(11;22)(q24;q12) translocation, involves *EWS* and *FLI-1* and is found in about 90–95% of the cases (Arvand and Denny, 2001). Identification of several breakpoints for both *EWS* and *FLI-1* demonstrated that *EWS/FLI-1* fusion genes are heterogeneous. Breakpoints of type 1 (exon 7 of *EWS*/exon 6 of *FLI-1*), type 2 (exon 7 of *EWS*/exon 5 of *FLI-1*) and type 3 (exon 10 of *EWS*/exon 6 of *FLI-1*) (Delattre *et al*., 1992) are most commonly detected in patients (Zucman *et al*., 1993). Although similar FLI-1 sequences present in distinct EWS/FLI-1 fusion proteins provide their DNA-binding specificity, they show different transactivation potential and their differential activities correlate with the malignant characteristics of the cells in culture and with the clinical presentation of the tumors (Lin *et al*., 1999). When detected early, localized ES/PNET tumors have a relatively good prognosis, with survival rates of up to 60–70%. However, these tumors metastasize quite efficiently and the treatment of metastatic disease has been rather unsuccessful to date (Rodriguez-Galindo *et al*., 2003). Consequently, understanding the mechanisms that sustain ES/PNET growth and promote metastasis may lead to the identification of novel therapeutic targets and agents that will help in devising more effective treatment protocols.

Recent studies have demonstrated essential roles for signaling pathways triggered by growth factors such as IGF-1 and PDGF-C and their receptors in maintaining the neoplastic phenotype and proliferation of ES/PNET cells (Scotlandi *et al*., 1998; Toretsky *et al*., 1999; Zwerner and May, 2001; Üren *et al*., 2003). Accordingly, it has been proposed (Hidalgo and Rowinsky, 2000) that inhibition of elements common to both signaling systems, such as PI3K, AKT or transducers further downstream, may provide effective antitumor activity in itself and/or enhance the chemotherapeutic efficacy of other agents. The fact that transformation by PI3K and AKT is dependent on the phosphorylation and activation of the 40S ribosomal protein S6 kinase ($p70^{S6K}$) and the phosphorylation of eucaryotic initiation factor 4E-binding protein (4E-BP1) by mTOR (Aoki *et al*., 2001) led us to explore the possible involvement of mTOR in ES/PNET cell proliferation as well as the potential use of the mTOR-specific inhibitor rapamycin

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(Abraham, 1998) as an antitumor agent for ES/PNET tumors. In this study, we assessed the activity of rapamycin against ES tumor cell lines representative of the three most common EWS/FLI-1 fusion types. We demonstrate that rapamycin inhibited the proliferation of all ES cell lines by downregulating the levels of the EWS/FLI-1 fusion proteins, independent of the fusion type involved.

In order to establish a possible role for mTOR in ES cell proliferation, we first determined whether the mTOR protein was expressed, and its phosphorylation status, in cell lines TC-71, SK-ES-1 and A4573, which carry type 1, 2 and 3 EWS/FLI-1 fusions (Delattre *et al*., 1992), respectively. Western analyses with anti-mTOR and anti-phospho-mTOR (Figure 1a) showed that, when growing exponentially, all three lines expressed various levels of mTOR protein and contained detectable levels of its phosphorylated, active form. Interestingly, the levels of phospho-mTOR observed in the three cell lines (greatest in TC71, intermediate in SK-ES-1 and lowest in A4573, Figure 1a) correlated with their growth rates (the respective doubling times were about 20, 31 and 41 h), thus supporting the notion that mTOR may participate in maintaining their proliferation potential. The three ES cell lines were then exposed to rapamycin (10 ng/ml) for different time periods, and the effect of this treatment on cell proliferation was followed by determining viable cell numbers using the trypan blue exclusion assay. Relative to time-matched, untreated controls, cell counts did not increase in treated cultures of any of the three cell lines. This time-dependent, relative decrease reached about 60% by 72 h after treatment (Figure 1b, top panel). This decrease could not be accounted for by a parallel increase in apoptosis, as cell death did not vary significantly during the

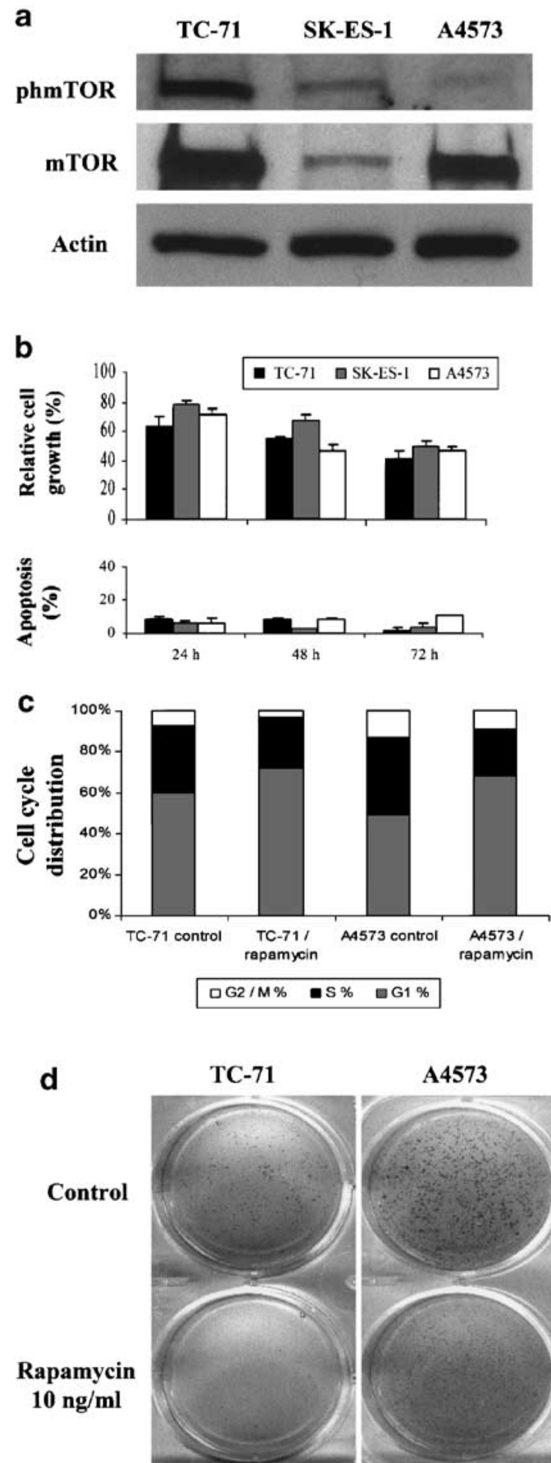

Figure 1 Rapamycin inhibits ES cell proliferation independent of their EWS/FLI-1 fusion type. ES cell lines differed in the content and phosphorylation status of the mTOR protein (**a**), but their proliferation was efficiently inhibited by rapamycin in all cases, with little apoptotic effect (**b**). Cell cycle arrest at G_1 is the mechanism underlying the growth-inhibitory effect (**c**), which is also reflected in the diminished ability of the cells to form colonies in soft agar (**d**). *Methods*: A4573 and SK-ES-1 cells were obtained from Dr Timothy J Kinsella (Case Western Reserve University). TC-71 cells were obtained from Dr Jeffrey A Toretsky (Georgetown University Medical Center). SK-ES-1 and A4573 cells were maintained in DMEM and TC-71 cells were maintained in RPMI 1640. These media (from Biofluids, Rockville, MD, USA) were supplemented with antibiotics and 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA). Cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. For proliferation assays and cell cycle analysis, 10^6 cells were seeded in 100-mm dishes. The following day, the medium was aspirated and replaced by a medium containing rapamycin (10 ng/ml). After different time periods, cells were harvested by trypsinization and washed once with PBS. The numbers of viable cells were determined using the trypan blue (Gibco, Invitrogen Corp., Grand Island, NY, USA) exclusion assay, with the help of a hemocytometer. For cell cycle analysis, cells were harvested 72 h after exposure to rapamycin, washed once in PBS, fixed in citrate buffer, pH 7.6, resuspended in PBS containing 20 μg/ml of propidium iodide (Calbiochem-Novabiochem Corp., San Diego, CA, USA), and incubated for 30 min at 37°C before flow cytometric analysis on a FACScan instrument (Becton Dickinson, San Jose, CA, USA), performed at the Flow Cytometry/Cell Sorting Shared Resource of the Lombardi Cancer Center. Anchorage-independent growth, a well-established criterion for neoplastic transformation, was evaluated by culturing the cells into 0.4% agarose (Invitrogen) in the appropriate complete medium, with a 0.6% agarose underlay. Cell suspensions (10^4 cells/dish) were plated with or without rapamycin (10 ng/ml) and incubated as above. Colonies were counted 14 days after seeding. Rapamycin was purchased from Sigma-Aldrich (St Louis, MO, USA). Western analysis was performed as described in the legend of Figure 2. Antibodies against phospho-mTOR and mTOR were purchased from Cell Signaling Technology (Beverly, MA, USA), and the anti-actin antibody was obtained from Abcam Ltd (Cambridge, UK)

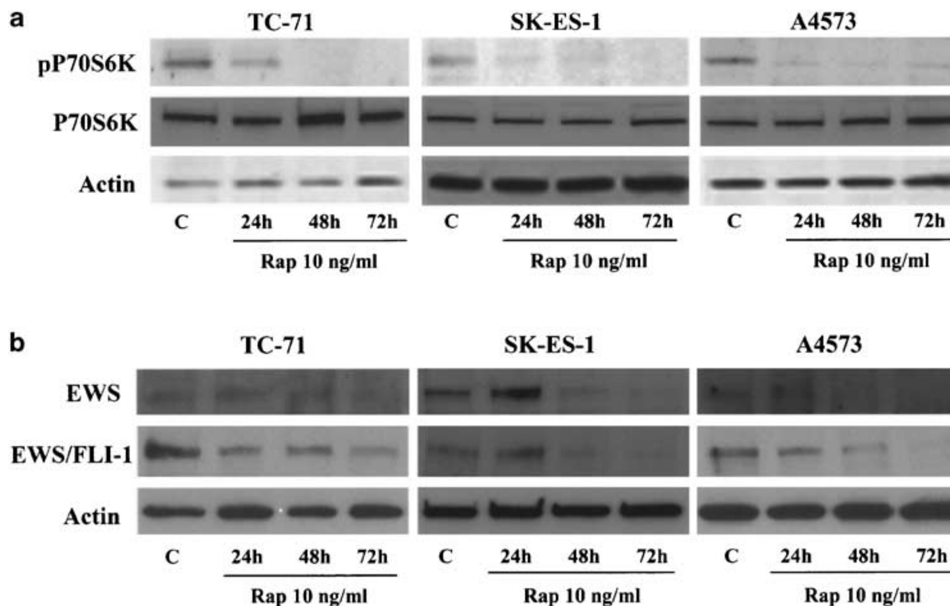

Figure 2 Rapamycin inhibited the phosphorylation of $p70^{S6K}$ (**a**) and downregulated the expression levels of the EWS and EWS/FLI-1 proteins (**b**) in ES cells, regardless of their fusion type. *Methods*: For Western blot analysis, ES cells treated with either rapamycin (10 ng/ml) or vehicle for 24, 48 or 72 h were lysed with RIPA buffer containing protease inhibitors (1 mM PMSF, 10 mg/ml aprotinin and 10 mg/ml leupeptin) and the lysates were centrifuged at 13 000 *g*, at 4°C, for 30 min. The protein contents of the supernatants were determined with the BCA protein assay system (Pierce Endogen, Rockford, IL, USA). Equal amounts of protein (50 μg) from each lysate were resolved by 10% SDS–PAGE and transferred onto nitrocellulose membranes. After blocking with 5% skim milk in PBS containing 0.2% Tween-20, at room temperature for 1 h, membranes were incubated overnight at 4°C with each of the antibodies. Next, the blots were incubated at room temperature for 1 h with an HRP-conjugated secondary antibody (1/2000) and the peroxidase activity was analysed with the ECL chemiluminescent substrate kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Antibodies against $p70^{S6K}$ and phospho-$p70^{S6K}$ were purchased from Cell Signaling Technology. Antibodies to FLI-1, used to detect the EWS/FLI-1 fusions, and EWS were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). β-Actin (Abcam Ltd) detection was used as a loading reference

rapamycin treatment (Figure 1b, bottom panel). Flow-cytometric analysis demonstrated that exposure to rapamycin resulted in cell cycle arrest. After 72 h of rapamycin treatment, about 70% of TC-71 and A4573 cells were arrested in the G_1 phase (Figure 1c). Furthermore, rapamycin treatment also inhibited significantly the anchorage-independent growth of ES cells (Figure 1d), causing a reduction of about 80 and 37% in the number of colonies of TC-71 and A4573 cells, respectively, which grew on soft-agar cultures.

To ascertain that mTOR inhibition mediated the action of rapamycin on ES cell proliferation, we examined its effect on $p70^{S6K}$, a known mTOR target involved in translation initiation and cell cycle G_1/S progression (Lane *et al*., 1993). Treatment of all three ES cell lines with rapamycin resulted in a time-dependent, progressive decrease in $p70^{S6K}$ phosphorylation (Figure 2a). However, rapamycin did not cause any significant alteration of their total $p70^{S6K}$ content. On the other hand, most interestingly, rapamycin exposure also resulted in the progressive decrease over time of the levels of the EWS/FLI-1 proteins present in the three ES cell lines, regardless of the fusion type involved. This effect was most evident after 72 h of rapamycin treatment (Figure 2b). In addition, similar to the EWS/FLI-1 hybrid proteins, the expression levels of the normal EWS protein were also downregulated by rapamycin (Figure 2b). The fact that RT–PCR analyses indicated that *EWS/FLI-1* and *EWS* mRNA levels did not change during rapamycin treatment (data not shown) suggested that a translational or post-translational mechanism mediates their downregulation.

TGF-β type 2 receptor (TGFβ RII) has been described as a direct target of EWS/FLI-1 (Hahm *et al*., 1999) and other EWS-ETS hybrid proteins (Im *et al*., 2000). TGFβ RII expression is transcriptionally repressed in ES cells and tumors as well as in other cell types ectopically expressing the fusion proteins. The repression of TGFβ RII makes ES cells less sensitive to the growth-inhibitory effect of TGFβ and, consequently, provides them with an important proliferative advantage in their conversion to malignancy (Hahm *et al*., 1999; Im *et al*., 2000). Therefore, we examined whether rapamycin treatment would restore TGFβ RII mRNA expression. Results showed that, in agreement with the downregulatory effect on the EWS/FLI-1 repressor molecules (Figure 2b), exposure to 10 ng/ml of rapamycin for 24 h resulted in a marked increase of the expression of TGFβ RII mRNA in the three ES cell lines tested (Figure 3).

Rapamycin is considered as an inhibitor specific for mTOR (Gingras *et al*., 2001). However, recent reports have identified certain effects of rapamycin that suggest the possibility that the drug may also act through alternative, mTOR-independent mechanisms (Van der Poel *et al*., 2003; Visner *et al*., 2003). Consequently, in

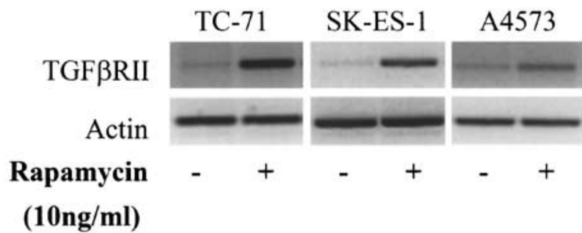

Figure 3 Downregulation of EWS/FLI-1 by rapamycin treatment restores TGFβ RII mRNA expression. TGFβ RII has been found to be repressed by EWS/FLI-1 in ES tumors. Results showed that the expression of TGFβ RII mRNA was increased in all three ES cells after exposure to 10 ng/ml of rapamycin for 24 h. *Methods*: Total RNA (3 μg), obtained from ES cells by means of the 'RNeasy Mini kit' (Qiagen Inc., Valencia, CA, USA), was reverse transcribed using 200 U of Superscript II RNase H-Reverse Transcriptase (Gibco-BRL, Bethesda, MD, USA) in a 20 μl reaction volume, in the presence of 25 μg/ml Oligo (dT), first strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT and 10 mM of each dATP, dGTP, dCTP and dTTP. The RNA and the Oligo (dT) mix were heated at 70°C for 10 min. After cooling to 4°C, the other reagents were added and reverse transcription was performed at 42°C for 50 min. TGFβ RII and Actin PCR primers were designed using Oligo 6.0 software (National Biosciences, Inc., Plymouth, MN, USA), based on GenBank published sequences. The TGFβ RII sequences were amplified using the oligos CTGGTGGGGAAAGGTCGC, as the upper primer, and AGGCAGCAGGTTAGGTCG as the lower. Human actin was used as a standard for competitive RT–PCR measurements and it was amplified using CACTGTGCCCATC-TACGAG and AGGGTGTAAAACGCAGC as the upper and lower primers, respectively. Amplifications were carried out in a 2700 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA, USA) and consisted of 35 cycles. Denaturation was performed at 94°C for 15 s, annealing at 57.4°C and extension at 72°C for 45 s. PCR products were separated on 1.5% agarose gels and quantified using the Molecular Analyst/Macintosh data analysis software and a Bio-Rad Image Analysis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the supplier's instructions and sequenced using an ABI Prism 310 genetic analyzer (Perkin-Elmer Corporation)

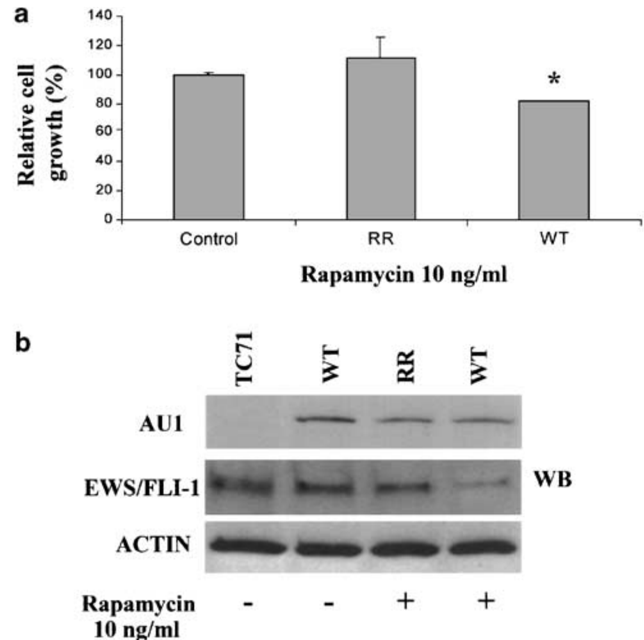

Figure 4 Expression of a rapamycin-resistant (RR) mTOR mutant in TC-71 cells prevented the inhibitory effect of rapamycin on cell proliferation (**a**) and the downregulation of the EWS/FLI-1 protein (**b**). As expected, relative to untreated, untransfected TC-71 cells (Control), the response to rapamycin exposure, for 24 h, of cells transfected with a wild-type (WT) mTOR construct agreed with the data shown in Figure 1, with regard to cell proliferation, and in Figure 2, with regard to the expression of EWS/FLI-1. *Methods*: Expression vectors encoding AU1-tagged wild-type ($AmTOR^{wt}$) and mutant (AmTOR-SI), rapamycin-resistant mTOR cDNAs were generously provided by Dr Robert T Abraham (The Burnham Institute, La Jolla, CA, USA). These vectors and the empty pcDNA3 control plasmid (4 μg of each) were transfected using Lipofectamine (Life Technologies) as recommended by the manufacturers. The transfection efficiency was monitored by Western analysis using a monoclonal antibody specific for the AU-1 tag (Covance, Berkeley, CA, USA). Cell proliferation assays were performed as described in the legend of Figure 1. Western analyses (WB) were carried out as described in the legend of Figure 2. Samples were run in triplicate and experiments were repeated at least three times. An ANOVA was used to assess the statistical significance of differences between group means, and groups were considered significantly different at $P \leqslant 0.02$. *Bars*, $\pm$s.d.; (*), $P \leqslant 0.02$ for test group versus untreated control group

order to demonstrate conclusively a role for mTOR in ES cell proliferation as well as the specificity of rapamycin in downregulating EWS/FLI-1, we transiently transfected TC-71 cells with AU1-tagged constructs for the expression of either wild-type ($AmTOR^{wt}$) or rapamycin-resistant (AmTOR-SI) mTOR, and then treated them with rapamycin (10 ng/ml) for 24 h. The proliferation of cells expressing the rapamycin-resistant mTOR mutant was not affected negatively by the treatment, whereas, relative to untreated control cells, a statistically significant decrease in cell number of about 20% ($P \leqslant 0.02$) was observed in the case of TC-71 cells transfected with wild-type mTOR (Figure 4a). Moreover, Western blot analyses showed no significant evidence of EWS/FLI-1 protein downregulation in cells transfected with rapamycin-resistant mTOR, while a marked decrease in EWS/FLI-1 was observed in TC-71 cells transfected with wild-type mTOR, relative to untransfected cells or to cells transfected with wild type and not treated with rapamycin (Figure 4b).

It is becoming increasingly apparent that rapamycin, or its more soluble analog CCI-779, is a promising tool for the treatment of several cancers (Daniels and Adjei, 2001; Luan *et al*., 2003). As such, they are currently undergoing clinical trials against various tumor types (Elit, 2002). Our results are consistent with the notion that rapamycin represents a novel class of cytostatic anticancer agents that induces G_1 cell cycle arrest of the target cells. Our finding that rapamycin is effective against ES cells, regardless of the type of EWS/FLI-1 that they contain, is particularly significant because ES tumors carrying non-type 1 fusions generally have poorer prognosis and clinical outcomes (De Alava *et al*., 2000). Our results strongly suggest that the inclusion of rapamycin in treatment protocols for patients with localized or metastatic ES tumors could have great beneficial effects.

Several studies reported that the induction of cell cycle arrest by rapamycin resulted from a decrease in the levels of cyclins (Seufferlein and Rozengurt, 1996; Decker *et al*., 2003). Moreover, it has been recently proposed that cyclin-dependent kinase inhibitors and other G_1 regulators are targets of the EWS/FLI-1 transcriptional activity (Matsumoto *et al*., 2001; Nakatani *et al*., 2003). Thus, it appears that, in ES cells, downregulation of EWS/FLI-1 expression may be an important contributor to the observed G_1 cell cycle arrest. This interpretation is in agreement with the previous observation that inhibition of the expression of the fusion protein with specific antisense oligodeoxynucleotides induced G_1 cell cycle arrest (Matsumoto *et al*., 2001). This does not exclude the possibility that abnormalities in G_1 checkpoint regulators known to occur in ES cells (Deneen and Denny, 2001) may also contribute to, and most likely increase their sensitivity to rapamycin. In this regard, the presence in ES cells of altered pRB, p16, p27 or cyclin D1 may be predictors of enhanced rapamycin efficacy (Sausville *et al*., 1999). As EWS/FLI-1 is the main determinant of the malignancy of ES tumors (Arvand and Denny, 2001), rapamycin treatment may have a dual effect as a cytostatic agent and suppressor of the neoplastic phenotype of ES cells.

It has been shown that the treatment of ES cells with antibodies specific to several cell surface receptors downregulated EWS/FLI-1 expression (Scotlandi *et al*., 1998; Girnita *et al*., 2000; Benini *et al*., 2001), suggesting that maintenance of its transforming potential requires specific growth factor-triggered signaling processes, such as the PI3K-AKT or the ERK1/2 MAP kinase pathways. Ours is the first study to investigate a possible role for mTOR signaling in ES cells. The mTOR-p70^{S6K} signaling pathway has been shown to be constitutively activated in several tumor cell lines, including small-cell lung cancer cells and pancreatic cancer cells (Seufferlein and Rozengurt, 1996; Shah *et al*., 2001). Our finding that the expression of a rapamycin-resistant mTOR mutant prevents the downregulation of EWS/FLI-1 and the proliferation blockade induced by rapamycin on ES cells, demonstrates that mTOR plays a central role in regulating the levels of EWS/FLI-1 protein expression. Results from a recent proteomics study on the specific translational control by rapamycin in T cells showed that most proteins totally inhibited by the drug were RNA-binding proteins (Grolleau *et al*., 2002). EWS was identified as a RNA-binding protein (Kim and Pelletier, 1999; Arvand and Denny, 2001) and, in agreement with that function, our results demonstrate that it is also downregulated by rapamycin treatment. However, because the EWS protein is dispensable for Ewing tumor growth (Kovar *et al*., 2001), it is unlikely that its downregulation may play a role in the observed inhibition of ES cell proliferation caused by rapamycin. Nevertheless, it appears that it is the invariable presence of EWS in all types of EWS/FLI-1 fusions that makes these proteins susceptible to downregulation by rapamycin.

One of the major problems in the treatment of the family of ES tumors is that fusion proteins of different types may act over different targets and/or respond to stimulation by different signaling pathways (Aryee *et al*., 2000). Our results demonstrate that mTOR signaling plays an essential role in cell proliferation in ES tumors and strongly support our proposal on the use of rapamycin as a cytostatic agent in the therapy in ES tumors. In addition, rapamycin may be an invaluable tool to identify molecular targets of EWS-FLI-1 proteins of different fusion types. Furthermore, because EWS and other members of its family have been described as partners in chromosomal translocations present in other tumor types (Kim and Pelletier, 1999), it is possible that rapamycin may be also useful in the treatment of those malignancies.

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