Published OnlineFirst November 24, 2009; DOI: 10.1158/1078-0432.CCR-09-1249

Targeted Inhibition of Mammalian Target of Rapamycin Signaling Inhibits Tumorigenesis of Colorectal Cancer

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Abstract

Purpose: The mammalian target of rapamycin (mTOR) kinase acts downstream of phosphoinositide 3-kinase/Akt to regulate cellular growth, metabolism, and cytoskeleton. Because ~60% of sporadic colorectal cancers (CRC) exhibit high levels of activated Akt, we determined whether downstream mTOR signaling pathway components are overexpressed and activated in CRCs.

Experimental Design: HCT116, KM20, Caco-2, and SW480 human CRC cells were used to determine the effects of pharmacologic (using rapamycin) or genetic (using RNAi) blockade of mTOR signaling on cell proliferation, apoptosis, cell cycle progression, and subcutaneous growth *in vivo*.

Results: We show that the mTOR complex proteins mTOR, Raptor, and Rictor are overexpressed in CRC. Treatment with rapamycin significantly decreased proliferation of certain CRC cell lines (rapamycin sensitive), whereas other cell lines were resistant to its effects (rapamycin resistant). Transient siRNA-mediated knockdown of the mTORC2 protein, Rictor, significantly decreased proliferation of both rapamycin-sensitive and rapamycin-resistant CRC cells. Stable shRNA-mediated knockdown of both mTORC1 and mTORC2 decreased proliferation, increased apoptosis, and attenuated cell cycle progression in rapamycin-sensitive CRCs. Moreover, stable knockdown of both mTORC1 and mTORC2 decreased proliferation and attenuated cell cycle progression, whereas only mTORC2 knockdown increased apoptosis in rapamycin-resistant CRCs. Finally, knockdown of both mTORC1 and mTORC2 inhibited growth of rapamycin-sensitive and rapamycin-resistant CRCs *in vivo* when implanted as tumor xenografts.

Conclusions: Targeted inhibition of the mTORC2 protein, Rictor, leads to growth inhibition and induces apoptosis in both rapamycin-sensitive and rapamycin-resistant CRCs, suggesting that selective targeting of mTORC2 may represent a novel therapeutic strategy for treatment of CRC. (Clin Cancer Res 2009;15(23):7207–16)

The activation of phosphatidylinositol 3-kinase (PI3K) by activating mutations in the *PIK3CA* gene (encoding the p110α catalytic subunit of PI3K) or loss of the tumor suppressor *PTEN* (encoding a lipid and protein phosphatase) is associated with growth and progression of a number of cancers, including colorectal cancer (CRC; refs. 1, 2). PI3K effects on tumor growth and progression are mediated by two key downstream effectors, Akt and mTOR (3, 4). Treatment with wortmannin or LY294002, general PI3K chemical inhibitors, increased apoptosis and inhibited growth of CRC cells (5, 6); however, use is limited due to toxicity *in vivo* (7). We have previously shown that targeted inhibition of upstream PI3K/Akt pathway components decreases growth, increases apoptosis, increases sensitivity to chemotherapy, and decreases metastatic capability of CRCs (8–12). Selective inhibition of downstream proteins that are directly involved in survival and proliferation may allow more targeted therapy with fewer toxicities.

mTOR, a serine/threonine kinase that regulates cell growth and metabolism (4), exists in two distinct functional complexes: mTORC1 and mTORC2. mTORC1 consists of mTOR, mLST8, PRAS40, and Raptor, whereas mTORC2 consists of mTOR, mLST8, Rictor, Sin1, and PROTOR (4). mTORC1 mediates phosphorylation and activation of the eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP1) and the p70S6 ribosomal kinase (S6K), which together control protein synthesis (13). mTORC2 plays a role in cell growth, in

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Received 5/16/09; revised 8/20/09; accepted 9/9/09; published OnlineFirst 11/24/09.

Grant support: RO1CA104748 and RO1DK48498 (B.M. Evers) and K01CA10209 (T. Gao) from the NIH and a Sealy Center for Cancer Cell Biology Fellowship (P. Gulhati).

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doi:10.1158/1078-0432.CCR-09-1249

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Translational Relevance

Colorectal cancer (CRC) is the second leading cause of cancer deaths in the United States. Because ~60% of sporadic CRCs exhibit high levels of activated Akt, we determined whether downstream mammalian target of rapamycin (mTOR) pathway components are overexpressed and activated in CRCs. Here, we show that the mTOR complex proteins mTOR, Raptor, and Rictor are overexpressed in CRC tissues. We define two subpopulations of CRC cells, rapamycin-sensitive and rapamycin-resistant, exhibiting differential patterns of feedback induction of $pAkt^{Ser473}$. We further show that targeted inhibition of the mTORC2 protein, Rictor, leads to growth inhibition and induces apoptosis in both rapamycin-sensitive and rapamycin-resistant CRCs, suggesting that selective targeting of mTORC2 may represent a novel therapeutic strategy for treatment of CRC. These findings have important ramifications for the treatment of patients with CRC because one can envision mTOR kinase inhibitors and/or dual phosphoinositide 3-kinase/mTOR inhibitors as one part of the treatment regimen for CRC patients.

addition to regulating cell cycle–dependent changes in the actin cytoskeleton (4, 14).

The bacterially derived drug, rapamycin, complexes with the FK506 binding protein (FKBP) 12, and the drug-receptor complex potently inhibits mTOR activity (4). mTORC1 is sensitive to rapamycin treatment; mTORC2 is thought to be rapamycin insensitive, but prolonged treatment inhibits its assembly in certain cells (4, 15). Despite the seemingly clear rationale for use of a mTOR inhibitor in cancers addicted to PI3K/Akt signaling, rapamycin and its analogues have only shown limited benefit in clinical trials. Inhibition of mTORC1 by rapamycin leads to activation of a negative feedback loop through S6K and insulin-like growth factor-I receptor (IGF-IR), which results in feedback activation of Akt (4, 16). This paradoxical Akt activation presents a problem as it promotes cell survival and resistance to the therapeutic benefits of mTORC1 inhibition (17).

mTORC2 has been implicated as the major hydrophobic kinase to phosphorylate the Ser^{473} residue of Akt, thus placing mTOR both upstream and downstream of Akt (4, 18). Because Akt activation is widespread in CRC, there is a rationale for inhibition of mTORC2 to prevent Akt^{Ser473} phosphorylation. In addition, the finding that mTORC2 is not essential in *Drosophila* but becomes essential for phenotypes reliant on elevated PI3K activity (4, 19) further supports the possibility that mTORC2 inhibition might have therapeutic potential, particularly in malignant states dependent on elevated Akt signaling. Here, we show that mTOR, Raptor, and Rictor are overexpressed in CRCs. Inhibition of mTORC1 and mTORC2 had pronounced effects on CRC proliferation and growth. Targeted inhibition of the mTORC2 protein, Rictor, proved to be effective in growth inhibition and inducing apoptosis in both rapamycin-sensitive and rapamycin-resistant CRCs, suggesting that selective targeting of mTORC2 may represent a novel therapeutic strategy for treatment of CRC.

Materials and Methods

Immunohistochemistry. Tissue microarrays containing normal and cancer tissues, A203 (VI), were purchased from ISUABXIS through Accurate Chemical & Scientific Corporation. Each array consisted of tissues derived from 45 patients: 90 tumor cores and 8 normal cores. Immunohistochemistry (IHC) was done as described previously (10). For negative controls, primary antibody was omitted from the above protocol. Scoring was done blindly by a pathologist according to a semiquantitative seven-tier system developed by Allred et al. (20). This system assesses the percentage of positive cells (none = 0; <10% = 1; 10-50%, = 2; >50% = 3) and intensity of staining (none = 0; weak = 1; intermediate = 2; and strong = 3). The intensity and percentage scores are added to give a final immunoreactivity score ranging from 0 to 6. mTOR, Raptor, and Rictor antibodies used for IHC in Fig. 1A were purchased from Bethyl Laboratories, whereas $pAkt^{Ser473}$ antibody was purchased from Cell Signaling. mTOR, Raptor, and Rictor antibodies used in Supplementary Fig. S1 were purchased from Abcam.

Cell lines, plasmid transfections, and lentiviral transductions. The human colon cancer cell lines HCT116, KM20, SW480, and Caco-2 were used in these studies. HCT116, SW480, and Caco-2 cells were obtained from the American Type Culture Collection; KM20 cells were kindly provided by Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center, Houston, TX). HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS; Hyclone). SW480 cells were cultured in DMEM supplemented with 10% FBS. Caco-2 cells were cultured in MEM supplemented with 10% FBS. KM20 cells were cultured in MEM supplemented with 10% FBS, 1% sodium pyruvate, 1% nonessential amino acids, and 2% MEM essential vitamins. All tissue culture media were purchased from Invitrogen. Nontargeting control (NTC) and SMARTPool Raptor and Rictor siRNA were purchased from Dharmacon.

For generation of stable knockdown HCT116 and SW480 cells, shRNAs for human mTOR, Raptor, and Rictor genes were constructed in pLKO.1-puro vector and purchased from Addgene. A plasmid carrying a nontargeting sequence was used to create the control cells. For virus packaging, the control or mTOR/Raptor/Rictor shRNA constructs were cotransfected with Mission lentiviral packing mix (Sigma-Aldrich) into HEK 293T cells using FuGene 6. The virus-containing medium was collected, filtered, and overlaid onto the parental cells in the presence of polybrene (10 μg/mL) for 24 h. The infected cells were then selected with puromycin (2.5 μg/mL).

Cell proliferation and apoptosis analyses. Equal numbers of cells were seeded onto 24-well plates at a density of 1×10^4 per well in the appropriate culture medium with supplements. For proliferation assays, cells were treated with varying doses of rapamycin for 24 to 96 h. Cells were trypsinized and counted using a cell counter (Beckman-Coulter) or Cell Proliferation ELISA (Roche). For apoptosis assays, cells were serum starved for 48 h and apoptosis was measured using the Cell Death Detection $ELISA^{plus}$ (Roche) as detailed in the manufacturer's instructions.

Cell cycle analysis. Cells (1×10^4) were trypsinized, washed with PBS, and fixed in 70% methanol. Fixed cells were then washed with PBS, incubated with 100 μg/mL RNase for 30 min at 37°C, and stained with propidium iodide (50 μg/mL). Cells were subjected to flow cytometry analysis using Becton Dickinson FACScanto. The percentages of cells in different cell cycle phases were analyzed using ModFit LT software (Verity Software House).

Western blot analysis. Western blot analysis was done as described previously (10). The following antibodies were from Cell Signaling: $pAkt^{Ser473}$, $pAkt^{Thr308}$, total Akt, β-actin, $p\text{-}mTOR^{Ser2448}$, tubulin, $p\text{-}p70S6K^{Thr389}$, and $pS6^{Ser235/236}$. Antibodies for mTOR, Raptor, and Rictor were obtained from Bethyl Labs.

In vivo *studies.* The stable knockdown HCT116 and SW480 CRC cells were collected in 50 μL of sterile PBS and inoculated s.c. into 6-wk-old male athymic nude mice at 2×10^6 cells per injection site ($n = 5$ for each group). The tumor size was measured every 3 to 5 d with a

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vernier caliper, and the tumor volume was defined as (longest diameter) × (shortest diameter)^2 / 2. At the end of the experiment on day 25 postinjection, mice were sacrificed and tumors were removed, weighed, and then extracted for protein analysis. All animal procedures were done in the nude mouse facility using protocols approved by the University of Texas Medical Branch Animal Care and Use Committee.

Statistical analysis. The association between IHC score and stage (Fig. 1B) was assessed using Fisher's exact test. The effects of (*a*) rapamycin dose on cell proliferation (Fig. 2), (*b*) siRNA treatment on cell proliferation (Fig. 3), (*c*) shRNA treatment on cell proliferation (Figs. 4B and 5B), and (*d*) shRNA treatment on tumor volume (transformed to cubic root) and tumor weight (Fig. 6) were analyzed using one-way ANOVA. Effects of combinations of shRNA treatment and serum (Figs. 4C and 5C) were analyzed using ANOVA for a two-factor experiment. All tests were assessed at the 0.05 level of significance (experiment wise). Multiple comparisons were conducted using Fisher's least significant difference procedure with Bonferroni adjustment for the number of comparisons. Statistical computations were carried out using SAS 9.1 (21).

Results

mTORC1 and mTORC2 are overexpressed in CRC tissues and cells. To determine whether mTORC1 and mTORC2 proteins are overexpressed in CRCs, we examined CRCs and adjacent normal colonic tissue for expression of mTOR, Raptor, and Rictor. Each sample was assigned an IHC immunoreactivity score ranging from 0 to 6. Representative patient samples for each protein are shown in Fig. 1A along with data analysis in Fig. 1B. The IHC score for tumor tissue was significantly higher than normal tissue for mTOR, Raptor, and Rictor ($P < 0.0001$). mTOR exhibits membranous and cytoplasmic staining, Raptor displays mixed cytoplasmic and nuclear staining of approximately equal intensity, and Rictor exhibits granular cytoplasmic staining. These findings were confirmed using another set of antibodies (Supplementary Fig. S1); we found no differences in the localization and intensity of staining between the two

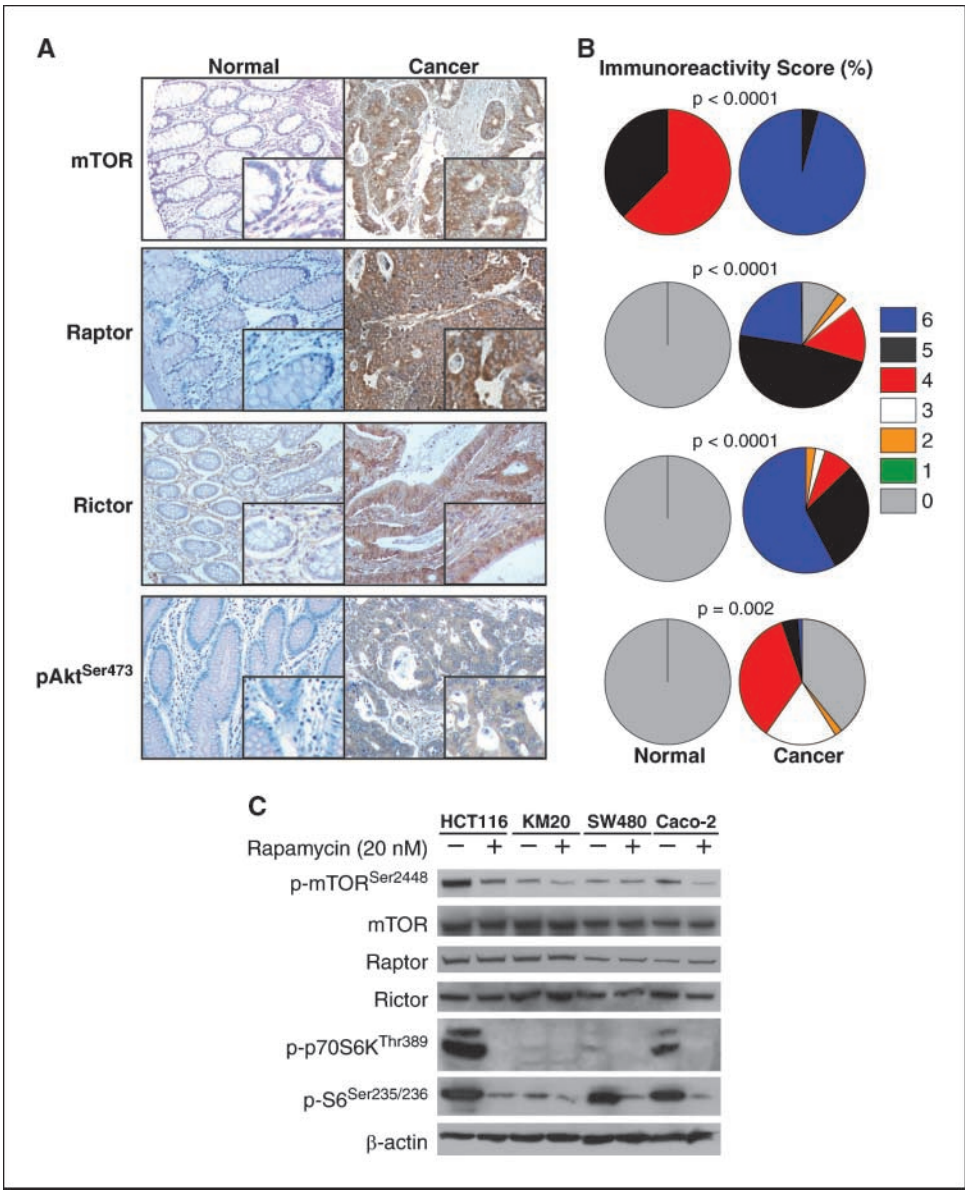

Fig. 1. Expression of mTORC1 and mTORC2 in CRC tissues and cell lines. *A* and *B*, immunohistochemical analysis of mTOR, Raptor, Rictor, and pAkt in representative colorectal adenocarcinomas and adjacent normal mucosa (tissue microarray, 10× magnification; $n = 45$ cases; 90 tumor cores, 8 nonneoplastic cores). *C*, expression and activation of mTOR signaling pathway components in HCT116, KM20, SW480, and Caco-2 CRC cells treated with 20 nmol/L rapamycin for 24 h.

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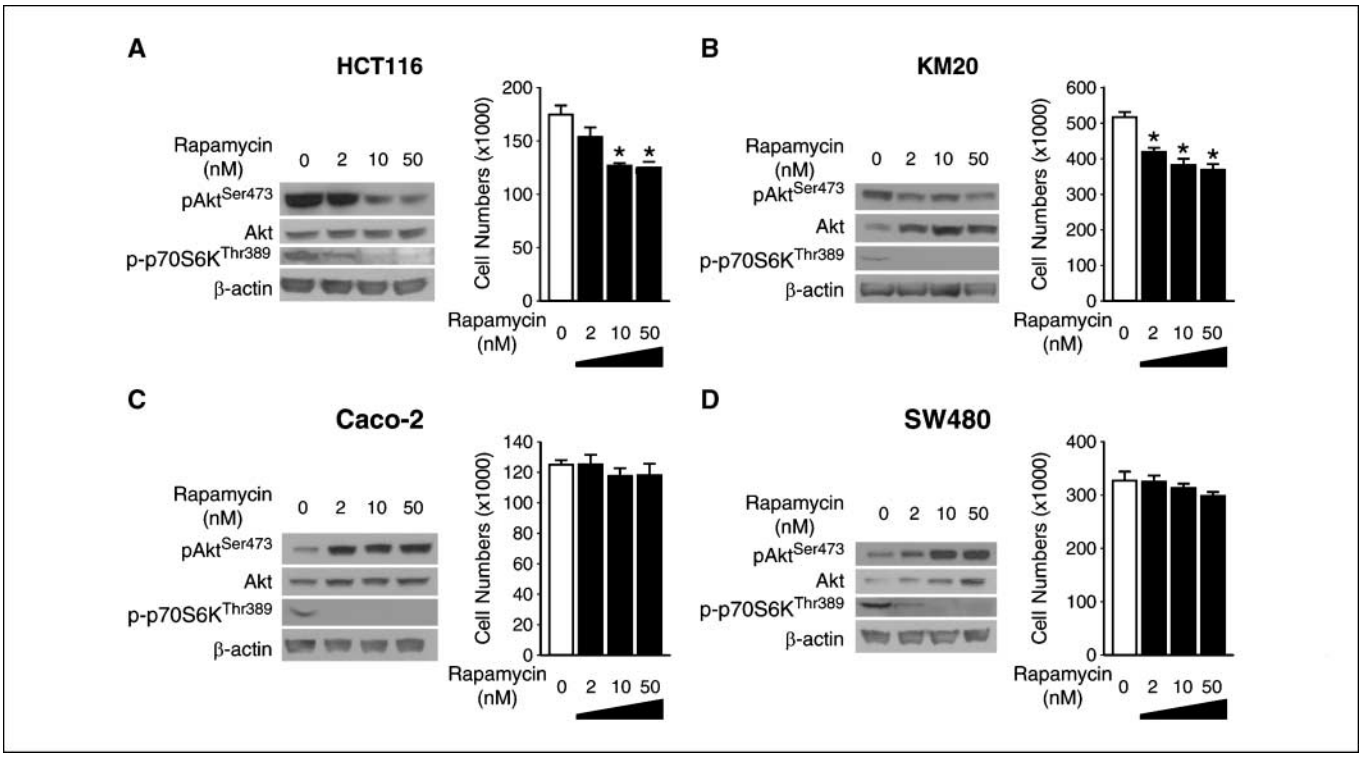

Fig. 2. HCT116 and KM20 cells are rapamycin sensitive whereas Caco-2 and SW480 cells are rapamycin resistant. Assessment of cell proliferation by counting cell numbers directly (*right*) after rapamycin treatment for 48 h in (*A*) HCT116, (*B*) KM20, (*C*) Caco-2, and (*D*) SW480 cells (*, $P < 0.05$ versus control). Western blot analysis (*left*) demonstrating expression patterns of $pAkt^{Ser473}$ and $p\text{-}p70S6K^{Thr389}$ after rapamycin treatment.

sets of antibodies used for analysis. Furthermore, because mTORC2 has been implicated as the major kinase to phosphorylate the Ser^{473} residue of Akt, we also examined the expression of $pAkt^{Ser473}$ in the same set of CRC tissues. The IHC score of tumor tissue was found to be significantly higher than normal tissue for $pAkt^{Ser473}$ ($P = 0.002$). Interestingly, expression of Rictor was found to correlate with elevated $pAkt^{Ser473}$ expression; all patient samples that stained positive for $pAkt^{Ser473}$ (regardless of staining intensity) exhibited elevated Rictor expression (IHC score = 5 or 6).

To further confirm our findings, we examined the expression levels of various mTOR complex components and their downstream effectors in a panel of CRC cell lines representing a spectrum of aberrations in this signaling pathway. Lysates were prepared from four human CRC cell lines (HCT116, KM20, SW480, and Caco-2) and probed with various antibodies using Western blot analysis. As shown in Fig. 1C, we found that all cell lines expressed the mTORC1 components, mTOR and Raptor, with highest levels detected in HCT116 and KM20 cells. Moreover, all four cell lines expressed the mTORC2 protein, Rictor. Furthermore, all cell lines exhibit a decrease in levels of $p\text{-}p70S6K^{Thr389}$ upon rapamycin treatment (Fig. 1C and Supplementary Fig. S2). Taken together with the IHC results, these findings suggest that mTORC1 and mTORC2 proteins are overexpressed in CRCs. Furthermore, the mTORC2 kinase may contribute to the elevated $pAkt^{Ser473}$ levels commonly seen in CRCs.

CRCs show differential sensitivity to rapamycin treatment. Many cancers show variable sensitivity to rapamycin treatment (4). The effects of mTOR inhibition on CRC growth are not well defined. Therefore, we determined the sensitivity or resistance of the aforementioned human CRC cell lines to rapamycin treatment. Cells were treated with increasing doses of rapamycin over a 48-hour time period and cell proliferation was quantitated. We found differential sensitivity to the effects of rapamycin with significant inhibition of proliferation noted in HCT116 and KM20 cells (rapamycin sensitive) as shown in Fig. 2A and B, respectively. Rapamycin treatment of both sensitive cell lines for 48 hours resulted in a dose-dependent decrease in $pAkt^{Ser473}$ levels. In contrast, increasing doses of rapamycin had no significant effect on Caco-2 or SW480 cell proliferation (rapamycin resistant) as shown in Fig. 2C and D, respectively. Interestingly, rapamycin treatment of both resistant cell lines for 48 hours resulted in a dose-dependent increase in $pAkt^{Ser473}$ levels. In summary, HCT116 and KM20 cells were sensitive to the antiproliferative effects of rapamycin and exhibited a decrease in $pAkt^{Ser473}$ levels with rapamycin treatment, whereas SW480 and Caco-2 cells were resistant to the antiproliferative effects of rapamycin and exhibited an increase in $pAkt^{Ser473}$ levels with rapamycin treatment.

Transient Rictor knockdown inhibits proliferation of both rapamycin-sensitive and rapamycin-resistant CRCs. We were interested in determining whether selective inhibition of either mTOR complex could inhibit proliferation of CRC cells. We first examined the rapamycin-sensitive cell lines, HCT116 and KM20, to determine the effects of Raptor or Rictor knockdown using siRNA (Fig. 3A and B). Specific knockdown of Raptor and Rictor by their corresponding siRNA was confirmed by Western blot analysis. Transfection with Raptor siRNA significantly decreased KM20 cell proliferation, but not HCT116, compared

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with NTC siRNA. In contrast, transfection with Rictor siRNA significantly inhibited cell proliferation in both cell lines. The levels of pAkt^{Ser473} were dramatically reduced with Rictor knockdown, but not with Raptor knockdown. Next, we looked at rapamycin-resistant CRCs, Caco-2 and SW480, to determine the effects of Raptor or Rictor knockdown using specific siRNA (Fig. 3C and D). Transfection with Raptor siRNA had no effect on proliferation of either cell line. Surprisingly, transfection with Rictor siRNA significantly inhibited cell proliferation in both rapamycin-resistant cell lines. Levels of pAkt^{Ser473} were

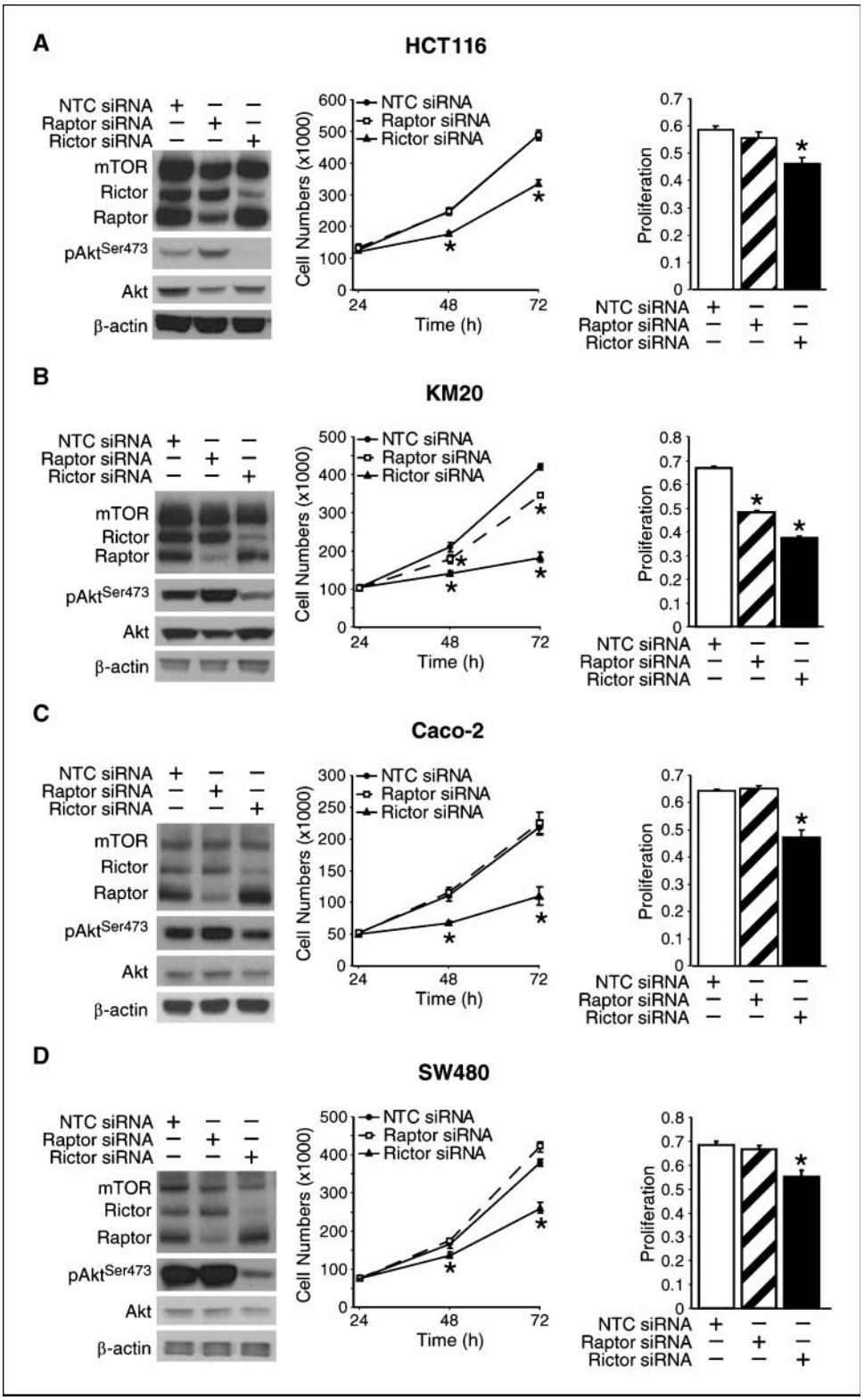

Fig. 3. Rictor siRNA decreases the proliferation of rapamycin-sensitive and rapamycin-resistant CRC cells. Assessment of cell proliferation by counting cell numbers directly (*middle*) or MTS cell proliferation assay (*right*) in (*A*) HCT116, (*B*) KM20, (*C*) Caco-2, and (*D*) SW480 cells transfected with Raptor, Rictor, or NTC siRNA and assessed by Western blotting (*left*) at 72 h after transfection (*, $P < 0.05$ versus NTC siRNA).

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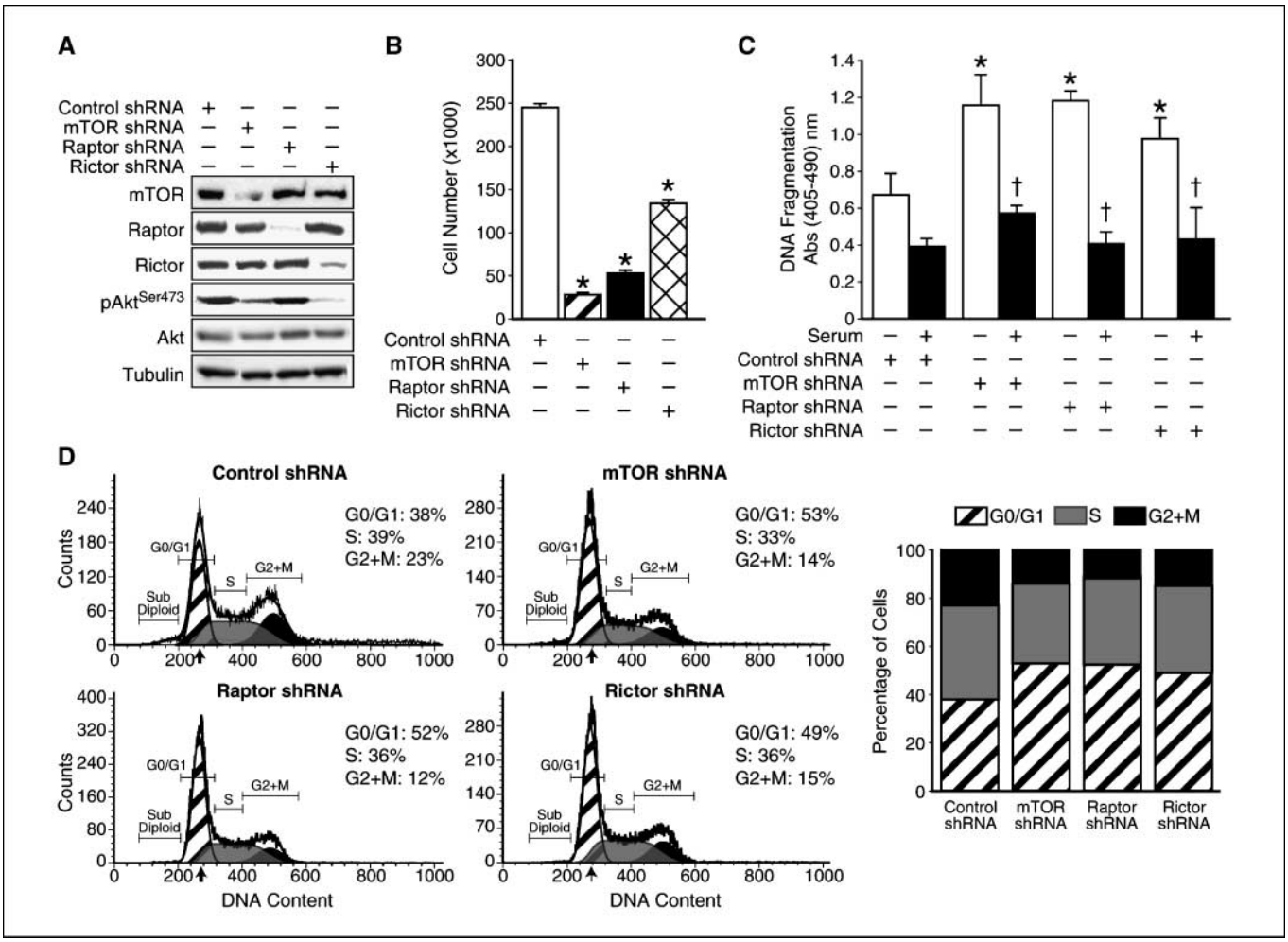

Fig. 4. Functional effects of shRNA-mediated stable inhibition of mTORC1 and mTORC2 in rapamycin-sensitive HCT116 CRC cells. *A,* assessment of specific protein knockdown using Western blot analysis. *B,* cell proliferation analysis by counting cell numbers directly (*, $P < 0.05$ versus control shRNA). *C,* apoptosis assessment using an ELISA detecting nucleosomes in the cytoplasm (*, $P < 0.05$ versus control shRNA; †, $P < 0.05$ versus –serum). *D,* cell cycle progression analysis using flow cytometric analysis.

dramatically reduced with Rictor knockdown but not with Raptor knockdown. These findings suggest that knockdown of Rictor can significantly inhibit the proliferation of both rapamycin-sensitive and rapamycin-resistant CRC cells.

Functional effects of stable inhibition of mTORC1 and mTORC2 in rapamycin-sensitive CRCs. To further confirm whether targeted inhibition of mTOR signaling affects the oncogenic properties of rapamycin-sensitive CRCs, we generated HCT116 cells with stable shRNA-mediated knockdown of mTOR, Raptor, or Rictor protein expression. Cells expressing shRNA targeting mTOR, Raptor, or Rictor had significantly reduced levels (>90% reduction) of each of the targeted proteins (Fig. 4A). Cells expressing shRNA targeting mTOR or Rictor had significantly reduced levels of $pAkt^{Ser473}$, whereas levels of $pAkt^{Ser473}$ in cells expressing shRNA targeting Raptor remained unaffected compared with control cells. Next, we determined whether knockdown of mTOR, Raptor, and Rictor affected proliferation of these cells. As shown in Fig. 4B, HCT116 cells with knockdown of mTOR, Raptor, or Rictor proliferate at significantly slower rates compared with control cells. The decreased cell proliferation produced by knockdown of these proteins may be due to decreased cell cycle progression and/or increased induction of apoptosis.

We then determined whether knockdown of mTOR, Raptor, and Rictor increased apoptosis in these cell lines. As shown in Fig. 4C, HCT116 cells expressing shRNA targeting mTOR, Raptor, or Rictor showed significantly increased rates of apoptosis compared with control cells. Furthermore, knockdown cells were sensitized to serum starvation–induced apoptosis compared with control cells. We also assessed the effect of knockdown of these mTOR complex proteins on cell cycle progression. As shown in Fig. 4D, the percentage of cells in the S and G_2-M phases decreased markedly and the percentage of cells in the G_0-G_1 phase increased markedly in the population of cells with reduced mTOR, Raptor, or Rictor compared with control cells. Taken together, these results suggest that inhibition of mTORC1 and mTORC2 proteins inhibits cell proliferation by inducing apoptosis as well as G_0-G_1 growth arrest in rapamycin-sensitive HCT116 cells.

Functional effects of stable inhibition of mTORC1 and mTORC2 in rapamycin-resistant CRCs. To determine the effects of inhibiting mTOR signaling on oncogenic properties of

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rapamycin-resistant CRCs, we generated SW480 cells with stable shRNA-mediated knockdown of mTOR, Raptor, or Rictor protein expression. Cells expressing shRNA targeting mTOR, Raptor, or Rictor had significantly reduced levels (>90% reduction) of each of the targeted proteins (Fig. 5A). Cells expressing shRNA targeting Rictor had significantly reduced levels of $pAkt^{Ser473}$, whereas levels of $pAkt^{Ser473}$ in cells expressing shRNA targeting mTOR and Raptor were increased compared with control cells. Next, we determined whether knockdown of mTOR, Raptor, and Rictor affected the proliferation of these cells. As shown in Fig. 5B, SW480 cells with knockdown of mTOR, Raptor, or Rictor proliferate at significantly slower rates compared with control cells.

We then determined whether knockdown of mTOR, Raptor, and Rictor increased apoptosis in these cells. Surprisingly, SW480 cells expressing shRNA targeting mTOR or Raptor had significantly reduced rates of apoptosis, whereas cells with stable Rictor knockdown showed increased apoptosis compared with control cells (Fig. 5C). Furthermore, Rictor knockdown sensitized cells to serum starvation–induced apoptosis compared with control cells. We also assessed the effect of knockdown of these mTOR complex proteins on cell cycle progression. As shown in Fig. 5D, the percentage of cells in the S phase decreased markedly and the percentage of cells in the G_0-G_1 phase increased markedly in the population of cells with reduced mTOR and Raptor compared with control cells. However, cells with reduced levels of Rictor did not undergo G_0-G_1 arrest; instead, the percentage of cells in the S phase was slightly decreased whereas the percentage of cells in the G_2-M phases was slightly increased. Taken together, these results suggest that inhibition of mTOR and Raptor components inhibits cell proliferation mainly by G_0-G_1 growth arrest, whereas inhibition of Rictor inhibits cell proliferation mainly by inducing apoptosis in rapamycin-resistant SW480 cells.

Stable knockdown of mTORC1 and mTORC2 inhibits xenograft tumor growth. To examine whether reduced mTOR, Raptor, and Rictor expression in rapamycin-sensitive and rapamycin-resistant CRC cells affects growth *in vivo*, we injected highly tumorigenic, rapamycin-sensitive HCT116 cells and moderately tumorigenic, rapamycin-resistant SW480 cells with stable knockdown of each of the mTOR complex proteins s.c. into athymic nude mice and monitored tumor growth over a period

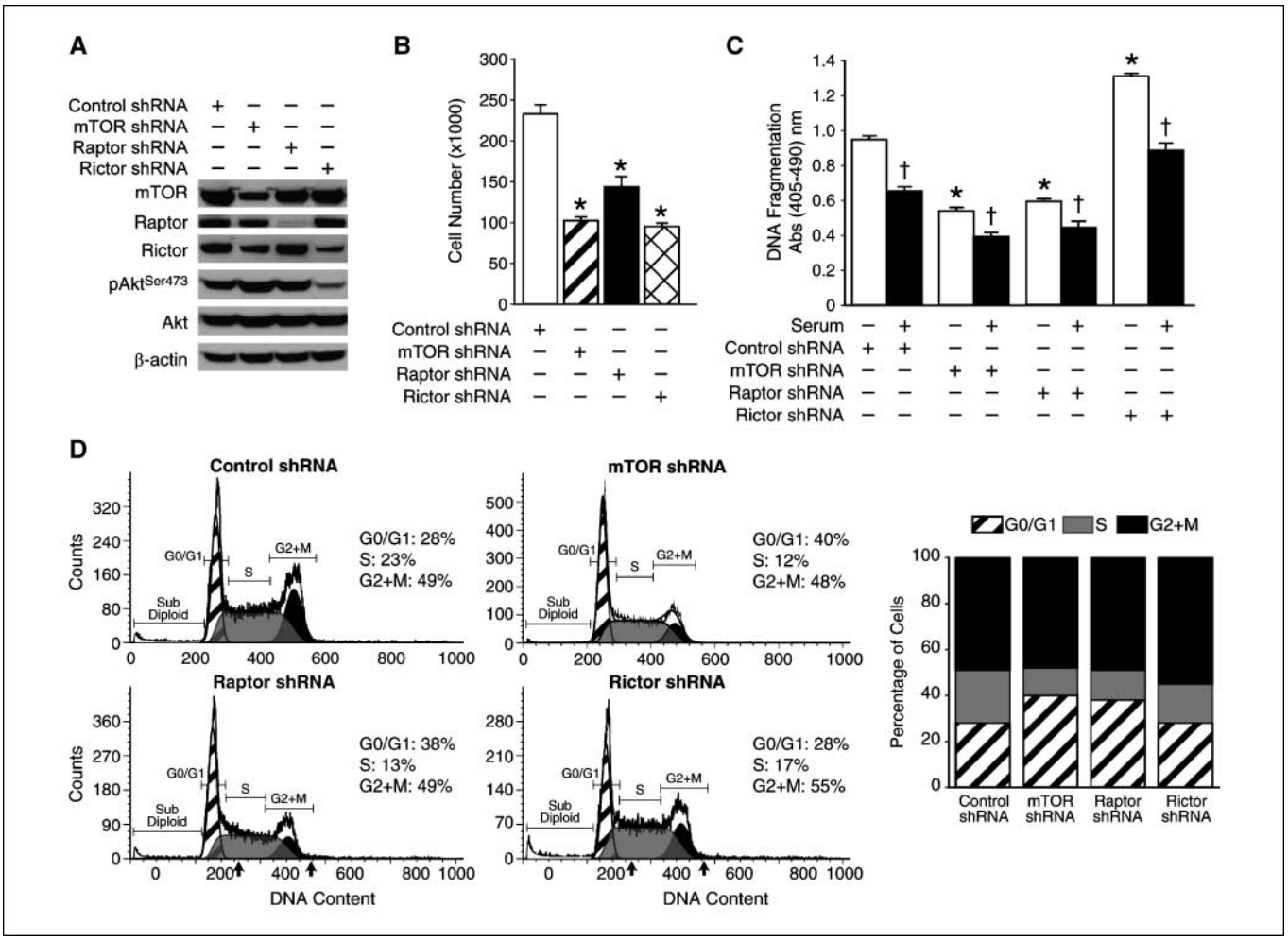

Fig. 5. Functional effects of shRNA-mediated stable inhibition of mTORC1 and mTORC2 in rapamycin-resistant SW480 CRC cells. *A,* assessment of specific protein knockdown using Western blot analysis. *B,* cell proliferation analysis by counting cell numbers directly (*, $P < 0.05$ versus control shRNA). *C,* apoptosis assessment using an ELISA detecting nucleosomes in the cytoplasm (*, $P < 0.05$ versus control shRNA; †, $P < 0.05$ versus –serum). *D,* cell cycle progression analysis using flow cytometric analysis.

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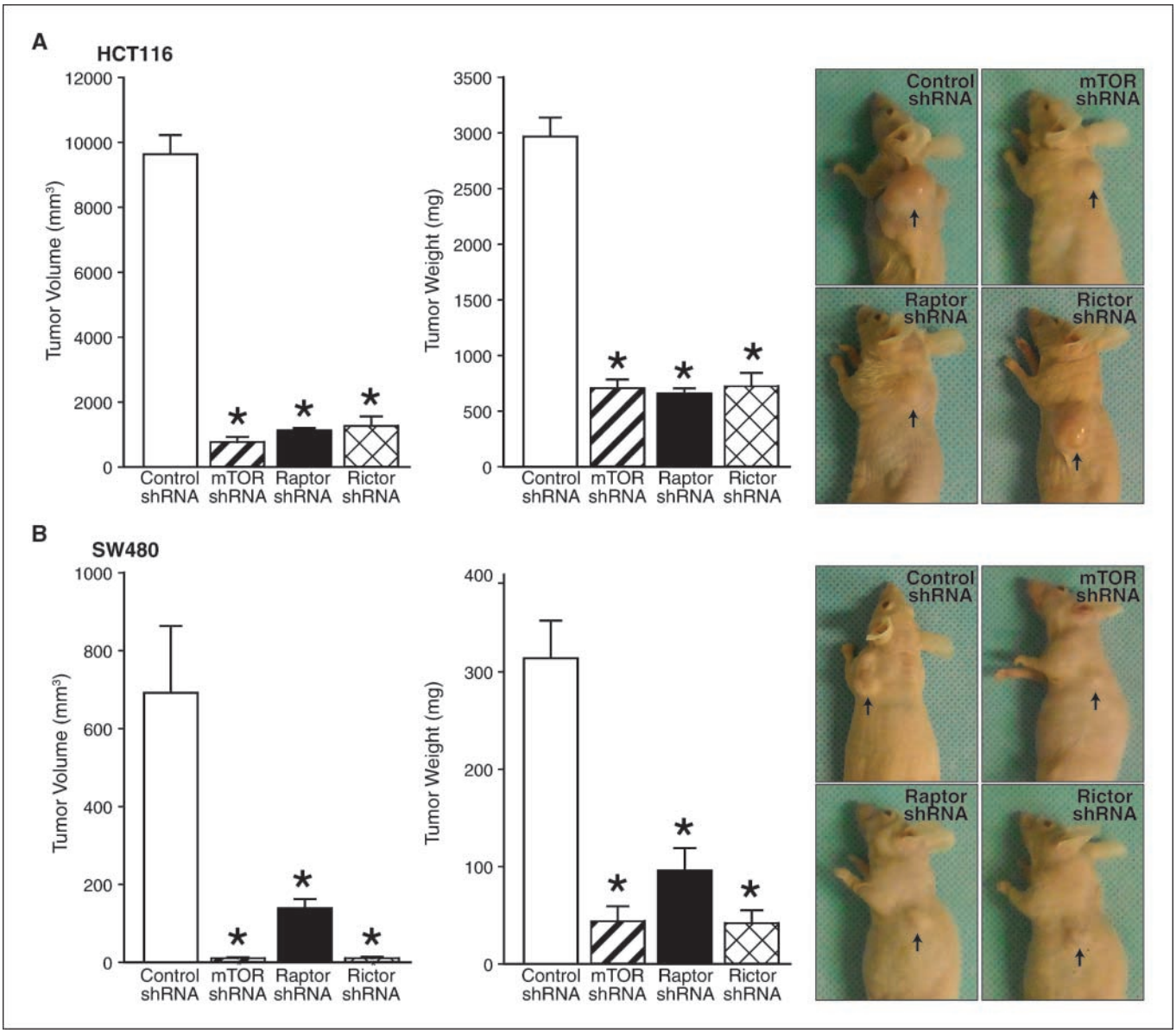

Fig. 6. Inhibition of mTORC1 and mTORC2 reduces the tumorigenic potential of rapamycin-sensitive and rapamycin-resistant CRC cells *in vivo*. Athymic nude mice were inoculated s.c. with (*A*) HCT116 shNTC, HCT116 shmTOR, HCT116 shRaptor, and HCT116 shRictor cells and (*B*) SW480 shNTC, SW480 shmTOR, SW480 shRaptor, and SW480 shRictor cells. The size of the tumors was measured after 25 d. Five mice were used in each group, and the cells were inoculated at one site in each mouse. Bar graphs showing tumor volume (*left*) and tumor weight (*middle*) are shown along with representative mice with tumors from each group (*right*; *, $P < 0.05$ versus control shRNA).

of 25 days. Tumors derived from control HCT116 cells formed tumors with sizes of ~9,500 mm^3 within 25 days (Fig. 6A). In contrast, knockdown of mTOR, Raptor, or Rictor significantly reduced tumor growth over the same time period; smaller tumors with sizes ranging from 750 to 1,250 mm^3 were detected at sacrifice (day 25). Lysates from HCT116-derived tumors expressing shRNA targeting mTOR or Rictor had significantly reduced levels of $pAkt^{Ser473}$, whereas levels of $pAkt^{Ser473}$ in lysates expressing shRNA targeting Raptor remained unaffected compared with the control (Supplementary Fig. S3A). Moreover, tumors derived from control SW480 cells formed tumors with sizes of ~690 mm^3 within 25 days (Fig. 6B). In contrast, knockdown of mTOR, Raptor, or Rictor significantly reduced tumor growth over the same period; smaller tumors with sizes ranging from 10 to 135 mm^3 were detected at sacrifice (day 25). Lysates from SW480-derived tumors expressing shRNA targeting Rictor had significantly reduced levels of $pAkt^{Ser473}$, whereas levels of $pAkt^{Ser473}$ in lysates expressing shRNA targeting mTOR and Raptor were increased compared with the control (Supplementary Fig. S3B). Taken together, our results show that knockdown of mTOR, Raptor, and Rictor inhibits growth of rapamycin-sensitive and rapamycin-resistant CRC xenografts in nude mice.

Discussion

In this study, we determined the role of mTORC1 and mTORC2 on CRC growth. First, we show that mTOR, Raptor, and Rictor are overexpressed in CRC specimens

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compared with normal colonic tissue. Furthermore, Rictor overexpression correlates with elevated pAkt^{Ser473} levels. Second, we found that transient inhibition of Rictor, an essential component of mTORC2, significantly decreased proliferation of both rapamycin-sensitive and rapamycin-resistant CRCs. Third, we found that stable inhibition of mTORC2 inhibited proliferation and induced apoptosis in both rapamycin-sensitive and rapamycin-resistant CRCs. Finally, we showed that *in vivo* growth of CRC xenografts was significantly reduced with targeted inhibition of both mTORC1 and mTORC2.

Cellular levels of phosphorylated Akt are suspected to be important determinants of rapamycin sensitivity of cancer cells (22, 23). The current dogma suggests that cancers addicted to elevated Akt signaling are dependent on downstream activation of mTORC1 to drive tumorigenesis (4). However, despite the seemingly clear rationale for the use of mTOR inhibitors in these cancers, clinical trials with rapamycin analogues have been, for the most part, disappointing (4). Rapamycin is a universal inhibitor of mTORC1-dependent S6K phosphorylation, but the existence of the negative feedback loop from S6K to Akt through IGF-IR presents a therapeutic problem as loss of feedback inhibition of Akt results in paradoxical Akt activation, which can promote cell survival and chemoresistance (4, 16, 17).

Consistent with these findings, our results show that rapamycin-resistant CRCs show a dose-dependent increase in pAkt^{Ser473} upon rapamycin treatment, whereas rapamycin-sensitive CRCs show a dose-dependent decrease in pAkt^{Ser473}. Moreover, both rapamycin-sensitive and rapamycin-resistant CRCs show a decrease in p-p70S6K^{Thr389} upon rapamycin treatment, but only the rapamycin-sensitive CRCs exhibit a significant decrease in proliferation upon rapamycin treatment. These results are consistent with the findings in other cancers in which rapamycin treatment shows variable sensitivity; induction of pAkt^{Ser473} by negative feedback is often noted in rapamycin-resistant cancers (24). There are at least three possible explanations to this unexpected pattern of change in pAkt^{Ser473} levels. First, intrinsic differences in upstream signaling between rapamycin-sensitive and rapamycin-resistant CRC cells may exist, which results in sustained feedback Akt activation by the negative feedback loop, thus leading to rapamycin resistance. Alternatively, there may be differential effects of rapamycin treatment on levels of the recently described PHLPP phosphatases, which regulate pAkt^{Ser473} and are lost or reduced in ~80% of CRC samples (25–27). Finally, it has been shown previously that long-term rapamycin treatment can lead to disassembly of mTORC2 in certain cells (15). It is interesting to speculate that rapamycin may be causing dissociation of mTORC2 only in the rapamycin-sensitive cell lines (HCT116 and KM20) but not in rapamycin-resistant cells (SW480 and Caco-2). Thus, changes in Akt^{Ser473} phosphorylation by mTORC2 may account for the differences in pAkt^{Ser473} levels noted in rapamycin-sensitive CRCs.

Targeting mTORC2 as an anticancer therapy is attractive for several reasons. First, almost 60% of CRCs show elevated Akt levels (2). In cultured cells and in the developing embryo, mTORC2 is a critical Akt^{Ser473} kinase (4, 18). Our results suggest that mTORC2 is the primary kinase that phosphorylates and activates Akt^{Ser473} in CRCs. We further show that targeted inhibition of Rictor can circumvent the paradoxical Akt activation noted with rapamycin treatment because there is no direct inhibition of mTORC1. Both transient and stable inhibition of the mTORC2 protein, Rictor, resulted in significantly reduced pAkt^{Ser473} levels in rapamycin-sensitive and rapamycin-resistant CRCs. Second, a mTORC2 inhibitor may not be toxic because mTORC2 activity is dispensable in normal epithelium or cultured mouse embryonic fibroblasts; rather, mTORC2 is required under conditions of elevated PI3K activity, which occurs in cancer (4, 28). This suggests the possibility that inhibition of mTORC2 could be more deleterious to cancer cells than to normal cells. Third, we found that transient inhibition of mTORC2, but not mTORC1, significantly reduced proliferation of all CRC cell lines tested, whereas stable inhibition of both mTORC1 and mTORC2 significantly reduced proliferation of the CRC cell lines and also reduced tumor growth *in vivo*. We hypothesize that CRC cells are more sensitive to inhibition of mTORC2 compared with mTORC1, such that even short-term loss of Rictor expression can inhibit proliferation of CRCs significantly. Finally, our results show that stable inhibition of Rictor, but not mTOR and Raptor, leads to significant induction of apoptosis in both rapamycin-sensitive and rapamycin-resistant CRCs. This is important from a therapeutic standpoint as most successful therapeutic targets have both antiproliferative and cytotoxic effects on cancer cells.

Recent studies suggest that a mTORC2-specific inhibitor may be a promising therapeutic agent for certain cancers driven by mutations promoting Akt signaling, such as activating mutations of PI3K or loss of PTEN. A recent study found that glioma cell lines and tissues exhibit Rictor overexpression, which results in elevated mTORC2 activity and promotes anchorage-independent growth, cellular motility, and *in vivo* growth (29). Another study showed that prostate cancers lacking PTEN require mTORC2 to form tumors when injected into nude mice (28). In addition, the development of prostate cancer caused by *Pten* deletion in prostate epithelium required mTORC2, whereas mTORC2 activity is not essential for maintaining the integrity of normal prostate epithelium. Another recent study found that muscle-specific deletion of the *Raptor* gene caused muscular dystrophy, whereas deletion of *Rictor* only had minor consequences (30).

Our studies on the effects of stable inhibition of mTORC1 and mTORC2 suggest that both complexes play a role in the proliferation and tumorigenesis of CRCs. Based on these results, it is tempting to speculate that the new generation of mTOR kinase inhibitors targeting the mTOR ATP-binding pocket, such as Torin1, PP242, and PP30, will hold greater therapeutic potential in the treatment of CRC than rapamycin analogues because they will inhibit a wider spectrum of functions downstream of both mTOR complexes (31–33). Moreover, there may be rationale for the use of dual PI3K/mTOR inhibitors, such as PI-103 and NVP-BEZ235, to avoid feedback activation of PI3K/Akt signaling, especially in rapamycin-resistant CRCs that display a marked increase in pAkt^{Ser473} levels after rapamycin treatment.

In conclusion, our data support a role for elevated mTORC1 and mTORC2 activity in CRC proliferation, apoptosis, cell cycle progression, and tumorigenesis *in vivo*. mTOR and its interaction partners, Raptor (mTORC1) and Rictor

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Published OnlineFirst November 24, 2009; DOI: 10.1158/1078-0432.CCR-09-1249

(mTORC2), were noted to be overexpressed in CRCs. Inhibition of mTORC1 and mTORC2 had pronounced effects on CRC growth and proliferation. Targeted inhibition of the mTORC2 component, Rictor, effectively inhibited growth and induced apoptosis in both rapamycin-sensitive and rapamycin-resistant CRCs, suggesting that selective targeting of mTORC2 may represent a novel therapeutic strategy for treatment of CRC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Mingjun Liao and Jun Song for technical assistance, Mark Griffin for assistance with flow cytometry, Karen Martin for manuscript preparation, and Tatsuo Uchida for help with statistical analysis.

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Clin Cancer Res 2009;15:7207-7216. Published OnlineFirst November 24, 2009.

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