Mammalian Target of Rapamycin Contributes to the Acquired Apoptotic Resistance of Human Mesothelioma Multicellular Spheroids*§

Dario Barbone†§, Tsung-Ming Yang†, Jeffrey R. Morgan§, Giovanni Gaudino†, and V. Courtney Broaddus†1

From the†Lung Biology Center, San Francisco General Hospital, University of California, San Francisco, California 94110, the§Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche, Università degli Studi del Piemonte Orientale ‘Amedeo Avogadro’, Novara 28100, Italy, and the¤Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, Rhode Island 02912

When grown as three-dimensional structures, tumor cells can acquire an additional multicellular resistance to apoptosis that may mimic the chemoresistance found in solid tumors. We developed a multicellular spheroid model of malignant mesothelioma to investigate molecular mechanisms of acquired apoptotic resistance. We found that mesothelioma cell lines, when grown as multicellular spheroids, acquired resistance to a variety of apoptotic stimuli, including combinations of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), ribotoxic stressors, histone deacetylase, and proteasome inhibitors, that were highly effective against mesothelioma cells when grown as monolayers. Inhibitors of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway, particularly rapamycin, blocked much of the acquired resistance of the spheroids, suggesting a key role for mTOR. Knockdown by small interference RNA of S6K, a major downstream target of the mTOR pathway; however, isolation of the apoptotic pathways by TRAIL-induced caspase-8 cleavage in spheroids, suggesting initial that mTOR inhibited apoptosis by actions at the death receptor pathway; however, isolation of the apoptotic pathways by means of Bid knockdown ablated this effect showing that mTOR actually controls a step distal to Bid, probably at the level of the mitochondria. In sum, mTOR and S6K contribute to the apoptotic resistance of mesothelioma cells in three-dimensional, not in two-dimensional, cultures. The three-dimensional model may reflect a more clinically relevant in vitro setting in which mTOR exhibits anti-apoptotic properties.

Resistance to apoptosis is a distinguishing characteristic of all tumors (1) and underlies their resistance to therapy (2). Understanding the molecular foundations of apoptotic resistance could lead to improvements in the utility of current therapies or provide new therapies altogether. In vitro, apoptotic resistance of tumor cells is usually studied using two-dimensional monolayers. However, cells can attain increased resistance when grown in three-dimensional structures, a phenomenon referred as the acquisition of multicellular resistance (3). This acquired property of tumor cells may help explain why promising findings from in vitro studies have not been easily translated into therapy (4). Consequently, in the last decade, multicellular spheroids have become a valuable tool in the study of solid tumors by representing a three-dimensional system of intermediate cellular complexity between monolayer cell cultures and tumors in vivo (5, 6).

Human malignant pleural mesothelioma, an aggressive thoracic cancer that arises from the pleural mesothelium, is characterized by a profound resistance to standard anti-neoplastic therapies. At present, no curative therapy is available (7). To investigate the apoptotic resistance of mesothelioma, we are increasingly utilizing in vitro three-dimensional models. Indeed, the use of a three-dimensional model appears highly appropriate to mesothelioma, a tumor that arises from a two-dimensional mesothelial layer into a solid, dense three-dimensional structure and that naturally forms aggregates in vivo, a feature once considered diagnostic (8, 9). In previous studies using three-dimensional tumor fragment spheroids grown from human mesothelioma tumor, we showed that mesothelioma cells demonstrated a high degree of resistance compared with mesothelioma cells in monolayer culture (10). Using these tumor fragment spheroids, we previously found that blockade of the Akt/mTOR pathway sensitized mesothelioma cells to treatment (10), suggesting that some of the resistance of tumor cells in their tissue environment derived from the PI3K/Akt/mTOR pathway. Limited by the complexity of tumor fragments, we decided to develop a more tractable in vitro model of multicellular spheroids to investigate the role of this pathway further.

The PI3K/Akt/mTOR pathway plays a pivotal role in tumor cell survival and resistance to chemotherapy (11) and is acti-

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 CA95671 (to V. C. B.). This work was also supported by a Buzzi-Unicem Foundation grant (to G. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

† To whom correspondence should be addressed: San Francisco General Hospital, University of California, 1001 Potrero Ave., Bldg. 1, Rm. 150, San Francisco, CA 94110. Tel.: 415-206-6656; Fax: 415-206-4123; E-mail: cbroaddus@medsfgh.ucsf.edu.

2 The abbreviations used are: mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; siRNA, small interference RNA; P-Akt, Akt phosphorylation; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly(ADP-Ribose)polymerase; Z, benzoxycarbonyl; fmk, fluoromethyl ketone; PTEN, phosphatase and tensin homolog.
mTOR Supports Acquired Survival of Mesothelioma Spheroids

vated in most tumors (12), including mesothelioma (13, 14). The roles of Akt and its myriad downstream targets are complex and interrelated (15), opening possibilities for specific targeting of certain functions. For example, in studies using animal models, the key tumorigenic and anti-apoptotic actions of Akt were shown to be mediated by its downstream kinase, mTOR (16, 17). From these and others studies, mTOR has been found to mediate survival, through either of its two downstream targets, S6K or 4E-BP1/elF4E (18, 19), suggesting that blockade of mTOR would be highly beneficial. Blockade of mTOR, however, can also lead to a rebound upstream activation of Akt that can perhaps negate or limit the effects of mTOR inhibition, especially if Akt has survival activity separate from its effects via mTOR (20). Nonetheless, finding an important role for Akt or for mTOR in mesothelioma cell survival would open the door to the use of promising highly specific and multitargeted inhibitors (21).

In this study, based on our prior work in human tumor fragments, we have developed an in vitro three-dimensional model using mesothelioma cell lines to determine whether mesothelioma cells acquire multicellular apoptotic resistance. Then, using pharmacologic inhibition and RNA interference, we evaluated the contribution of the PI3K/Akt/mTOR pathway to this resistance and discovered an important role for the kinase, mTOR. Finally, we investigated the step(s) in the apoptotic pathways where mTOR exerts its anti-apoptotic effects.

**EXPERIMENTAL PROCEDURES**

**Reagents and Inhibitors**

Inhibitors and apoptotic agents were purchased and prepared in DMSO as follows, with the stock concentration shown: rapamycin (5 mM), LY294002 (10 mM), MG-132 (20 mM), trichostatin A (10 mM), anisomycin (5 mg/ml) (all from Sigma-Aldrich) and PI-103 (1 mM) (a kind gift of Dr. Kevan Shokat, San Francisco, CA). Sodium butyrate (1 mM stock solution) (Sigma-Aldrich) was prepared in water. Final concentrations of inhibitors and apoptotic agents were used at recommended dilutions.

**Spheroid Generation**

Spheroids of a consistent cell number and size were generated in non-adhesive round bottomed 96-well plates. The 96-well plates were rendered non-adhesive by coating them with a 1:24 dilution of polyHEMA (120 mg/ml, Sigma-Aldrich) in 95% ethanol and drying them at 37 °C for 48 h (27). Plates were sterilized by UV light for 30 min prior to use. To generate spheroids and monolayers, cells were plated 24 h before each experiment; cells were added into each well of the 96-well plates to form spheroids or into each well of cell culture-treated 6-well plates to form monolayers. Then, before each experiment, 15–20 spheroids were transferred to each well of a 24-well polyHEMA-coated plate. The number of spheroids per well was chosen to match the numbers of cells plated per well in the monolayers (150,000–200,000). Apoptotic agents with or without inhibitors (and the appropriate DMSO vehicle control) were added to spheroids and to monolayers at the same time.

Microspheroids generated from an average of 300 cells per spheroid were produced as described (28). In brief, 3% agarose gels (Ultrapure agarose, Invitrogen) were cast using micromolds (Dr. Jeffrey Morgan, Brown University). After setting, gels (Ultrapure agarose, Invitrogen) were cast using micromolds by centrifugation were then transferred to 24-well plates to form spheroids or into each well of cell culture-treated 6-well plates to form monolayers. Then, before each experiment, 15–20 spheroids were transferred to each well of a 24-well polyHEMA-coated plate. The number of spheroids per well was chosen to match the numbers of cells plated per well in the monolayers (150,000–200,000). Apoptotic agents with or without inhibitors (and the appropriate DMSO vehicle control) were added to spheroids and to monolayers at the same time.

Microspheroids generated from an average of 300 cells per spheroid were produced as described (28). In brief, 3% agarose gels (Ultrapure agarose, Invitrogen) were cast using micromolds (Dr. Jeffrey Morgan, Brown University). After setting, gels were equilibrated overnight with culture medium. Cells were trypsinized, counted, and resuspended to the desired cell density and then overlaid onto the gel, where cells sedimented into calibrated recesses. Microspheroids recovered from micromolds by centrifugation were then transferred to 24-well polyHEMA-coated plates for experiments, as above.

**Antibodies**

For immunoblotting, antibodies used to detect phospho-Akt (Ser-473, #4056, Thr-308 #2972), phospho-S6K (Thr-389, #9206), phospho-4E-BP1 (Thr-37/46, #9459), phospho-PTEN (Ser-380, #9551), phospho-Bad (Ser-136, #9295), Akt (#9272), S6K (#9202), 4E-BP1 (#9452), PTEN (#9559), Bad (#9292), cleaved caspase-8 (#9496), Bcl-2 (#2872), Bcl-XL (#2762), Mcl-1 (#4572), Bax (#2772), Bak (#3792), PARP (#9532), and survivin (#2808) were from Cell Signaling Technology (Beverly, MA). α-Tubulin antibody (#T-6074) was from Sigma-Aldrich. The anti-FLIP<sub>S</sub> antibody (#5276) was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-BIM antibody (#559685) was from BD Pharmingen (San Jose, CA). Antibodies were used at recommended dilutions.

For immunohistochemistry, antibodies used to detect phospho-Akt (Ser-473, #3787, 1:50) or phospho-S6K (Thr-389, #9206, 1:100) were from Cell Signaling Technology, and the antibody to detect TRAIL (biotinylated anti-rhTRAIL, #BAF375, 1:50) was from R&D Systems.

**Cell Cultures**

The human mesothelioma cell lines M28 (from Dr. Brenda Gerwin, NCI, National Institutes of Health, Bethesda, MD), REN (from Dr. Roy Smythe, University of Texas M.D. Anderson Cancer Center, Houston, TX), and VAMT (from Dr. B. Gerwin, NCI, NIH) were all cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a 37 °C humidified incubator with 5% CO<sub>2</sub>. Normal human mesothelial cells were cultured from ascites fluid from patients without infection or malignancy according to a protocol approved by the UCSF Committee on Human Research. All cells were confirmed to be negative for mycoplasma every 2 months by PCR analysis as previously described (26).

**RNA Interference**

M28, REN, or VAMT cells (5 × 10<sup>6</sup>) were pelleted and resuspended in 100 μl of nucleofection buffer (solution V, Amaxa Biosystems, Cologne, Germany) with 3 μg of the appropriate siRNA duplex. This suspension was transferred to a sterile cuvette and nucleofected using program T-20 on a Nucleofector II device (Amaxa Biosystems). After recovery for 30 min in complete Dulbecco’s modified Eagle’s medium, the cells were...
plated and allowed to grow for 24 h. Cells were then trypsinized, counted, and plated as monolayers and spheroids for 24 h and exposed to apoptotic stimuli (48 h after transfection). The siRNA sequences (Ambion, TX) were: S6K #1, CUG UUA GUU UCA AUU GAC CaG CdTdT; S6K #2 (to a non-overlapping sequence), AAA CAC UGG UGc CAU GcU GUC CdTdT; Bid, UAU UCC GGA UGG UGc UUU CdTdT; and scramble, ACG UGA CAC GUU CGG AGA AdTdT.

**Analysis of Apoptosis**

*Annexin V Binding*—Monolayers and spheroids were exposed to apoptotic stimuli, trypsinized under identical conditions and then resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Cells were stained with annexin V and propidium iodide and analyzed, using standard techniques as described previously (29).

*Hoechst Staining*—After the indicated apoptotic stimuli, monolayers and spheroids were disaggregated to single cells with trypsin under identical conditions, taking ~5 min (Invitrogen). Trypsin was inactivated with serum-containing medium, and cells were pelleted at 2000 revolutions/minute for 10 min at 4 °C, fixed with 2.5% glutaraldehyde (Sigma-Aldrich), stained with 8 µg/ml Hoechst 33342 (Molecular Probes, Invitrogen), and placed onto slides. For each condition, at least 100 cells were counted in triplicate by investigators blinded to the experimental conditions. Cells with distinctive signs of nuclear condensation were considered apoptotic. After direct comparison with the annexin V assay, we found Hoechst staining to be a more consistent and reliable quantitative method for assessing apoptosis in both spheroids and monolayers and used it for the analysis of most experiments.

**Immunohistochemistry**

Spheroids were collected, fixed in 10% formalin in PBS overnight at 4 °C, and set in 5% agar pellets that were embedded in paraffin. From the paraffin blocks, 8 µm sections were cut and mounted on glass slides, deparaffinized, rehydrated, and boiled in sodium citrate solution (pH 6.0) with 0.1% Tween 20 in a pressure cooker for 10 min for antigen retrieval. After cooling 30 min at room temperature, sections were blocked with hydrogen peroxide solution for 20 min to remove endogenous peroxidase. Primary antibodies to P-Akt or P-S6K were applied overnight at 4 °C. The secondary antibody conjugated to a horseradish peroxidase-labeled polymer was applied for 30 min and detected by the 3,3-diaminobenzidine tetrahydrochloride hydroperoxide (Pierce). For each experiment, a representative blot is shown as a loading control; a representative blot is shown.) 1B. Spheroids generated with 10,000 cells per spheroid showed consistent shape and size. Representative images of spheroids formed by 10,000 M28, REN, and VAMT cells at 24 h, viewed from above by inverted phase contrast microscopy of spheroids growing in wells and from the side (at left) by light microscopy of a paraffin-embedded section of an M28 spheroid. Spheroids were between 500 and 1000 µm in greatest width, and 100 µm in thickness. REN and VAMT spheroids were of a similar thickness, despite their smaller widths.

**Statistical Analysis**

Data from each experiment are expressed as mean ± one S.D. of at least three different experiments. One- or two-way analysis of variance was used to evaluate statistical significance, and the Tukey test was performed to detect where the differences lay (Prism version 4.0, GraphPad Software, Inc.). A p value of <0.05 was considered significant; the significance reached is specified in the text.

**RESULTS**

Mesothelioma Cells Readily Formed Multicellular Spheroids within 24 h—To determine the characteristics and kinetics of spheroid formation by mesothelioma cells, we used two epithe-
Spheroids can be generated by various methods (30); after evaluating several techniques, we found that plating cells in polyHEMA-coated non-adhesive 96-well plates generated the most reproducible and consistent spheroids (27). When seeded in these non-adhesive conditions, cells from all tested mesothelioma cell lines aggregated and formed multicellular spheroids within 24 h. Spheroids of a range of cell numbers were tested for the presence of baseline apoptosis. Spheroids formed from 25,000 cells or greater showed baseline cleavage of PARP in M28 and REN cell lines as evidence of apoptosis (Fig. 1A). In addition, because spheroids formed from 5,000 or fewer cells by this technique were loosely formed, we chose spheroids formed from 10,000 cells each for further experiments (Fig. 1B). Spheroids assumed a discoid shape with a width of 500–1000 μm and an average thickness of 100 μm, a shape we considered an advantage for minimizing diffusion distances. To confirm that acquired resistance was not dependent on a particular size or shape, we also generated microspheroids of ~300 cells per spheroid by means of micromolded non-adhesive hydrogels (28) and utilized them in some of the apoptosis studies (supplemental Fig. S1A). These microspheroids were spherical in shape, with most in the range of 50–100 μm in diameter and a few with a maximal diameter of 150 μm. Thus, in both three-dimensional structures, the mean maximal diffusion distance would be 25–75 μm, a distance in which cells can be sustained by diffusion alone (31).

Mesothelioma Cells in Spheroids Acquire Resistance to Diverse Apoptotic Stimuli—Our group and others have found that mesothelioma cells in monolayer culture undergo apoptosis in response to combinatorial strategies using TRAIL plus either DNA or other damaging agents (23, 32) or histone deacetylase or proteasome inhibitors (33, 34). To assess whether mesothelioma three-dimensional cell structures acquired multicellular resistance, we tested these combinations of apoptotic agents on cells grown as monolayers and as spheroids. Compared with monolayers, spheroids from all cell lines showed a significant resistance to these TRAIL-containing regimens (Fig. 2A).

To determine whether apoptotic resistance was limited to TRAIL and its engagement of the extrinsic apoptotic pathway, we sought non-TRAIL agents that would induce apoptosis in mTOR Supports Acquired Survival of Mesothelioma Spheroids

M28, REN, and VAMT monolayers and spheroids exposed to TRAIL (1 ng/ml) plus the proteasome inhibitor MG-132 (2.5 μM) or the histone deacetylase inhibitor trichostatin A (250 nM) for 24 h were studied for apoptosis using annexin V staining and confirmed by examination of condensed nuclei following Hoechst staining. Spheroids consistently showed resistance to the different apoptotic stimuli in all cell lines tested (n = 3, *p < 0.005 monolayer versus spheroids, mean ± S.D.). B, TRAIL diffuses uniformly within spheroids. After M28 spheroids were exposed to TRAIL (1 ng/ml) for 6 h, immunohistochemistry was performed to detect TRAIL within spheroids. TRAIL diffusion into the spheroids was complete within 6 h as demonstrated by the uniform staining of spheroids exposed to TRAIL, with no staining seen in unexposed spheroids (control) (representative images of four separate experiments). C, spheroids also display resistance to non-TRAIL combinations at 24 h. Combinations of non-TRAIL apoptotic agents that were found to induce apoptosis in mesothelioma cells in monolayers were then tested on monolayers and spheroids of M28, REN, and VAMT cells. Monolayer cells underwent apoptosis following MG-132 (2.5 μM) together with either sodium butyrate (10 mM) or trichostatin A (250 mM), whereas the same cells grown as spheroids were relatively resistant. Each agent, MG-132, trichostatin A, and sodium butyrate, had little effect alone, with apoptosis being <10% (not shown). Apoptosis was measured by quantification of nuclear condensation of Hoechst-stained cells, in this and remaining apoptosis studies (n = 3, *p < 0.01 monolayer versus spheroids, mean ± S.D.). D, spheroids are also resistant to TRAIL plus the sensitizing agent, anisomycin, at 6 h. After exposure to TRAIL (1 ng/ml) plus anisomycin (25 ng/ml) for 6 h, all three mesothelioma cell lines showed apoptosis, whereas spheroids displayed resistance. The same resistance was seen at 12 and 24 h (not shown) (n = 3, *p < 0.001 monolayer versus spheroids, mean ± S.D.).
mTOR Supports Acquired Survival of Mesothelioma Spheroids

For further studies of acquired resistance, we chose to use the combination of TRAIL with anisomycin at a low concentration (25 ng/ml) that we have shown induces JNK activation without toxicity (23) (see also supplemental Fig. S5). The combination of TRAIL plus anisomycin induces apoptosis rapidly and consistently in our mesothelioma cells (23). In response to TRAIL plus anisomycin, monolayers demonstrated extensive apoptosis after only 6 h, whereas spheroids, whether treated for 6 h or up to 24 h (data not shown), failed to undergo apoptosis (Fig. 2D). TRAIL plus anisomycin was chosen for further experiments.

Inhibitors of PI3K/Akt/mTOR Reduce the Apoptotic Resistance of Multicellular Spheroids—Our laboratory has previously reported that inhibitors of the PI3K/Akt/mTOR pathway are able to sensitize spheroids obtained from actual human mesothelioma tumor to TRAIL-containing apoptotic combinations (10). Thus, we aimed to investigate the effectiveness of these inhibitors in the multicellular spheroids. Inhibitors included rapamycin, a highly specific inhibitor of one complex of mTOR (TORC1), PI-103, a novel dual inhibitor of PI3K and both mTOR complexes (TORC1 and TORC2) (22), and LY294002, a broader and less specific inhibitor of PI3K (37). At baseline, although all three cell lines showed high levels of S6K phosphorylation (P-S6K), only two of the three showed Akt phosphorylation (P-Akt) (Fig. 3A). Rapamycin completely blocked P-S6K, as evidence of effective mTOR inhibition and consistently resulted in increased P-Akt (Ser-473), a positive feedback described in other systems (38, 39). PI-103 completely blocked both P-S6K and P-Akt. LY294002 inhibited P-Akt and reduced but did not completely block P-S6K, suggesting other inputs to S6K activation other than Akt (40). Similar findings were observed in spheroids (data not shown).

To learn whether inhibition of the PI3K/Akt/mTOR pathway reduced apoptotic resistance of spheroids, we then tested the inhibitors in monolayers and spheroids of the three cell lines exposed to TRAIL plus anisomycin. The inhibitors had little effect on the apoptotic response of monolayers but consistently reduced the resistance of spheroids to TRAIL plus anisomycin and tested them in spheroids. As in our prior experience, no single agent (MG-132, gemcitabine, trichostatin A, sodium butyrate, or etoposide) was found to induce apoptosis in mesothelioma cell monolayers (data not shown). Combinations of every two agents were then tried; MG-132 plus trichostatin A or MG-132 plus sodium butyrate were the most effective at inducing apoptosis of cells in monolayers. When these apoptotic stimuli were tested in spheroids, all cell lines consistently showed resistance (Fig. 2C), indicating that acquired resistance was a general feature and was not limited to TRAIL-containing therapies.

For further studies of acquired resistance, we chose to use the combination of TRAIL with anisomycin at a low concentration (25 ng/ml) that we have shown induces JNK activation without toxicity (23) (see also supplemental Fig. S5). The combination of TRAIL plus anisomycin induces apoptosis rapidly and consistently in our mesothelioma cells (23). In response to TRAIL plus anisomycin, monolayers demonstrated extensive apoptosis after only 6 h, whereas spheroids, whether treated for 6 h or up to 24 h (data not shown), failed to undergo apoptosis (Fig. 2D). TRAIL plus anisomycin was chosen for further experiments.

Inhibitors of PI3K/Akt/mTOR Reduce the Apoptotic Resistance of Multicellular Spheroids—Our laboratory has previously reported that inhibitors of the PI3K/Akt/mTOR pathway are able to sensitize spheroids obtained from actual human mesothelioma tumor to TRAIL-containing apoptotic combinations (10). Thus, we aimed to investigate the effectiveness of these inhibitors in the multicellular spheroids. Inhibitors included rapamycin, a highly specific inhibitor of one complex of mTOR (TORC1), PI-103, a novel dual inhibitor of PI3K and both mTOR complexes (TORC1 and TORC2) (22), and LY294002, a broader and less specific inhibitor of PI3K (37). At baseline, although all three cell lines showed high levels of S6K phosphorylation (P-S6K), only two of the three showed Akt phosphorylation (P-Akt) (Fig. 3A). Rapamycin completely blocked P-S6K, as evidence of effective mTOR inhibition and consistently resulted in increased P-Akt (Ser-473), a positive feedback described in other systems (38, 39). PI-103 completely blocked both P-S6K and P-Akt. LY294002 inhibited P-Akt and reduced but did not completely block P-S6K, suggesting other inputs to S6K activation other than Akt (40). Similar findings were observed in spheroids (data not shown).

To learn whether inhibition of the PI3K/Akt/mTOR pathway reduced apoptotic resistance of spheroids, we then tested the inhibitors in monolayers and spheroids of the three cell lines exposed to TRAIL plus anisomycin. The inhibitors had little effect on the apoptotic response of monolayers but consistently reduced the resistance of spheroids to TRAIL plus anisomycin and tested them in spheroids. As in our prior experience, no single agent (MG-132, gemcitabine, trichostatin A, sodium butyrate, or etoposide) was found to induce apoptosis in mesothelioma cell monolayers (data not shown). Combinations of every two agents were then tried; MG-132 plus trichostatin A or MG-132 plus sodium butyrate were the most effective at inducing apoptosis of cells in monolayers. When these apoptotic stimuli were tested in spheroids, all cell lines consistently showed resistance (Fig. 2C), indicating that acquired resistance was a general feature and was not limited to TRAIL-containing therapies.
mTOR Supports Acquired Survival of Mesothelioma Spheroids

FIGURE 4. In spheroids, the baseline activity of Akt/mTOR pathway is down-regulated but remains more activated than in non-malignant mesothelial cells. A, Akt/mTOR activity in spheroids is reduced compared with monolayers. Akt/mTOR pathway activity of M28, REN, and VAMT mesothelioma cells was reduced in the spheroids (s) as demonstrated by a lower expression of P-Akt (Ser-473), P-S6K (Thr-389), and P-4E-BP1 (Thr-37/46) compared with that in the monolayers (m). The total levels of Akt, S6K, and 4E-BP1 show no consistent change, and the tubulin levels confirm equal loading. B, Akt/mTOR activity in spheroids is greater than in normal mesothelial cells. Normal mesothelial cells (NMC) monolayers and both monolayers and spheroids from M28, REN, and VAMT cells were analyzed by immunoblot for P-Akt (Ser-473 or Thr-308) and P-S6K. In this experiment, cells were grown in reduced serum (2% fetal bovine serum) to reduce growth factor stimulation. P-Akt (Ser-473), P-S6K, and P-4E-BP1 show no consistent change, and the tubulin levels confirm equal loading. The P-Akt panel demonstrates a greater activation of the pathway than in their non-malignant counterparts. Below, the P-S6K panel has been overexposed to show more clearly the higher level of P-S6K in spheroids than in normal mesothelial cells. C, Akt and S6K phosphorylation is uniform throughout the spheroid. Staining of M28 spheroids with antibodies against P-Akt (Ser-473) and P-S6K (Thr-389) shows a uniform pattern of Akt and S6K phosphorylation throughout the spheroid. For the P-Akt, specificity is shown by a lack of staining with inclusion of a P-Akt Ser-473 blocking peptide (negative control). For the P-S6K staining, no primary antibody was used as a control and resulted in no staining (not shown). Two magnifications (20× and 40×) are shown.

somycin (Fig. 3B). Apoptosis was also confirmed with analysis of PARP cleavage by immunoblot (Fig. 3C). As expected, rapamycin also reduced apoptotic resistance of M28 cells when grown as microspheroids (supplemental Fig. S1B).

We concluded from these results that the PI3K/Akt/mTOR pathway contributes to acquired apoptotic resistance and that, because rapamycin was as effective as other inhibitors with a broader range of targets, the resistance due to PI3K/Akt/mTOR was mostly mediated by mTOR. A major role for mTOR was further supported by considering that mTOR inhibition with rapamycin reduced acquired apoptotic resistance despite an associated increase of P-Akt (see Fig. 3A).

The apoptotic resistance was calculated as the difference between the percentage of apoptosis for monolayer and spheroid divided by that of the monolayer and shown for TRAIL plus ansomycin (the experiments presented in Fig. 3B) and for additional experiments using trichostatin A plus MG-132. Overall, rapamycin reduced the apoptotic resistance of spheroids by ~40%, from 73 ± 14% to 44 ± 17% (Fig. 3D). Because PI-103, despite its broader inhibitory effect, was not significantly more effective than rapamycin, we decided to focus on mTOR by using rapamycin for further studies.

PI3K/Akt/mTOR Activity Is Down-regulated in Spheroids Compared with Monolayers but Remains Higher Than in Normal Mesothelial Cells—Because inhibitors had a greater effect in spheroids than in monolayers, we evaluated the activity of the PI3K/Akt/mTOR pathway in spheroids. Somewhat surprisingly, the baseline phosphorylation of PI3K/Akt/mTOR pathway members in all spheroids was reduced compared with that of the monolayers (Fig. 4A). Nonetheless, the levels of P-Akt, detected by phosphorylation at either Ser-473 or Thr-308, and P-S6K in spheroids were clearly elevated compared with that of normal mesothelial cells (Fig. 4B).

To determine whether spheroids displayed regional variation in activity of this pathway, we stained M28 spheroids with phospho-specific antibodies to localize P-Akt and P-S6K. P-Akt and P-S6K were found to be uniform throughout the spheroid (Fig. 4C).

S6K Mediates Apoptotic Resistance in Spheroids—To identify the molecular pathway underlying the resistance to apoptosis displayed by spheroids and to confirm a role for mTOR, we interrupted mTOR signaling more specifically by ablating the activity of one of its downstream effectors. We selected S6K as the target for ablation, because it has been recognized as a major kinase involved in many diseases, including cancer (41), and is known to be the target of mTOR that is fully inhibited by rapamycin (42). The other major target of mTOR, 4E-BP1, has rapamycin-insensitive phosphorylation sites and thus may not be fully inhibited by rapamycin (43). Furthermore, interference with 4E-BP1/eIF4E results in phosphorylation events downstream of mTOR by unknown mechanisms (19, 44).

We utilized an RNA interference strategy to knock down S6K for the time needed for spheroid formation and for exposure to
TRAIL plus anisomycin. Knockdown of S6K significantly increased the apoptotic response of spheroids to TRAIL plus anisomycin when compared with control spheroids (Fig. 5). Moreover, the S6K knockdown reproduced the effects that rapamycin had on control spheroids, and the addition of rapamycin to S6K-kd spheroids did not further increase their apoptotic rate, suggesting that the effects of S6K-kd and rapamycin were identical. The same results were obtained using an siRNA duplex targeting a separate, non-overlapping region of S6K mRNA (data not shown). Data are shown for M28 cells; similar results were seen in REN and VAMT cells (not shown).

Bcl-2 and FLIPs Are Up-regulated in Some Spheroids but Are Not Modulated by Rapamycin or S6K Knockdown—To find possible mediators of the mTOR-dependent resistance, we measured the levels of a panel of pro-/anti-apoptotic proteins known to be important in mesothelioma resistance to apoptosis (45). Two anti-apoptotic proteins, Bcl-2 and FLIPs, which could account for acquired resistance, were up-regulated in two of the three cell lines displaying increased levels of FLIPs and Bcl-2 proteins. The levels of other proteins did not show a consistent change that would account for acquired resistance in spheroids. mTOR inhibition does not modulate FLIPs and Bcl-2 levels. Neither pharmacological nor molecular inhibition of mTOR signaling modified FLIPs and Bcl-2 protein levels as evidenced by immunoblot analysis of monolayers and spheroids formed from control or S6K-kd M28 cells, treated with or without rapamycin (5 nM) for 4 h.

Reduced caspase-8 cleavage compared with monolayers; mTOR inhibition restored caspase-8 cleavage and apoptosis (Fig. 7A). We considered that the increase in TRAIL-induced caspase-8 cleavage after mTOR inhibition could indicate either an action of mTOR at the level of the death receptor or alternatively at the level of the mitochondria, where amplification of apoptotic signals leads to activation of multiple caspases leading to a feedback activation and cleavage of caspase-8 (47) (Fig. 8).

We then ablated the pro-apoptotic protein Bid (Bid-kd) using RNA interference in order to block the mitochondrial amplification step downstream of TRAIL and to assess whether mTOR was acting proximal to Bid, at the level of death receptors, or distal to Bid, at the mitochondria (see Fig. 8). In control cells, as expected, cells in monolayer culture were sensitive to TRAIL plus anisomycin-induced apoptosis, whereas cells in spheroids were resistant but rendered more sensitive after rapamycin. In cells without Bid, however, monolayers and spheroids failed to undergo apoptosis after TRAIL plus anisomycin, and rapamycin did not increase the apoptotic rate in the spheroids (Fig. 7B). We then evaluated caspase-8 cleavage in Bid-kd spheroids following TRAIL plus anisomycin with or without rapamycin. Importantly, the ability of rapamycin to increase caspase-8 cleavage in the control spheroids following...
mTOR Supports Acquired Survival of Mesothelioma Spheroids

FIGURE 7. Rapamycin increases caspase-8 cleavage in spheroids by actions distal to Bid, presumably at the mitochondria. A, rapamycin increases TRAIL-induced caspase-8 cleavage in spheroids. Spheroids but not monolayers grown from M28 cells displayed increased caspase-8 cleavage when rapamycin (5 nM) was added to TRAIL plus anisomycin, as shown by the appearance of p26 and p41/43 caspase-8 fragments in the immunoblot performed after 6 h. The increase in caspase-8 cleavage was associated with increases in apoptosis, as shown by PARP cleavage. B, rapamycin increases apoptosis in spheroids by actions requiring Bid. Bid was knocked down by siRNA to isolate the death receptor from the mitochondrial apoptotic pathways. Upper panel, in control M28 cells, as expected, monolayer cells were sensitive to TRAIL plus anisomycin-induced apoptosis, whereas spheroid cells were resistant but rendered more sensitive after rapamycin. In cells without Bid, however, monolayers and spheroids failed to undergo apoptosis after TRAIL plus anisomycin, and rapamycin did not increase the apoptotic rate in the spheroids (n = 3, *p ≤ 0.001 no inhibitor versus rapamycin, mean ± S.D.). Lower panel, Bid was effectively ablated by siRNA, as measured 48 h after transfection, the time when agents are added. C, rapamycin increases caspase-8 cleavage in spheroids by actions distal to Bid. Bid was knocked down by siRNA to permit identification of the site of action of rapamycin. Bid-kd prevented the increase in caspase-8 cleavage induced by rapamycin in control spheroids treated with TRAIL plus anisomycin. Because Bid is necessary for the rapamycin-induced increase in caspase-8 cleavage, mTOR is acting, not at the death receptor pathway, but distal to Bid. Caspase-8 cleavage paralleled apoptosis as shown by PARP cleavage. Addition of z-VAD-fmk (50 μM) inhibited both caspase-8 cleavage and apoptosis.

FIGURE 8. mTOR/S6K contributes to acquired multicellular apoptotic resistance at the level of the mitochondria. Scheme of the apoptotic signaling circuitry activated by TRAIL plus mitochondria sensitzers or stressors. Extrinsic (death receptor) and intrinsic (mitochondrial) apoptotic pathways, linked by the BH3-molecule Bid, can activate an effective apoptosis in mesothelioma cells (47). Truncated Bid (tBid), generated by caspase-8 activated at the death-inducing signaling complex (DISC) by TRAIL, signals to the mitochondria. When the mitochondrial threshold for apoptosis is lowered by concurrent DNA damaging or other sensitizers such as anisomycin, the mitochondria can respond to tBid and activate downstream caspases and activate caspase-8 by feedback. In our study, Bid was necessary for the ability of rapamycin to enhance caspase-8 cleavage and apoptosis indicating that mTOR/S6K acted distal to Bid. (Schematic diagram modified from Refs. 23 and 47).

TRAIL plus anisomycin was lost in the Bid-kd spheroids (Fig. 7C). The fact that Bid was absolutely required in order for rapamycin to enhance apoptosis and caspase-8 cleavage indicates that mTOR inhibits an apoptotic step distal to Bid, presumably at the level of the mitochondria, not at the death receptor.

DISCUSSION

A compelling strategy for cancer therapy is to undermine cancer’s extensive defenses against apoptosis, thereby either killing cancer cells outright or lowering their defenses against standard therapies (48). Understanding the molecular mechanisms of apoptotic resistance thus assumes an important role in counteracting cancer resistance to therapy. It has become apparent in recent years that the standard two-dimensional monolayer in vitro model does not exhibit the full range of resistance to apoptosis seen in human cancers (49). Cancer cells have been found to acquire apoptotic resistance when grown as three-dimensional structures that may model the apoptotic resistance of human solid tumors (3).

In our study, by using a three-dimensional system, we have identified a role for mTOR in cell survival that was not apparent in the same cells grown as monolayers. Indeed, this strategy has allowed us to identify mTOR as a contributor to multicellular resistance to apoptosis of mesothelioma thereby suggesting that mTOR could be a useful target in this highly resistant tumor. It is true that mTOR/S6K inhibition did not induce apoptosis by itself, but it enhanced the response to other apoptotic stimuli, presumably by releasing an inhibitory step at the level of the mitochondria. It is also worth pointing out that mTOR/S6K inhibition did not remove all acquired resistance, but it did account for a significant portion (roughly 40%) that would be amenable to currently available inhibitors. Our results provide a fresh insight into the functions of mTOR/S6K in mesothelioma and perhaps in other refractory solid tumors.

mTOR has been increasingly recognized as a crucial kinase in cancer and metabolic diseases because of its role in the integration of diverse mitogenic and metabolic inputs (41) and its recognition as a mediator of survival (16, 17, 19, 50, 51). In addition, with its own inputs and restraints, mTOR can act independently of the PI3K/Akt pathway (52, 53). Several of our observations point to the importance of mTOR signaling, as apart from Akt signaling, in mesothelioma cells. For one, mTOR/S6K was activated, as judged by an elevated P-S6K, in all our cells, whereas P-Akt was elevated only in M28 and VAMT (see Fig. 4A). For another, despite the increase in P-Akt with
rapamycin (see Fig. 3A). rapamycin was as effective as the other inhibitors in reducing apoptotic resistance. This suggests that, within the PI3K/Akt/mTOR pathway, mTOR is the major mediator of the acquired apoptotic resistance in mesothelioma spheroids. Finally, knockdown of S6K reproduced the effect of rapamycin, confirming the role of the mTOR/S6K arm in the survival of spheroids.

By silencing Bid expression and removing an essential amplification step between the death receptor and mitochondrial apoptotic pathways (54), we localized the anti-apoptotic activity of mTOR/S6K to the level of the mitochondria. Indeed, inhibition of apoptosis at the mitochondrial level would account for the observed resistance to a wide array of apoptotic stimuli involving the intrinsic and extrinsic pathways (see Fig. 8). mTOR has also been shown to associate with mitochondria (55) possibly serving as a modulator of stress signals (56) and cell fate (57). Although we were able to localize the resistance to the mitochondria, we were not able to identify a specific molecule that transduced the survival function of mTOR/S6K in spheroids. In particular, with the development of a three-dimensional structure, there was no clear change in the abundance of many pro-/anti-apoptotic proteins thought to be important for mesothelioma apoptotic resistance (45) or for molecules known to be regulated by S6K such as phospho-Bad (supplemental Fig. S4) (18). Anti-apoptotic Bcl-2 and FLIPs, which were up-regulated in the transition from two-dimensional to three-dimensional, were found to be independent of mTOR/S6K activity, suggesting that they may account for resistance that is not controlled by mTOR/S6K, at least in two of the cell lines. mTOR may also contribute to survival in general by its support of metabolism and energy (56) or of protein translation, key functions to which the cells may become reliant or “addicted” (58).

The survival function of mTOR became evident only in the three-dimensional setting. This may represent a redirection of the function of mTOR in three-dimensional, as has been noted for other signaling pathways that, with a transition from two- to three-dimensional, can be spatially reorganized (59, 60), coupled to other pathways (61), or redirected downstream to different functions (59). In fact, mTOR has previously been noted to have a greater effect on survival in three-dimensional than in two-dimensional breast cancer cell cultures (62). The Akt/mTOR pathway was clearly altered by the three-dimensional environment as seen in the reduction in phosphorylation of Akt and S6K (see Fig. 4, A and B). Because the signals can respond to stresses (see supplemental Fig. S2), we consider the reduction in phosphorylation to represent a down-regulation, instead of a suppression, of the pathway. Indeed, down-regulation may provide an improved signal to noise ratio, as can be achieved with modulation from feedback loops (63). An increase in PTEN activity in spheroids, as suggested by our finding of a decrease in the inhibitory phosphorylation at Ser-380 (see supplemental Fig. S3), might account for down-regulation by providing a brake on unrestrained PI3K activity. Such an increase in PTEN may parallel a general increase in phosphatases that has been described in spheroids compared with monolayers (64). Like other pathways, the Akt/mTOR pathway may thus be differently regulated and assume different functions in the three-dimensional setting.

Limited diffusion of macromolecules and the presence of a hypoxic core have been proposed to drive resistance of three-dimensional cell models (35, 36). We did not find evidence that the spheroids utilized in this study were affected by an impaired diffusion of agents. They had no baseline apoptosis, they showed homogeneous TRAIL diffusion and P-Akt/P-S6K staining, and they responded similarly as microspheroids of a different size and shape. Of note, the estimated maximal diffusion distance of the spheroids was 25–75 μm, below the distance of 100–200 μm described as limiting for the diffusion of oxygen in vivo (31). Clearly though, there will be diffusion gradients in three-dimensional models that may be relevant to gradients existing in avascular units of tumor units (36).

In this study of three-dimensional spheroids, we have discovered a role for mTOR in survival that could provide a therapeutic rationale for the use of mTOR inhibitors against mesothelioma, probably as an adjunct to current therapies. Inhibitors of mTOR are currently in use or in clinical trials for several tumors (65). If inhibition of mTOR is found to be useful in mesothelioma, it will underscore the value of three-dimensional studies for revealing underlying causes of acquired multicellular resistance in tumors.

Acknowledgments—We thank Dr. Luciano Mutti for help in initiating this study and Erica L. Elford for help in the editing process.

REFERENCES

1. Hanahan, D., and Weinberg, R. A. (2000) Cell 100, 57–70
2. Johnstone, R. W., Rueffli, A. A., and Lowe, S. W. (2002) Cell 108, 153–164
3. Desoie, B., and Jardillier, J. (2000) Crit. Rev. Oncol. Hematol. 36, 193–207
4. Johnson, J. L., Toh, J., Smith, S., Zhavezvit, D., Rubinstein, L. V., Venditti, J. M., Schepartz, S., Kalyandurg, S., Christian, M., Arbuck, S., Hollingshead, M., and Sausville, E. A. (2001) Br. J. Cancer 84, 1424–1431
5. Kim, J. B. (2005) Semin. Cancer Biol. 15, 365–377
6. Pampaloni, F., Reynaud, E. G., and Stelzer, E. H. (2007) Nat. Rev. Mol. Cell. Biol. 8, 839–845
7. Steele, J. P., and Klabatsa, A. (2005) Ann. Oncol. 16, 345–351
8. Michael, C. W., King, J. A., and Hester, R. B. (1997) Diagn. Cytopathol. 17, 272–279
9. Whitaker, D. (1977) Acta Cytol. 21, 236–239
10. Kim, K. U., Wilson, S. M., Abayasiriwardana, K. S., Collins, R., Fjellbirke-land, L., Xu, Z., Jablons, D. M., Nishimura, S. L., and Broaddus, V. C. (2005) Am. J. Respir. Cell Mol. Biol. 33, 541–548
11. Testa, J. R., and Tsichlis, P. N. (2005) Oncogene 24, 7391–7393
12. Cheng, J. Q., Lindsley, C. W., Cheng, G. Z., Yang, H., and Nicosia, S. V. (2005) Oncogene 24, 7482–7492
13. Altomare, D. A., You, H., Xiao, G. H., Ramos-Nino, M. E., Skele, K. L., De Rienzo, A., Ihanwar, S. C., Mossman, B. T., Kane, A. B., and Testa, J. R. (2005) Oncogene 24, 6080–6089
14. Cacciotti, P., Barbone, D., Porta, C., Altomare, D. A., Testa, J. R., Mutti, L., and Gaudino, G. (2005) Cancer Res. 65, 5256–5262
15. Manning, B. D., and Cantley, L. C. (2007) Cell 129, 1261–1274
16. Wendel, H. G., De Stanchina, E., Friman, J. S., Malina, A., Ray, S., Kogan, S., Cordon-Cardo, C., Pelletier, J., and Lowe, S. W. (2004) Nature 428, 332–337
17. Wendel, H. G., Malina, A., Zhao, Z., Zender, L., Kogan, S. C., Cordon-Cardo, C., Pelletier, J., and Lowe, S. W. (2006) Cancer Res. 66, 7639–7646
18. Harada, H., Andersen, J. S., Mann, M., Terada, N., and Korsmeyer, S. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9666–9670
19. Panner, A., James, C. D., Berger, M. S., and Pieper, R. O. (2005) Mol. Cell.
mTOR Supports Acquired Survival of Mesothelioma Spheroids

Biol. 25, 8809–8823

20. Shi, Y., Yan, H., Frost, P., Gera, J., and Lichtenstein, A. (2005) Mol. Cancer Ther. 4, 1533–1540

21. Knight, Z. A., Gonzalez, B., Feldman, M. E., Zander, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B., Balla, T., Weiss, W. A., Williams, R. L., and Shokat, K. M. (2006) Cell 125, 733–747

22. Fan, Q. W., Knight, Z. A., Goldenberg, D. D., Yu, W., Mostov, K. E., Stokoe, D., Shokat, K. M., and Weiss, W. A. (2006) Cancer Cell 9, 341–349

23. Abayasiriwardana, K. S., Barbone, D., Kim, K. U., Vivo, C., Lee, K. K., D, Shokat, K. M., and Weiss, W. A. (2006) J. Cell. Physiol. 215, 144–152

24. Reddy, R. M., Yeow, W. S., Chua, A., Nguyen, D. M., Baras, A., Ziauddin, M. F., Shamimi-Noori, S. M., Maxhimer, J. B., Schrump, D. S., and Nguyen, D. M. (2007) Apoptosis 12, 55–71

25. Emanuele, S., D’Anneo, A., Bellavia, G., Vassallo, B., Lauricella, M., De Blasio, A., Vento, R., and Tesoriere, G. (2004) Eur. J. Cancer 40, 1441–1452

26. Torres Filho, I. P., Leunig, M., Yuan, F., Intaglietta, M., and Jain, R. K. (2000) J. Biol. Chem. 275, 25461–25467

27. Napolitano, A. P., Chai, P., Dean, D. M., and Morgan, J. R. (2007) Mol. Cancer Ther. 6, 2766–2776

28. Ivascu, A., and Kubbies, M. (2006) J. Biolog. Chem. 281, 1098–1101

29. Broaddus, V. C., Yang, L., Scavo, L. M., Ernst, J. D., and Boylan, A. M. (1999) Science 286, 252–259

30. Santini, M. T., and Rainaldi, G. (1999) J. Pathobiology 67, 148–157

31. Torres Filho, I. P., Leunig, M., Yuan, F., Intaglietta, M., and Jain, R. K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2081–2085

32. Liu, W., Bodle, E., Chen, J. Y., Gao, M., Rosen, G. D., and Broadus, V. C. (2001) Am. J. Respir. Cell Mol. Biol. 25, 111–118

33. Neuzil, J., Swettenham, E., and Gellert, M. (2004) Biochem. Biophys. Res. Commun. 314, 186–191

34. Bartalone-Bianchi, A., Gasparri, F., Galvani, A., Nici, L., Darnowski, J. W., Barbone, D., Fennell, D. A., Gaudino, G., Porta, C., and Mutti, L. (2007) Clin. Cancer Res. 13, 5942–5951

35. Fracasso, G., and Colombatti, M. (2000) Crit. Rev. Oncol. Hematol. 36, 159–178

36. Minchinton, A. I., and Tannock, I. F. (2006) Nat. Rev. Cancer 6, 583–592

37. Gharbi, S. I., Zvelebil, M. J., Shuttleworth, S. J., Hancox, T., Saghir, N., Timms, J. F., and Waterfield, M. D. (2007) Biochem. J. 404, 15–21

38. Wan, X., Harkavy, B., Shen, N., Grohar, P., and Helman, L. J. (2007) Oncogene 26, 1932–1940

39. Sarbasov, D. D., Guerin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Science 307, 1098–1101

40. Corradetti, M. N., and Guan, K. L. (2006) Oncogene 25, 6347–6360

41. Dann, S. G., Selvaraj, A., and Thomas, G. (2007) Trends Mol. Med. 13, 252–259

42. Wang, X., Beugnet, A., Murakami, M., Yamanaka, S., and Proud, C. G. (2005) Mol. Cell. Biol. 25, 2558–2572

43. Edinger, A. L., Linardic, C. M., Chiang, G. G., Thompson, C. B., and Abraham, B. T. (2003) Cancer Res. 63, 8451–8460

44. Wang, X., Beugnet, A., Murakami, M., Yamanaka, S., and Proud, C. G. (2005) Mol. Cell. Biol. 25, 2558–2572

45. Fennell, D. A., and Rudd, R. M. (2004) Lancet Oncol. 5, 354–362

46. Wan, X., Beugnet, A., Murakami, M., Yamanaka, S., and Proud, C. G. (2005) Mol. Cell. Biol. 25, 25461–25467

47. Li, M., Howes, A., Lesperance, J., Stallcup, W. B., Hauser, C. A., Kadoya, Y., Kitamura, M., and Shimizu, T. (2004) Oncogene 23, 6436–6446

48. Lowe, S. W., Cepero, E., and Evan, G. (2004) Nature 432, 307–315

49. Malley, K. S., Lioni, M., and Herlyn, M. (2006) In Vitro Cell. Dev. Biol. Anim. 42, 242–247

50. Majumder, P. K., Febbo, P. G., Bikoff, R., Berger, R., Xue, Q., McMahon, L. M., Manola, J., Brugarolas, J., McDonnell, T. J., Golub, T. R., Loda, M., Lane, H. A., and Sellers, W. R. (2004) Nat. Med. 10, 594–601

51. Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruettz, S., O’Reilly, T., Natt, F., Hall, J., Lane, H. A., and Thomas, G. (2005) Cell 120, 747–759

52. Reddy, R. M., Yeow, W. S., Chua, A., Nguyen, D. M., Baras, A., Ziauddin, M. F., Shamimi-Noori, S. M., Maxhimer, J. B., Schrump, D. S., and Nguyen, D. M. (2007) Apoptosis 12, 55–71

53. Santini, M. T., and Rainaldi, G. (1999) Pathobiology 67, 148–157

54. Torres Filho, I. P., Leunig, M., Yuan, F., Intaglietta, M., and Jain, R. K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2081–2085

55. Liu, W., Bodle, E., Chen, J. Y., Gao, M., Rosen, G. D., and Broadus, V. C. (2001) Am. J. Respir. Cell Mol. Biol. 25, 111–118

56. Neuzil, J., Swettenham, E., and Gellert, M. (2004) Biochem. Biophys. Res. Commun. 314, 186–191

57. Bartalone-Bianchi, A., Gasparri, F., Galvani, A., Nici, L., Darnowski, J. W., Barbone, D., Fennell, D. A., Gaudino, G., Porta, C., and Mutti, L. (2007) Clin. Cancer Res. 13, 5942–5951

58. Fracasso, G., and Colombatti, M. (2000) Crit. Rev. Oncol. Hematol. 36, 159–178

59. Minchinton, A. I., and Tannock, I. F. (2006) Nat. Rev. Cancer 6, 583–592

60. Gharbi, S. I., Zvelebil, M. J., Shuttleworth, S. J., Hancox, T., Saghir, N., Timms, J. F., and Waterfield, M. D. (2007) Biochem. J. 404, 15–21

61. Wan, X., Harkavy, B., Shen, N., Grohar, P., and Helman, L. J. (2007) Oncogene 26, 1932–1940

62. Sarbasov, D. D., Guerin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Science 307, 1098–1101

63. Corradetti, M. N., and Guan, K. L. (2006) Oncogene 25, 6347–6360

64. Dann, S. G., Selvaraj, A., and Thomas, G. (2007) Trends Mol. Med. 13, 252–259

65. Easton, J. B., and Houghton, P. J. (2006) Oncogene 25, 6436–6446