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Regulation of mTOR Complex 2 Signaling in Neurofibromatosis 2–Deficient Target Cell Types

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Abstract

Inactivating mutations in the neurofibromatosis 2 (*NF2*) tumor suppressor gene results in the development of schwannomas and meningiomas. Using *NF2*-deficient meningioma cells and tumors, together with the normal cellular counterparts that meningiomas derive, arachnoid cells, we identified merlin as a novel negative regulator of mTOR complex 1 (mTORC1). We now show that merlin positively regulates the kinase activity of mTORC2, a second functionally distinct mTOR complex, and that downstream phosphorylation of mTORC2 substrates, including Akt, is reduced upon acute merlin deficiency in cells. In response to general growth factor stimulation, Akt signaling is attenuated in merlin RNA interference-suppressed human arachnoid and Schwann cells by mechanisms mediated by hyperactive mTORC1 and impaired mTORC2. Moreover, Akt signaling is impaired differentially in a cell type–dependent manner in response to distinct growth factor stimuli. However, contrary to activation of mTORC1, the attenuated mTORC2 signaling profiles exhibited by normal arachnoid and Schwann cells in response to acute merlin loss were not consistently reflected in *NF2*-deficient meningiomas and schwannomas, suggesting additional genetic events may have been acquired in tumors after initial merlin loss. This finding contrasts with another benign tumor disorder, tuberous sclerosis complex, which exhibits attenuated mTORC2 signaling profiles in both cells and tumors. Finally, we examined rapamycin, as well as the mTOR kinase inhibitor, Torin1, targeting both mTOR complexes to identify the most efficacious class of compounds for blocking mTOR-mediated signaling and proliferation in merlin-deficient meningioma cells. These studies may ultimately aid in the development of suitable therapeutics for *NF2*-associated tumors. *Mol Cancer Res*; 10(5): 649–59. ©2012 AACR.

Introduction

Germline mutations of the neurofibromatosis 2 (*NF2*) gene are associated with *NF2*, a severe, inherited tumor syndrome characterized by bilateral vestibular schwannomas, often in combination with other cranial and spinal schwannomas and meningiomas (1). Biallelic inactivation of *NF2* is also the initiating event in the development of the majority (~60%) of sporadic meningiomas and almost all schwannomas, tumors that arise from cell types of neural crest origin. Specifically, meningiomas and schwannomas

develop respectively from arachnoid cells of the meninges covering the brain and spinal cord, and from Schwann cells that ensheath and myelinate peripheral nerves. Meningiomas comprise approximately 30% of all brain neoplasms and although some can often be effectively treated with surgery and radiation, an important subset remains inoperable or has recurrence rates of up to 20% over 10 years. To date, most medical therapies have generally been ineffective indicating that an improved understanding of the molecular pathogenesis of these tumors is needed to benefit the development of new treatments (1).

NF2 encodes the tumor suppressor product, merlin, a member of the ezrin, radixin, moesin (ERM) family of membrane-cytoskeletal linker proteins (2, 3), which regulates membrane organization and numerous actin cytoskeletal-based cellular processes including cell adhesion, cell–cell contact, membrane transport, and signal transduction pathways (4). Although merlin overlaps functionally in part with ERM proteins, merlin is distinguished by its tumor suppressor activity. Merlin is suggested to control cell proliferation by mediating contact inhibition of growth through mechanisms that include formation of stable adherens junctions or promoting efficient activity, expression, and/or transport of specific growth factor receptors (5, 6). Consequently, merlin loss induces signaling of numerous

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mitogenic pathways including Ras/mitogen-activated protein kinase (MAPK), Rac, phosphoinositide 3-kinase (PI3K), Hippo/Mst1 (7–12), as well as E3 ubiquitin ligase activity (13) in multiple cell types. Although merlin is implicated in a wide range of cellular activities in different cell types, it is unclear which of these functions are essential for inhibiting cell proliferation and tumor growth in meningeal and Schwann cells.

To investigate the mechanism(s) by which merlin loss results in tumor growth, we have developed *in vitro* NF2 model systems using human meningeal- and Schwann-derived cells to more accurately reflect the environment of NF2 tumorigenesis. Using primary human merlin-deficient meningioma and merlin RNA interference (RNAi)-suppressed arachnoid cells, we recently identified merlin as a novel negative regulator of the mTOR complex 1 (mTORC1; ref. 14), a large multisubunit protein complex that integrates signals from growth factors, nutrients and energy to coordinate many key cellular processes including growth (cell mass gain), and proliferation. mTOR, an evolutionarily conserved Ser/Thr kinase exists in one of 2 distinct functional complexes, mTORC1 and mTORC2, which consists of discrete sets of proteins and appears to carry out nonoverlapping functions (15). mTORC1 regulates cell growth and proliferation by promoting increased translation and protein synthesis through phosphorylation of effector proteins, S6 kinase (S6K), and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; ref. 16). mTORC1 is potently inhibited by the tuberous sclerosis complex (TSC) tumor suppressors TSC1 and TSC2 that together regulate the GTP-loading state of Rheb (Ras homologue enriched in brain), a key upstream activator of mTORC1 (15, 17). Rapamycin acutely and specifically inhibits mTORC1, whereas its effects on mTORC2 are more variable and generally requires prolonged treatment. In addition to their differential sensitivity to rapamycin, mTORC1 and mTORC2 are activated in different ways and possess distinct substrate specificity (18).

Although much less is known about the regulation and function of mTORC2, its best characterized function is the phosphorylation of Akt on S473 that lies within a hydrophobic motif conserved among AGC (protein kinase A, G, and C) family kinases (19). Similar hydrophobic motifs on other AGC kinases including PKC α (S657), and the serum and glucocorticoid-induced protein kinase (SGK1; S422), are also phosphorylated by mTORC2 (20, 21). The phosphorylation of a second conserved motif on Akt, referred to as the turn motif (T450 on Akt), is also dependent on mTORC2. Although the kinase activity of mTORC2 can be stimulated by growth factors, perhaps downstream of PI3K, some functions of mTORC2, such as the phosphorylation of PKC α S657 or Akt T450, are independent of growth factor signaling (19).

Activation of mTORC1 has been found to negatively impact Akt phosphorylation in response to insulin or insulin-like growth factor (IGF)1 through negative feedback loops at multiple levels. Inhibition of PI3K signaling by mTORC1 can be attributed to the phosphorylation and

degradation of insulin receptor substrate 1 (IRS1) by active S6K or by inhibition of platelet-derived growth factor (PDGF) receptors through an unknown mechanism (22–24). In addition, very recent phosphoproteome studies have identified Grb10 as an mTORC1 substrate that mediates feedback inhibition of PI3K and ERK-MAPK pathways (25, 26). The TSC1-TSC2 complex, in addition to its role in inhibiting mTORC1, was shown to interact with mTORC2 and positively regulates its kinase activity (17). These findings help to explain the enhanced Akt inhibition as well as reduced activation of mTORC2 in TSC1-TSC2 deficiency (27, 28).

Previously, we showed that merlin loss results in hyperactivation of mTORC1 *in vitro* and *in vivo* and that Akt signaling is impaired in response to insulin stimulation through an mTORC1-mediated negative feedback loop (14). Deregulation of mTORC1 signaling in merlin-deficient arachnoid cells is reminiscent of TSC deficiency in cells/tumors suggesting that growth control mechanisms may be overlapping in TSC and NF2 tumor suppressor syndromes. Here, we examined the regulation of mTORC2 signaling by merlin in NF2 target cell types and tumors. We identify both parallels and distinctions in mTORC1/2 downstream signaling events and in negative feedback regulation to Akt between NF2-deficient Schwann and arachnoid cells. Our data also indicate that acute merlin suppression in arachnoid cells may not completely reflect the landscape of NF2-associated meningiomas. In addition, we evaluated the efficacy *in vitro* of mTOR and dual PI3K/mTOR inhibitors in blocking activated mTOR signaling and proliferation/survival.

Materials and Methods

Antibodies and reagents

Antibodies to phospho-S6 (S240/244), S6, p70 S6K, phospho-p70 S6K (T389), phospho-Akt (T450; S473), Akt, phospho-p44/42 MAPK (ERK1/2) (T202/Y204), p44/42 MAPK (ERK1/2), phospho-NDRG1 (T346), PKC α , preimmune rabbit immunoglobulin G (IgG) were obtained from Cell Signaling Technology. An antibody to Rictor was obtained from Bethyl Laboratories; phospho-PKC α (S657) from Upstate; NDRG1 from Abcam; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Chemicon International; and α -tubulin from Sigma. Merlin rabbit polyclonal antibody (C26) was described previously (29). Growth factors were from Sigma (insulin, EGF, PDGF-BB) and Austral Biologicals (IGF1). Rapamycin and PI-103 were purchased from Calbiochem. Torin1 was kindly provided by Dr. David Sabatini (Whitehead Institute/MIT, Cambridge, MA). Commercial inhibitors were reconstituted in dimethyl sulfoxide (DMSO) as per manufacturer's recommendations.

Sample collection and cell culture

Fresh tissues were collected at the time of clinically indicated surgery for tumor resection (excess discarded tissues) or from patients who underwent autopsy by the

MGH neurooncology tumor repository in accordance with an Institutional Review Board–approved protocol. Informed consent was obtained from all study subjects. Tissue was flash frozen in liquid nitrogen or fixed in formalin to use for histology and immunohistochemical analyses. NF2-deficient tumors were classified as sporadic tumors or NF2-associated tumors based on diagnoses of referring clinicians. Unless otherwise specified, meningiomas and meningioma cells described in text refer to benign or World Health Organization (WHO) grade I meningioma tumors and cells. Atypical meningioma cells were established from atypical (WHO grade II) meningiomas.

Cultures of primary meningioma and normal arachnoid cells from fresh tissues were established as described previously (29). Primary meningioma cells and the immortalized arachnoid cell line, AC007-hTERT (14), were maintained in Dulbecco's Modified Eagles's Medium (DMEM) with a 4.5 g/L glucose solution containing 15% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin (full serum medium). Primary normal arachnoid cells were maintained in a mixture of improved minimum essential medium with L-glutamine [Richter's Modified Medium (Cellgro; Mediatech, Inc.)] supplemented with 15% FBS (Hyclone) insulin (4 mg/L; Gibco Life Technologies, Inc.) and 100 U/mL penicillin and 100 µg/mL streptomycin (full medium; refs. 14, 29). The immortalized human Schwann cell line, pn02.3, provided by Dr. Wallace of the University of Florida, was established from a primary human Schwann cell culture derived from the sciatic nerve of a healthy, unaffected individual (30). A full description of this immortalized cell line will be described in detail elsewhere (H. Li, personal communication; ref. 31). In brief, Schwann cells were immortalized by lentiviral-mediated expression of Cdk4 (murine) and telomerase (human, hTERT). The immortalized pn02.3 Schwann cells have been passed well beyond the capacity of the original culture to proliferate (p6). They are uniformly Schwann-like in appearance (spindle-shaped cells) and express S100 by immunocytochemistry. They are maintained in DMEM supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin but without neuregulin (GGF2), and seeded in the absence of matrix-coating on tissue culture plates.

For growth factor stimulation studies, control (scr) and merlin (m5) knockdown arachnoid cells (AC007-hTERT) and Schwann cells (pn02.3) were serum-deprived in 0.1% FBS for 18 hours before growth factor stimulation for 30 minutes at the indicated concentrations. For mTORC2 substrate phosphorylation analyses, cells were serum deprived for 18 or 24 hours as described in the text, or cultured in full serum conditions with or without rapamycin (20 nmol/L) for variable time periods (1, 18, or 24 hours) before cell lysis. For signaling studies in response to mTOR or dual PI3K/mTOR inhibitor treatment, cells were cultured in full serum medium in the presence of rapamycin (50 nmol/L), PI-103 (500 nmol/L), Torin1 (250 nmol/L), or vehicle only (DMSO) for 1 or 24 hours before cell lysis.

Constructs, virus production, and lentiviral infections

Previously, we employed 3 NF2 short hairpin RNAs (shRNA) specific for merlin and one nonspecific shRNA control in pLKOpuro.1 to establish that merlin suppression in human arachnoid cells resulted in almost complete loss of merlin expression and consequent aberrant activation of mTORC1 signaling (14, 29). Here, we have chosen one representative NF2 shRNA (m5) and one nonspecific scramble control (scr) pLKOpuro.1 construct and generated lentivirus as described previously (29). Isogenic pairs of scr and m5 merlin knockdown arachnoid and pn02.3 Schwann cells were established by lentiviral infections at a multiplicity of infection of 10, and harvested for cell lysates 7 to 10 days postinfection.

Cell lysis and immunoblotting

Protein lysates were harvested from subconfluent cultures because many growth factor–mediated signaling events are inhibited upon cell confluence. Control (scr) and NF2/merlin (m5) knockdown arachnoid and pn02.3 Schwann cell lysates were prepared from subconfluent (80%–90%) cultures in NP-40 lysis buffer (20 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl₂, 1% Nonidet p-40 (NP-40) containing calyculin (50 nmol/L; Cell Signaling Technology), ×1 HALT phosphatase inhibitor cocktail (Thermo Scientific) and ×1 Complete protease inhibitor cocktail (Roche Diagnostics). Subconfluent primary merlin-deficient meningioma cell cultures were harvested in RIPA buffer for protein lysates as described previously (14). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and subjected to immunoblot analysis using indicated antibodies, horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology) and the enhanced chemiluminescence (ECL) detection systems (Amersham Pharmacia Biotechnology).

mTORC2 kinase assays

Kinase assays on endogenous mTORC2 were conducted as described previously with minor modifications (17, 32). Near-confluent, 150-mm plates of control (scr) or NF2/merlin knockdown (m5 RNAi) arachnoid cells were stimulated with EGF (5 ng/mL) for 30 minutes before lysis in 1 mL mTORC lysis buffer (40 mmol/L HEPES (pH 7.5), 120 mmol/L NaCl, 1 mmol/L EDTA, 0.3% CHAPS {3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate} containing phosphatase and protease inhibitors as described above, and immunoprecipitations conducted with 1.5 µg Rictor or control IgG antibodies. The soluble fraction of the lysates was precleared for 1 hour with nonspecific IgG antibodies with rotation at 4°C and then incubated with the precipitating antibody for 2 hours. Immunoprecipitates were captured with protein A/G-agarose (Amersham) for 1.5 hours and then washed 3 times with mTORC lysis buffer and twice with the Rictor-mTOR kinase buffer (25 mmol/L HEPES, pH 7.5, 100 mmol/L potassium acetate, 2 mmol/L MgCl₂). For mTORC2 kinase reactions, immunoprecipitates were incubated in a final volume of 15 µL for 20

minutes at 32°C in the Rictor-mTORC kinase buffer containing 500 ng inactivate Akt1/PKB1 (Upstate Biotechnology) as the substrate and 500 µmol/L ATP (32). The reaction was stopped by placing samples on ice and immediately adding SDS sample buffer. The supernatant was removed from protein A/G agarose and analyzed by SDS-PAGE and immunoblotting as indicated.

Immunohistochemistry

Five benign NF2-deficient meningiomas and 4 vestibular schwannomas were formalin and paraffin embedded (27). Antigen unmasking was achieved by microwaving in 10 mmol/L sodium citrate (pH 6.0) followed by immersion in 3% hydrogen peroxide for 20 minutes and 5% goat serum for 1 hour to block endogenous peroxidase and nonspecific antibody binding, respectively. After incubation with the appropriate primary antibodies overnight at 4°C [total PKCα at 1:20 dilution and phospho-Akt S473 at 1:50 dilution (27)], secondary antibodies and avidin-biotin-peroxidase complex were applied according to the manufacturer's protocol (ABC Elite Staining; Vector Labs). Visualization was achieved by incubating with 3,3'-diaminobenzidine tetrahydrochloride (Pierce), and the sections were counterstained with hematoxylin. Control tissues for antibodies include TSC-associated kidney angiomyolipomas (AML) (negative control).

Cell proliferation/viability assays

Cell proliferation/viability was assessed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega) that determines the number of viable cells based on quantitation of ATP present. On day 0, 96-well plates were seeded with 1,000 cells per well in triplicate and grown overnight. On days 1 and 4, cells were treated with the appropriate compounds and viability assays were conducted on days indicated. Plates were incubated with the CellTiter-Glo reagent according to manufacturer's suggestions, shaken on an orbital shaker for 2 minutes, and incubated 10 minutes further in darkness at room temperature to stabilize the luminescent signal. Luminescence was detected on a MicroLumatPlus LB 96V Luminometer (Berthold Technologies) and average values depicted as relative luminescent units (RLU).

Results

General attenuation of Akt phosphorylation in merlin-deficient arachnoid and Schwann cells

We previously reported that arachnoid cells deficient for merlin show constitutive phosphorylation of S6, a marker of mTORC1 activity, but impaired Akt S473 phosphorylation in response to insulin stimulation (14). Attenuated Akt S473 phosphorylation in merlin knockdown arachnoid cells is consistent with a negative feedback mechanism mediated by mTORC1/S6K phosphorylation of IRS proteins and consequent inhibition of PI3K-Akt signaling (15, 33). To determine whether merlin knockdown arachnoid cells show defective Akt signaling in response to

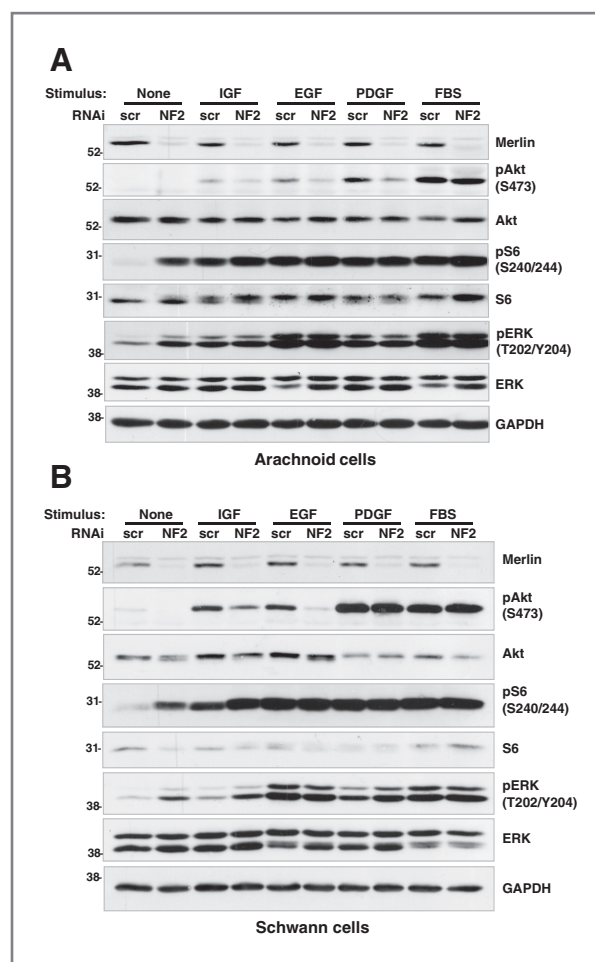


Figure 1. General attenuation of Akt phosphorylation upon decreased merlin expression. Phosphorylation of Akt S473 (pAkt S473) is impaired differentially in response to growth factor stimuli in merlin-suppressed arachnoid cells (A) and Schwann cells (B). A, arachnoid cells stably expressing either control (scr) or merlin (NF2) RNAi transcripts were serum deprived (0.1% FBS) 18 hours and stimulated with or without IGF1 (50 ng/mL), EGF (5 ng/mL), PDGF-BB (5 ng/mL), or 15% FBS, as indicated, for 30 minutes before cell lysis. Akt S473 phosphorylation is attenuated in response to IGF1, EGF, PDGF, but not FBS. Total protein and/or phosphorylation levels for merlin, Akt, phospho-S6 (pS6 S240/244), S6, phospho-ERK (pERK T202/Y204), ERK, and GAPDH as a loading control, were determined by immunoblotting with indicated antibodies. B, control and merlin-suppressed immortalized Schwann cells (pn02.3) were stimulated as described in (A), substituting 10% FBS for 15% FBS stimulation. Akt S473 phosphorylation is attenuated in response to IGF1 and EGF but not PDGF or FBS stimulation. ERK1/2 phosphorylation (T202/Y204) was elevated in serum deprived, IGF, and PDGF stimulated pn02.3 immortalized Schwann cells.

stimulation by additional growth factors, we examined Akt S473 phosphorylation in response to IGF1, EGF, PDGF-BB, and FBS stimulation. Akt S473 phosphorylation was markedly reduced in merlin knockdown arachnoid cells compared with control cells stimulated with IGF1, EGF, and PDGF, but not appreciably in response to FBS (Fig. 1A).

We next examined mTORC1 signaling and Akt S473 phosphorylation in Schwann cells, a major target cell type in

NF2 tumorigenesis. Consistent with arachnoid cells, merlin deficiency by RNAi in an immortalized human Schwann cell line (pn02.3) resulted in elevated mTORC1 signaling as indicated by constitutive S6 phosphorylation. In further agreement, Akt S473 phosphorylation was not observed in merlin knockdown Schwann cells under serum-deprived conditions compared with control cells. In response to growth factor stimulation by IGF1 and EGF, but not PDGF or FBS, merlin knockdown Schwann cells exhibited impaired Akt S473 phosphorylation relative to control cells (Fig. 1B). The lack of attenuated Akt S473 phosphorylation in response to PDGF stimulation in merlin-suppressed Schwann cells is in sharp contrast to merlin knockdown arachnoid cells. Our data are in agreement with a general loss of Akt stimulation observed characteristically in TSC-null cells in response to a variety of growth factors including those that signal independent of IRS proteins (27). These findings indicate that in addition to mTORC1/S6K-dependent feedback inhibition of IRS, other mechanism(s) may be functional in merlin-deficient NF2 target cells to dampen Akt signaling.

Although ERK (T202/Y204) phosphorylation is elevated in serum-deprived conditions in merlin-deficient arachnoid and Schwann cells compared with control cells (Fig. 1A and B), we and others previously showed it was not responsible for mTORC1 activation (14, 34). Interestingly, we observed an increase in ERK phosphorylation in merlin-suppressed Schwann cells in response to IGF and PDGF stimulation. To assess whether this increase in ERK phosphorylation might be due to disruption of receptor signaling, we examined total and phosphorylated levels of the PDGF receptor beta (PDGFR β) and IGF1 receptor beta (IGF1R β) in control and NF2 RNAi Schwann cells. In response to PDGF stimulation, NF2 knockdown Schwann cells showed an increase in both total and phosphorylated PDGFR β levels compared with control cells. This finding is in agreement with previous reports that showed elevated PDGFR β levels in schwannoma cells and tissues compared with Schwann cells (35, 36), and that schwannoma cells exhibit elevated ERK1/2 activation compared with Schwann cells in response to PDGF stimulation (36). On the contrary, we noted a decrease in total and phosphorylated IGF1R β levels in response to IGF1 stimulation in NF2 knockdown Schwann cells compared with control cells suggesting that activation of IGF1R β is not responsible for ERK activation (Supplementary Fig. S1).

Loss of merlin leads to a decrease in mTORC2 signaling and kinase activity

To determine whether decreased mTORC2 signaling contributes to general Akt attenuation in response to growth factors in merlin-deficient arachnoid and Schwann cells, we examined phosphorylation sites of several mTORC2-dependent substrates of the AGC family of kinases including Akt (S473 and T450), PKC α (S657), and SGK1 (S422). Because mTORC2 phosphorylates the hydrophobic motif of Akt S473 and SGK1 S422 in a PI3K-dependent manner, and Akt T450 and PKC α S657 sites independent of growth

factor signaling, we examined the phosphorylation of mTORC2 substrates in the absence and presence of full serum growth conditions.

Consistent with our earlier findings, Akt S473 phosphorylation was not observed under serum-deprived conditions in either control or merlin-suppressed arachnoid and Schwann cells. Although no difference in Akt phosphorylation was detected in NF2-deficient cells in response to acute serum stimulation (Fig. 1), steady-state levels of Akt phosphorylation were reduced in these cells grown in full serum (Fig. 2A and B). Prolonged rapamycin treatment (18 hours) had varying effects on the 2 cell types, increasing Akt-S473 phosphorylation in arachnoid cells but decreasing it in Schwann cells. Surprisingly, we detected increased phosphorylation of N-Myc downstream regulated gene 1 (NDRG1) T346, a specific marker for SGK1 signaling (19, 21), in both merlin knockdown arachnoid and Schwann cells compared with controls. In a growth factor-independent manner, we observed reduced levels of Akt T450 and PKC α S657 phosphorylation upon merlin suppression in arachnoid and Schwann cells compared with controls. In addition, PKC α total protein levels were reduced in merlin knockdown cells reflecting the significance of this residue to PKC α stability (19). Collectively, these findings indicate that merlin regulates mTORC1 as well as mTORC2 signaling.

To more fully understand whether the attenuated Akt phosphorylation in merlin-deficient cells is due to mTORC1 as well as mTORC2 signaling, we treated NF2 knockdown arachnoid and Schwann cells with rapamycin for short-term (1 hour) and long-term (24 hours) time periods, which specifically blocks mTORC1 signaling (1 hour), or mTORC1 and mTORC2 signaling (24 hours). In response to short-term rapamycin treatment, arachnoid cells show an increase in Akt S473 phosphorylation indicating relief of the negative feedback regulation from mTORC1-S6K signaling (Fig. 2C). An increase in Akt S473 phosphorylation was not observed in Schwann cells indicating cell type-dependent differences in response to rapamycin (Fig. 2D). In response to long-term exposure to rapamycin, Schwann cells, unlike arachnoid cells, exhibited a further decrease in Akt S473 phosphorylation indicating that mTORC2 assembly may be affected in Schwann cells and not in arachnoid cells (Fig. 2C and D). Our data are in agreement with the established notion that long-term rapamycin treatment does not always lead to the total loss of mTORC2 activity, and that mTORC2 assembly is not completely blocked in all cell types (18).

To determine whether the attenuation of mTORC2 signaling observed in merlin knockdown arachnoid cells and Schwann cells could be due to diminished mTORC2 kinase activity, we directly assayed mTORC2 kinase activity. Endogenous mTORC2 was isolated from control and merlin knockdown arachnoid cell lysates by immunoprecipitating Rictor, and its kinase activity was assayed using an exogenous Akt1 substrate. Similar amounts of mTOR were immunoprecipitated with Rictor in control and merlin-deficient cells stimulated with EGF, however, mTORC2

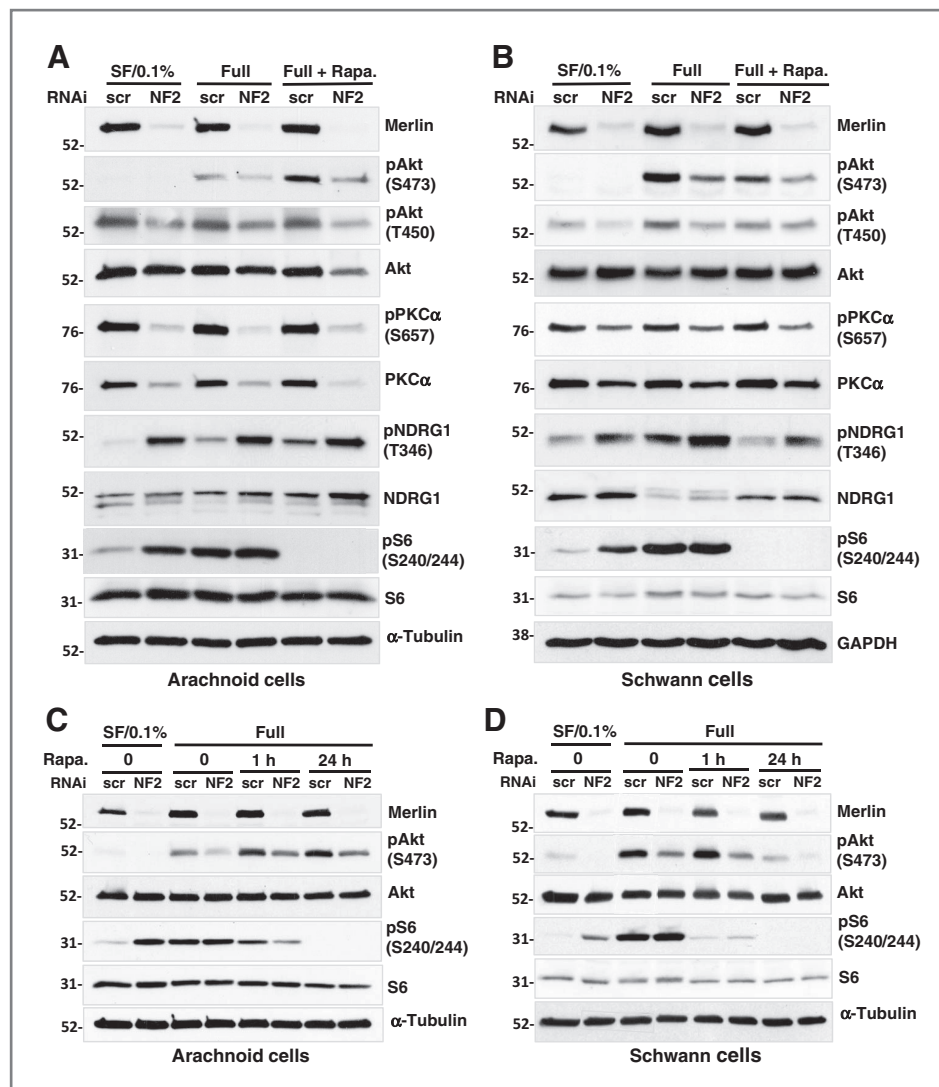


Figure 2. Phosphorylation of mTORC2 substrates in merlin-deficient arachnoid and Schwann cells. Control (scr) and merlin knockdown (NF2) arachnoid (A and C) or immortalized Schwann (pn02.3; B and D) cells were serum starved (0.1% FBS) or cultured in full serum with and without rapamycin for 18 hours (A and B) or 1 and 24 hours (C and D). A and B, phosphorylation of Akt S473, Akt T450, and PKCα S657 is impaired in merlin knockdown arachnoid and pn02.3 Schwann cells compared with control cells. Phosphorylation of NDRG1 T346, a direct substrate of SGK1, is increased in merlin knockdown cells compared with control cells. Expression and/or phosphorylation levels also were determined for merlin, Akt, PKCα, NDRG1, phospho-S6 (pS6 S240/244), and S6 by immunoblotting. Expression of α-tubulin or GAPDH was used as loading controls. C and D, Akt S473 phosphorylation is differentially sensitive to short-term (1 hour) and long-term (24 hours) rapamycin treatment in arachnoid and immortalized pn02.3 Schwann cells.

kinase activity was greatly impaired in merlin knockdown arachnoid cells indicating that merlin loss inhibits mTORC2 kinase activity (Fig. 3A). Akt S473 phosphorylation levels for control and NF2 RNAi samples from 2 independent mTORC2 kinase assays were quantified. The average decrease in EGF-stimulated mTORC2 kinase activity in merlin knockdown arachnoid cells compared with control cells was approximately 50% (Fig. 3B).

Heterogeneous expression of PKCα and Akt S473 phosphorylation in merlin-deficient meningiomas and schwannomas

Our data indicate that acute merlin downregulation by RNAi in NF2 target cell types such as normal arachnoid and Schwann cells results in elevated mTORC1 signaling and decreased mTORC2 signaling. We have shown earlier that mTORC1 signaling is activated in NF2-deficient meningiomas and schwannomas (14). To determine whether NF2 tumors exhibit reduced mTORC2 signaling, we examined

PKCα and phospho-Akt S473 expression levels in merlin-deficient meningioma and schwannoma tumors compared with normal arachnoid tissue by immunohistochemical staining. We detected heterogeneity in both PKCα and phospho-Akt S473 expression levels across multiple benign meningioma ($n = 5$) and schwannoma ($n = 4$) samples compared with TSC AML (Fig. 4 and Table 1). Previous studies showed that TSC AMLs display high phospho-S6 levels but do not express detectable levels of PKCα or phospho-Akt (27). PKCα immunoreactivity was moderate to strongly positive in normal arachnoid tissue as well as benign meningiomas and schwannomas. Weak to moderate levels of phospho-Akt S473 positivity were detected in both normal arachnoid and benign meningioma samples relative to TSC AML tissue. In schwannomas, however, phospho-Akt S473 staining was uniformly reduced across tumor samples relative to normal arachnoid and meningioma tissues, and more comparable with TSC AMLs. The lack of detectable Akt S473 phosphorylation

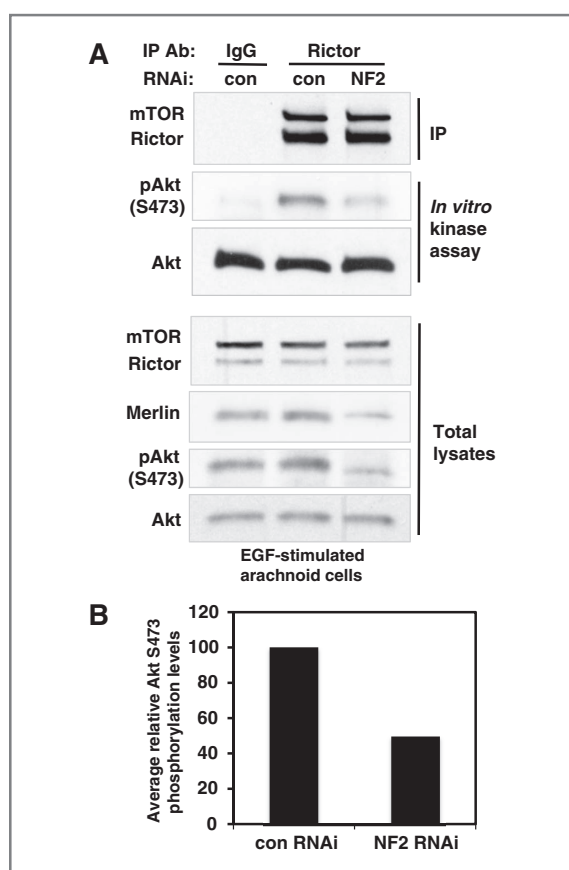


Figure 3. mTORC2 kinase activity is impaired in cells deficient for merlin. Control (con) and merlin knockdown (NF2) arachnoid cells were serum deprived (0.1% FBS) overnight and stimulated with EGF (5 ng/mL) for 30 minutes before lysis and immunoprecipitation (IP) with control IgG or Rictor antibodies (Ab). mTORC2 kinase activity was assessed in these immunoprecipitates using inactive Akt1 as a substrate. Immunoblotting for mTOR, Rictor, phospho-Akt (S473), Akt, and merlin was conducted with appropriate antibodies. B, the average relative phospho-Akt (S473) levels for control (con) and NF2 RNAi samples were quantified from 2 independent mTORC2 kinase assays. Phospho-Akt (S473) levels were adjusted to total inactive Akt substrate levels, and phospho-Akt (S473) levels from NF2 RNAi samples (49.9%) were represented as percentage values relative to con RNAi levels that were arbitrarily set to 100%.

in schwannoma tissue by immunohistochemistry is in agreement with the study of Ammoun and colleagues (2010), which reports that Akt phosphorylation was not observed in vestibular schwannoma specimens ($n = 10$) by phosphokinase profiling array analysis (37). However, other earlier studies showed positive Akt S473 phosphorylation in vestibular schwannoma samples and/or increased Akt protein levels (38, 39). These differences could be due to tumor heterogeneity or variations in sensitivity of the methods/antibodies employed. Our data show that reduced signaling events downstream of mTORC2 in response to acute merlin suppression in arachnoid and Schwann cells are not necessarily reflective of mTORC2 signaling in merlin-deficient meningiomas and schwannomas with chronic merlin loss.

mTOR signaling and proliferation in merlin-deficient meningioma cells in response to mTOR and dual PI3K/mTOR inhibitors

Treatments directed against mTOR signaling pathways have shown promise in the management of solid tumors and therefore may constitute a potential target for the treatment of NF2 meningiomas. However, rapamycin and its analogs effectively inhibit S6K phosphorylation, but not 4E-BP1 phosphorylation and therefore incompletely inhibit mTORC1-dependent protein synthesis (40). In addition, a major concern is that rapalogs also cause activation of prosurvival and oncogenic pathways such as PI3K/Akt. These trepidations have fueled the development of mTOR kinase inhibitors and therefore incompletely inhibit mTORC1-dependent protein synthesis (40). In addition, a major concern is that rapalogs also cause activation of prosurvival and oncogenic pathways such as PI3K/Akt. These trepidations have fueled the development of mTOR kinase inhibitors and therefore incompletely inhibit mTORC1-dependent protein synthesis (40). Therefore, in addition to rapamycin, we have evaluated the effectiveness of the dual PI3K/Akt-mTOR inhibitor, PI-103, as well as the ATP-competitive inhibitor, Torin1, targeting both mTOR complexes (41), in blocking mTOR-dependent signaling and proliferation/prosurvival pathways in merlin-deficient meningioma cells. In the first set of experiments, we evaluated the effect of short (1 hour) and prolonged exposures (24 hours) of mTOR or PI3K/mTOR inhibition on primary benign and atypical meningioma cells by western analysis. Inhibition of S6 (S240/244) or S6K (T389) phosphorylation was observed in merlin-deficient benign or WHO grade I meningioma cells (Fig. 5A and C), and atypical (WHO grade II; Fig. 5B and D) meningioma cells in response to low doses (50 nmol/L; ref. 41) of rapamycin at both time points, showing that rapamycin effectively suppresses the mTORC1 pathway in these cells *in vitro*. As expected, blocking mTORC1 with rapamycin resulted in elevated Akt S473 phosphorylation above levels of untreated (vehicle) cells under short (Fig. 5A and B) and long (Fig. 5C and D) treatment periods.

Torin1, used at a concentration (250 nmol/L) reported to overcome hyperactivated PI3K due to the negative feedback between mTORC1 and PI3K under conditions of prolonged mTORC1 inhibition (>5 hours; ref. 41), inhibited S6 or S6K phosphorylation at both treatment times, similar to rapamycin treatment (Fig. 5A–D). However, in contrast to rapamycin, Torin1, effectively blocked Akt S473 phosphorylation at both treatment periods.

Similar to rapamycin and Torin1, PI-103 (500 nmol/L) inhibited S6 or S6K phosphorylation after short exposure periods (Fig. 5A and B), but in contrast, failed to sufficiently inhibit S6 or S6K phosphorylation after prolonged time periods in meningioma cells regardless of tumor grade (Fig. 5C and D). Likewise, PI-103 efficiently blocked Akt S473 phosphorylation after short exposure times in all cells examined, but was ineffective in inhibiting Akt phosphorylation after a prolonged exposure period. The general ineffectiveness of PI-103 in blocking

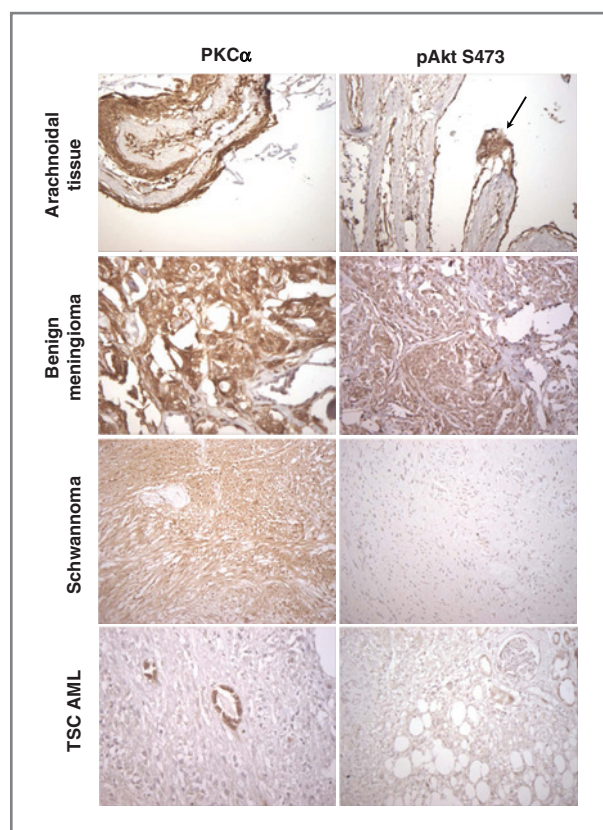


Figure 4. Normal arachnoid and benign (WHO grade I) merlin-deficient meningioma and vestibular schwannoma sections were immunostained for PKC α and phospho-Akt (S473) expression. Normal arachnoid, benign merlin-deficient meningioma, and vestibular schwannoma tissues exhibit similar, moderate to strong PKC α expression. Phospho-Akt (S473) (pAkt S473) staining is weak to moderate in normal arachnoid and meningioma tissue, and negative to weak in vestibular schwannomas. Data are representative of results for 5 benign merlin-deficient meningiomas and 4 vestibular schwannomas (Table 1). Sections of TSC AML (negative controls) show negative to weak PKC α and phospho-Akt (S473) staining consistent with previous reports (27). Arrow indicates normal arachnoid "cap" cells [magnification, $\times 20$, except PKC α staining of benign meningioma and TSC AML ($\times 40$)].

phosphorylation of Akt and S6K/S6 after 24 hours may be, at least in part, due to the short half-life and rapid metabolism of this drug that has been reported *in vivo* (42).

We next evaluated the effect of rapamycin or Torin1 on cell proliferation/viability of merlin-deficient meningioma cells ($n = 4$) treated over a period of 6 days. In comparison with untreated cells, we observed that Torin1 (250 nmol/L) was most effective in inhibiting proliferation of all meningioma cells tested, and rapamycin (50 nmol/L) significantly reduced the rate of proliferation. These data are provided in a representative growth curve of a benign meningioma (Fig. 6A). When we compared the average proliferative rates of 2 independent cultures of benign meningioma or atypical meningioma cells at one time point (day 7), we observed that Torin1 elicited the greatest inhibitory response in all cell lines (Fig. 6B).

Discussion

Our previous work showed that merlin deficiency in NF2 target cells results in aberrant activation of mTORC1-mediated signaling as well as attenuation of Akt S473 phosphorylation upon insulin treatment (14), consistent with negative feedback regulation of IRS1 by activated mTORC1 (22, 23, 43). In this study, we show that Akt S473 phosphorylation is impaired in merlin-deficient human arachnoid cells in response to stimulation with growth factors such as EGF and PDGF, and in merlin-deficient human Schwann cells, in response to EGF, but not PDGF. These results clearly show that merlin-deficient arachnoid and Schwann cells may differ from each other in PDGF-mediated signaling. Attenuation of Akt S473 phosphorylation by growth factors other than insulin/IGF suggest that in NF2, similar to TSC, more than one mechanism could be operative to downregulate Akt activation. Furthermore, recent studies have shown that mTORC1 activation, functioning through S6K phosphorylates Rictor to inhibit mTORC2 (44, 45).

Here, we examined signaling downstream of mTORC2, including phosphorylation of Akt (S473, T450) as well as other well-characterized substrates of mTORC2 such as PKC α (S657) and NDRG1, the latter being a specific readout for mTORC2 and SGK1 activation (21). The observed decrease in phospho-AKT (S473, T450) and phospho-PKC α (S657) in merlin-suppressed arachnoid and Schwann cells resemble TSC1- or TSC2-deficient cells (27).

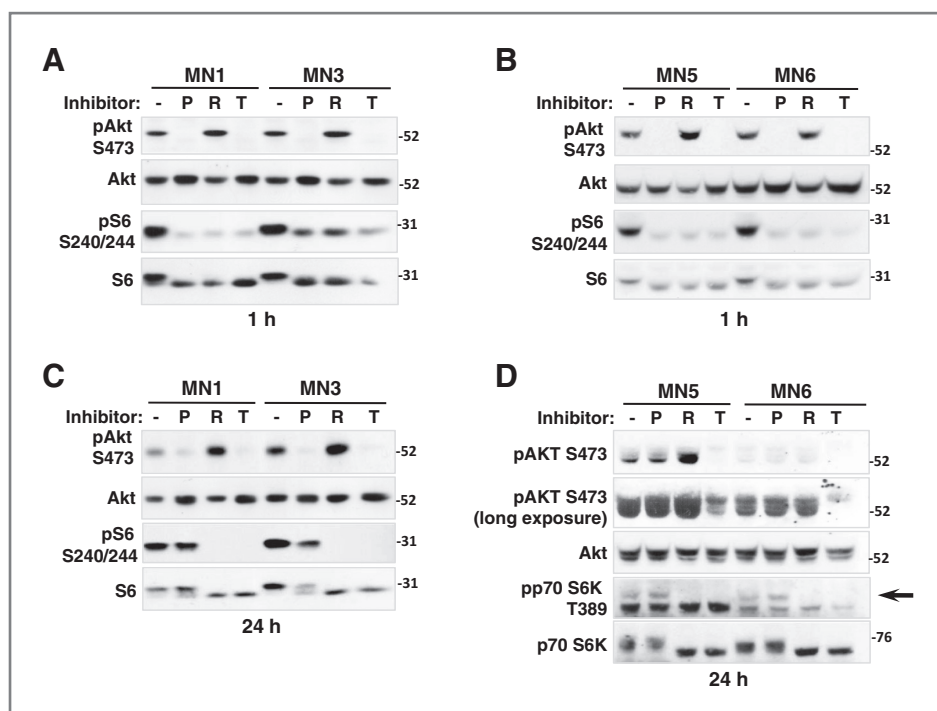
Table 1. Summary of results from immunostaining of PKC α and phospho-Akt S473 in 5 benign (WHO grade I) merlin-deficient meningiomas and 4 vestibular schwannomas

Case no.	PKC α	Phospho-Akt (S473)
MN 1034	++	++
MN 2805	++	+
MN 2896	++	+ / ++
MN 3633	++ / +++	+
MN 1670	++	+
VS 3924	++	-
VS 4206	+ / ++	-
VS 4231	++	-
VS 4787	++	- / +
Arachnoid	++	+ / ++
TSC-AML	-	-

NOTE: Staining was scored semiquantitatively: -, negative; +, weak; ++, medium; +++, strong.

Abbreviations: MN, merlin-deficient meningiomas; VS, vestibular schwannomas.

Figure 5. Torin1 blocks mTORC1 and mTORC2 signaling in benign and atypical merlin-deficient meningioma cells. Immunoblot analysis of phospho-S6 (pS6 S240/244) and phospho-Akt S473 (pAkt S473) in primary cell cultures of benign (A and C) and atypical (B and D) meningioma cells treated with PI-103 (P; 500 nmol/L), rapamycin (R, 50 nmol/L), Torin1 (T; 250 nmol/L), or vehicle (-; DMSO) for 1 hour (A and B) or 24 hours (C and D). Arrow indicates phospho-p70 S6K (T389) signal.



The decrease in mTORC2 kinase activity observed in merlin knockdown cells is consistent with growth factor-independent targets of mTORC2 (Akt T450, PKC α S657) being defective in these cells. Future studies are necessary to understand the precise molecular mechanisms of mTORC2 regulation by NF2 and whether this regulation is dependent or independent of TSC proteins. It is intriguing that contrary to other mTORC2 targets, NDRG1 is aberrantly activated (independent of growth factors) in merlin knockdown arachnoid and Schwann cells. Interestingly, a very recent study has shown that knockout of Protor-1, an interactor of Rictor results in a decrease in phosphorylation of SGK and its physiologic substrate NDRG1, without

influencing the phosphorylation of Akt and PKC α at their hydrophobic or turn motifs (46). It is therefore tempting to speculate whether Protor-1 is involved in mediating NDRG1 activation in merlin deficiency, and further studies are necessary to understand the mechanism and consequences of NDRG1 activation in merlin-deficient cells.

Our results suggest that primary meningioma cells and meningioma tumors with long-term (chronic) loss of merlin may exhibit heterogeneity in mTORC2 signaling when compared with acute loss of merlin achieved by RNAi in control normal arachnoid and Schwann cells *in vitro*. Phosphorylation of Akt S473 is not dramatically reduced in meningiomas when compared with normal arachnoid tissue.

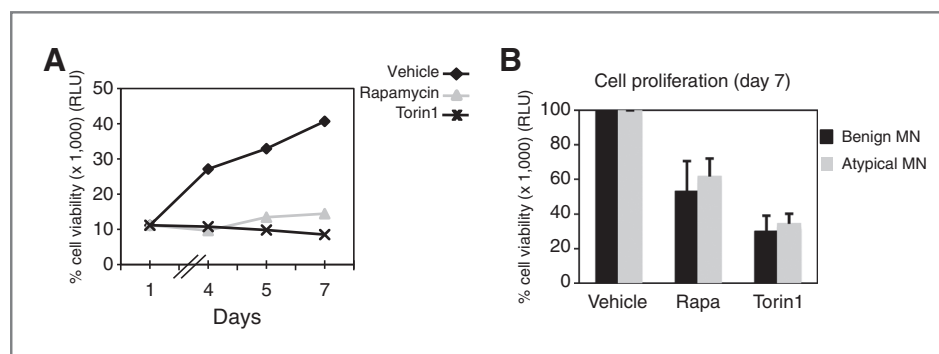


Figure 6. Torin1 effectively inhibits proliferation of merlin-deficient meningioma cells. A and B, mTOR inhibition by Torin1 prevents the proliferation of merlin-deficient meningioma cells more effectively than rapamycin at concentrations tested. A, representative growth curve of a benign merlin-deficient meningioma cell line (MN1) grown in the presence of vehicle, 50 nmol/L rapamycin, or 250 nmol/L Torin1 for 6 days. Cell proliferation was measured in triplicate at indicated time points using the CellTiterGlo viability assay. B, cell proliferation of benign and atypical meningioma cells are inhibited most effectively in response to Torin1. Bar graphs represent the average RLU values of 2 benign and 2 atypical meningiomas treated for 6 days with indicated compounds as described in (A). Data are presented as average RLU values \pm SE from 2 individual cultures treated in triplicate.

However, interestingly, phospho-Akt S473 staining in schwannomas is dramatically reduced, more closely resembling the staining pattern of TSC tumors than meningiomas. The lack of strong attenuation of mTORC2 signaling, particularly Akt S473 phosphorylation, which is a driver of cell proliferation and survival in primary meningiomas, suggests that either compensation mechanisms exist or additional genetic events cooperate with merlin loss in meningiomas. This is in agreement with a recent study, which elegantly documents frequent chromosome alterations in NF2-associated grade 1 meningiomas (47). We also speculate that similar to TSC lesions, the lack of Akt S473 phosphorylation in schwannomas may explain the benign nature of these tumors as well as the difficulty in establishing cells from them in culture. On the contrary, the atypical features commonly seen in benign meningiomas may be elicited by additional genetic events and/or Akt activation.

Clinical trials with the allosteric inhibitor, rapamycin and its analogs, known as rapalogs, are shown to be effective for various TSC-associated benign tumors including subependymal giant cell astrocytomas (48, 49). However, the feedback activation of oncogenic pathways by rapamycin and rapamycin-resistant phosphorylation of 4E-BP1 has led to the development of kinase inhibitors of mTOR as well as dual PI3K/Akt and mTOR inhibitors (50). We tested the efficacy of mTORC1 inhibitor rapamycin; an ATP-competitive mTOR inhibitor Torin1, which is shown to be a potent inhibitor of both mTORC1 and mTORC2 complex; and the dual PI3K/mTOR inhibitor PI-103 on primary benign and atypical meningioma cells. We conclude that Torin1 is more effective in blocking mTORC1 and Akt activation in meningioma cells *in vitro* than rapamycin and PI-103 and more effective than rapamycin in inhibiting cell

proliferation. Although benign in nature, we believe that heterogeneity exists in NF2-associated tumors adding complexity to signaling events. Therefore, therapeutic strategies employing Torin1 or equivalent mTOR kinase inhibitors in combination with other pathway inhibitors may be promising for treating NF2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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