Tuberous Sclerosis Complex Suppression in Cerebellar Development and Medulloblastoma: Separate Regulation of Mammalian Target of Rapamycin Activity and p27Kip1 Localization

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Abstract

During development, proliferation of cerebellar granule neuron precursors (CGNP), candidate cells-of-origin for the pediatric brain tumor medulloblastoma, requires signaling by Sonic hedgehog (Shh) and insulin-like growth factor (IGF), the pathways of which are also implicated in medulloblastoma. One of the consequences of IGF signaling is inactivation of the mammalian target of rapamycin (mTOR)–suppressing tuberous sclerosis complex (TSC), comprised of TSC1 and TSC2, leading to increased mRNA translation. We show that mice, in which TSC function is impaired, display increased mTOR pathway activation, enhanced CGNP proliferation, glycosyl synthase kinase-3α/β (GSK-3α/β) inactivation, and cytoplasmic localization of the cyclin-dependent kinase inhibitor p27Kip1, which has been proposed to cause its inactivation or gain of oncogenic functions. We observed the same characteristics in wild-type primary cultures of CGNPs in which TSC1 and/or TSC2 were knocked down, and in mouse medulloblastomas induced by ectopic Shh pathway activation. Moreover, Shh-induced mouse medulloblastomas manifested Akt-mediated TSC2 inactivation, and the mutant TSC2 allele synergized with aberrant Shh signaling to increase medulloblastoma incidence in mice. Driving exogenous TSC2 expression in Shh-induced medulloblastoma cells corrected p27Kip1 localization and reduced proliferation. GSK-3α/β inactivation in the tumors in vivo and in primary CGNP cultures was mTOR-dependent, whereas p27Kip1 cytoplasmic localization was regulated upstream of mTOR by TSC2. These results indicate that a balance between Shh mitogenic signaling and TSC function regulating new protein synthesis and cyclin-dependent kinase inhibition is essential for the normal development and prevention of tumor formation or expansion. [Cancer Res 2009;69(18):7224–34]

Introduction

Medulloblastoma is the most common malignant solid pediatric tumor. These tumors arise in young children in the cerebellum, a dorsal brain structure. Current treatment regimens for medulloblastoma cause developmental, behavioral, and cognitive disturbances in long-term survivors. These devastating side effects underscore the need to understand the basic mechanisms underlying medulloblastoma initiation, expansion, and recurrence, so that novel treatments can be developed which specifically target tumor cells without damaging the developing brain.

Cerebellar granule neuron precursors (CGNP), a proposed cell-of-origin for medulloblastomas (1), proliferate rapidly during a transient postnatal expansion phase in the cerebellar external granule layer a (EGLa). CGNPs become postmitotic in the EGLb, then migrate inward and terminally differentiate in the internal granule layer (IGL). The CGNP expansion phase requires signaling by Sonic hedgehog (Shh) and insulin-like growth factor (IGF; refs. 2, 3). Aberrant Shh pathway activation is found in sporadic medulloblastomas and in Gorlin’s syndrome, in which patients carry mutations or deletions in the Patched (Ptc) gene (4, 5), a key inhibitory component of the Shh receptor complex.

IGF pathway activity is also found in medulloblastomas in both mice and humans (6, 7). IGF signaling activates the kinase mammalian target of rapamycin (mTOR), which promotes mRNA translation. Growth factors activate mTOR by turning off the tuberous sclerosis complex (TSC), composed of TSC1 (hamartin) and TSC2 (tuberin), which normally keeps mTOR in check (8, 9). TSC1 stabilizes TSC2 protein; TSC2’s GTPase-activating protein function inhibits mTOR. TSC2 overexpression results in decreased mTOR activity, whereas abrogating TSC1 or TSC2 function results in increased mTOR activity and excessive cell growth. Conversely, TSC1 or TSC2 overexpression impedes cell cycle progression (10, 11). TSC2 positively regulates the cyclin-dependent kinase inhibitor p27Kip1 by preventing its degradation (12) and promoting its nuclear localization (13). Akt-mediated TSC2 phosphorylation impairs its ability to inhibit mTOR/S6K, but does not affect TSC2-mediated control of p27Kip1, indicating that TSC2’s regulation of mTOR and p27Kip1 are separable functions.

In the developing cerebellum, p27Kip1 is expressed in the EGLb, the molecular layer, and the IGL (14, 15). In adult cereella, p27Kip1 is expressed in the cells of the IGL. p27Kip1 heterozygous or null mice possess cerebella that are larger than those of wild-type mice (14). Importantly, loss of p27Kip1 in Patched+/- mice increased the incidence of medulloblastoma (16). CGNPs prepared from p27Kip1-/- mice show enhanced proliferation compared with CGNPs from wild-type mice (14). The Roussel and Eisenman groups have shown an antagonistic relationship between p27Kip1 and the Shh target N-myc during cerebellar development (17, 18). N-myc and D-type cyclins are destabilized by glycogen synthase kinase (GSK)-3α/β activity (2, 19).

We used transgenic mice constitutively expressing a dominant negative TSC2 allele (20) to investigate whether TSC inhibition...
plays a role(s) in CGNP expansion and medulloblastoma. These mice possess clusters of granule neurons that fail to migrate to the IGL, and they have cerebellar development abnormalities consistent with enhanced CGNP proliferation. CGNPs from these mice had mTOR signaling pathway activity resembling that of mouse Shh-driven medulloblastomas, including enhanced rpS6 phosphorylation, inactivation of GSK-3α/β, and cytoplasmically localized p27Kip1. In Shh-driven medulloblastoma cells, introduction of wild-type TSC2 caused p27Kip1 to go to the nucleus and reduced proliferation. Interestingly, p27Kip1 was not relocalized to the nucleus by treatment with mTOR inhibitors, indicating that its subcellular localization is regulated by TSC2 upstream of mTOR. Our results suggest that loss of TSC activity can synergize with Shh signaling to alter cerebellar development and enhance medulloblastoma expansion via combined effects on mTOR activation, GSK-3α/β inactivation, and p27Kip1 cytoplasmic localization.

Materials and Methods

Mice. TSC2-RGΔ, Ptc1−/−, and NeuroD2-SmoA1 mice were generated and maintained on a C57/BL6 background. Mice were managed according to the Memorial Sloan Kettering Cancer Center policies described in A.M. Kenney’s Institutional Animal Care and Use Committee–approved protocol. NeuroD2-SmoA1 mice were provided by Dr. Jim Olson (Fred Hutchinson...
Figure 2. TSC inactivation increases the basal proliferative capacity of CGNPs through increased mTOR signaling and inactivating phosphorylation of p27Kip1 and GSK-3β/α. A, quantification of BrdUrd incorporation in wild-type and TSC2-RGΔ CGNPs treated with Shh or vehicle alone (left). Vehicle-treated mutant CGNPs have significantly higher levels of BrdUrd incorporation than wild-type CGNPs (*, \(P = 0.0004\)). In the presence of Shh, TSC2-RGΔ CGNP BrdUrd incorporation is significantly greater than that of Shh-treated wild-type CGNPs (**, \(P = 0.0245\)). Two-tailed t tests were used to test significance. Right, quantification of staining for the mitotic marker phosphorylated histone H3 in wild-type and TSC2-RGΔ CGNPs treated with Shh or Shh vehicle alone. TSC2-RGΔ CGNPs have increased levels of phosphorylated histone H3 in comparison with wild-type CGNPs even in the absence of Shh (*, \(P = 0.0021\)). Levels of phosphorylated histone H3 are significantly increased in mutant CGNPs in the presence of Shh (**, \(P = 0.0108\)). Two-tailed t tests were used to test significance. B, Western blot shows that TSC2-RGΔ CGNPs have higher endogenous levels of cyclin D2 and N-myc in comparison with wild-type freshly isolated CGNPs. β-Tubulin was used as a loading control. Representative data from four wild-type postnatal day 4/5 mice and four TSC2-RGΔ postnatal day 4/5 mice. C, Western blot analysis of wild-type Shh-treated CGNPs infected with TSC shRNA lentiviruses. Pooled TSC shRNA lentiviruses increased the phosphorylation of rpS6, phosphorylation of GSK-3β/α, mislocalized p27Kip1, and proliferation due to TSC knockdown. Short-term treatment with mTOR inhibitor rapamycin blocks S6 phosphorylation but does not significantly affect phosphorylated p27Kip1 and cyclin D2 levels. GFP shRNA lentiviruses were used as controls to knock down GFP in CGNPs derived from Math1-GFP mice. Protein levels from TSC1, TSC2, phosphorylated rpS6, and phosphorylated GSK-3β/α protein levels were unaffected. D, knockdown of TSC significantly increases CGNP proliferation (*, \(P < 0.0001\)). Ki67 staining was quantified using automated detection and scoring. One-way ANOVA test was used to test significance.
Cancer Center, Seattle, WA). Math1-GFP mice were provided by Jane Johnson (UT Southwestern, Dallas, TX).

Cell culture and lentivirus production. For CGNP culture, postnatal day 4 or 5 mice were used as previously described (21). The CGNPs were treated with Shh (1 μg/mL; R&D Systems) to promote proliferation. Where indicated, rapamycin (Calbiochem) and wortmannin (Calbiochem) were used at 10 nmol/L for 6 h. Proteasome inhibitor lactacystin (Calbiochem) was used at 10 mmol/L for 4 h.

Pzp53med cells (kindly provided by Dr. Matt Scott, Stanford University, Stanford, CA) were grown in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin (B. Hhatia and A.M. Kenney). Pzp53med cells were transfected with either pcDNA3.1/TSC2 or pRK-TSC2 (both plasmids kindly provided by Dr. Pier Paolo Pandolfi, Harvard Medical School, Boston, MA) using Fugene 6. Cells were fixed after 48 h of posttransfection.

TSC1, TSC2, and green fluorescent protein (GFP) short hairpin RNA (shRNA) lentiviral constructs were obtained from Sigma. Four constructs of each were kindly provided by Dr. Matt Scott, Stanford University, Stanford, CA using Fugene 6. Cells were fixed after 48 h of posttransfection.

Western blot analysis. Protein extraction and Western blotting were carried out as previously described (21). Primary antibodies were TSC1 (Cell Signaling), TSC2 (Cell Signaling), GSK-3α/β (Upstate Biotechnology), p-GSK-3α/β Ser21/9 (Cell Signaling), cyclin D2 (Santa Cruz Biotechnology), N-myc (Santa Cruz Biotechnology), p27Kip1 (BD Transduction Laboratories), p-p72Rheb Ser20/24 (BD Transduction Laboratories), p-p72Rheb Ser20/24 (BD Transduction Laboratories), p-TOR Ser481 (Cell Signaling), mTOR (Cell Signaling), proliferating cell nuclear antigen (Calbiochem), GFP (Calbiochem), vascular endothelial growth factor (Santa Cruz Biotechnology), and β-tubulin (Sigma). Horseradish peroxidase–conjugated secondary antibodies were goat anti-rabbit IgG (H + L) at 1:3,000 (Pierce Labs) and donkey anti-mouse IgG (H + L) at 1:5,000 (The Jackson Laboratory).

Immunohistochemistry and in situ hybridization. Postnatal day 7 brains were fixed in 4% paraformaldehyde at 4°C, processed for paraffin-embedding, and sectioned at 10 μm. Immunohistochemistry and RNA in situ hybridization were performed using standard methods. Immunohistochemistry and in situ hybridization (cyclin D2) were performed on sagittal sections. Detailed protocols are available at the Memorial Sloan-Kettering Cancer Center web site.¹

Immunocytochemistry. CGNPs and Pzp53med cells were fixed in 4% paraformaldehyde for 10 min. Postnatal days 7 and 15, adult brains, and tumors were fixed in 4% paraformaldehyde at 4°C, processed for paraffin-embedding, and sectioned at 10 μm. Immunocytochemistry and bromodeoxyuridine (BrdUrd) incorporation detection were carried out as described (21). Primary antibodies are listed above, with the exception of BrdUrd (BD Transduction Laboratories), Ki67 (Vector Labs), p-H3Ser10 (Cell Signaling), GFP (Cell Signaling), and Bmi1 (Upstate Biotechnology). Secondary antibodies were AlexaFlour 555 goat anti-rabbit IgG (H + L) at 1:1,000 (Invitrogen) and AlexaFlour 488 goat anti-mouse IgG (H + L) at 1:1,000 (Invitrogen). BrdUrd incorporation was measured using the MetaMorph imaging system.

Results

TSC inactivation leads to abnormal cerebellar development and p27Kip1 mislocalization. We wished to determine how the TSC complex might be regulated in the context of normal and oncogenic Shh signaling. However, TSC1-null and TSC2-null mice die in utero; TSC1−/− and TSC2−/− mice are not reported to have cerebellar abnormalities (22), suggesting that the remaining TSC alleles are sufficient to support TSC function in the developing brain in those mice. Therefore, we made use of mice transgenic for a TSC2 allele lacking Rheb-GTPase–activating protein function (20), termed TSC2-RGα. The cerebellum of these mice were reported to contain “rests,” which may represent failed pneuomeplastic lesions (23, 24), but they do not develop medulloblastomas (20), consistent with the observation that humans with TSC mutations are not predisposed to medulloblastoma.

We evaluated TSC2-RGα mice at three stages of cerebellar development: postnatal day 7, postnatal day 15, and in adults. RT-PCR analysis confirmed the expression of the dominant negative TSC2 transgene (Supplementary Fig. S1A). TSC2-RGα mice have a significantly thicker EGL compared with wild-type controls in postnatal day 7 (Fig. 1A, arrow, quantification shown in Supplementary Fig. S1B). At postnatal day 15, when CGNPs from wild-type mice have left the EGL, TSC2-RGα mice still possessed an EGL several cells thick. Rests were observed at the cerebellar surface in TSC2-RGα adult cerebella as previously reported (20).

We focused on postnatal day 7, the stage of development at which CGNP proliferation is at its peak, to identify mechanisms through which TSC inactivation contributes to CGNP proliferation. The cyclin-dependent kinase (CDK) inhibitor p27Kip1 is an essential negative regulator of CGNP proliferation (14). In cell lines, p27Kip1 has been reported to interact with TSC2, which retains p27Kip1 in the nucleus (10, 13). When we analyzed p27Kip1 protein in wild-type and TSC2-RGα cerebella, we detected nuclear p27Kip1 in postnatal day 7 wild-type mice (Fig. 1B). In the EGLb, where CGNP proliferation is at its peak, we identified p27Kip1 that was entirely located in the cytoplasm, where other studies have proposed that it becomes degraded or takes on inappropriate functions (25–27).

The TSC2-RGα cerebella display widespread rpS6 phosphorylation in the mitotic region EGLa and differentiated region IGL (Supplementary Fig. S1C), indicating up-regulation of mTOR activity. Consistent with impaired cell cycle exit, the EGL of TSC2-RGα mice had increased Ki67 levels (Fig. 1C) at postnatal days 7 and 15. Next, we examined the expression of cyclin D2, an effector of N-myc activity in the developing mouse cerebellum (28). The expanded EGL at postnatal day 7 and the retained EGL at postnatal day 15 of TSC2-RGα cerebella have increased cyclin D2 expression (Fig. 1D). These findings suggest that TSC inactivation leads to abnormal cerebellar development due to increased proliferation in the EGL, associated with mislocalized p27Kip1.

TSC inactivation increases the basal proliferative capacity of CGNPs. Addition of Shh to the medium permits the proliferation of CGNP primary cultures (29–31) although exogenous IGF is required for their survival (32, 33). Shh treatment elicited a significantly higher level of proliferation in TSC2-RGα CGNPs as compared with wild-type CGNPs (Fig. 2A) as determined by BrdUrd incorporation and histone H3 phosphorylation. In comparison with wild-type CGNPs, TSC2-RGα CGNPs have increased endogenous levels of N-myc and cyclin D2 (Fig. 2B). These results indicate that TSC2-RGα CGNPs have an increased intrinsic capacity for proliferation and that TSC inhibition may cooperate with Shh signaling to further enhance CGNP proliferation.

Because Shh-mediated medulloblastomas and TSC2-RGα mice might have mutations in other pathways that result in p27Kip1 inactivation, we wished to determine how acute shRNA-mediated disruption of TSC signaling in wild-type CGNPs affects p27Kip1 and GSK-3α/β inactivation, we wished to determine how acute shRNA-mediated disruption of TSC signaling in wild-type CGNPs affects p27Kip1 and GSK-3α/β inactivation. As shown in Fig. 2C, infection with shRNA-carrying lentiviruses targeting TSC1 and/or TSC2 effectively reduced their protein levels in infected CGNPs. GFP knockdown in CGNPs derived from Math1-GFP mice did not affect mTOR pathway activity (Fig. 2C).

¹ http://www.mskcc.org/mskcc/html/77387.cfm
TSC knockdown resulted in increased rpS6 phosphorylation. Compared with control-infected CGNPs, there was an increase of phosphorylated p27Kip1 (Ser10) levels following TSC knockdown. Phosphorylation at this site promotes p27Kip1 export to the cytoplasm (34, 35). Treatment with mTOR inhibitor rapamycin blocked S6 phosphorylation, but only modestly changed phosphorylated p27Kip1 levels compared with control CGNPs, suggesting that regulation of p27Kip1 in CGNPs depends on the TSC, and is not downstream of mTOR. These results contrast with a recent study carried out in cell lines, wherein p27Kip1 localization was found to be regulated downstream of mTOR (36). We also observed increased GSK-3α/β phosphorylation following TSC knockdown, which was reduced by rapamycin treatment. Cyclin D2, a CGNP proliferation marker, and Ki67 immunostaining show that proliferation is increased by TSC knockdown (Fig. 2C and D).

**TSC2 inactivation increases Shh-mediated medulloblastoma incidence.** TSC2-RGΔ CGNPs showed increased phosphorylation of the serine/threonine kinase mTOR and rpS6 (Fig. 3A), consistent with maintained mTOR activation, in comparison with wild-type localization was found to be regulated downstream of mTOR (36). We also observed increased GSK-3α/β phosphorylation following TSC knockdown, which was reduced by rapamycin treatment. Cyclin D2, a CGNP proliferation marker, and Ki67 immunostaining show that proliferation is increased by TSC knockdown (Fig. 2C and D).

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**Figure 3.** TSC2 inactivation in mice leads to p27Kip1 mislocalization, GSK-3α/β destabilization, and decreased survival latency. A, protein lysates extracted from age-matched postnatal days 4/5 wild-type and TSC2-RGΔ CGNPs, and medulloblastomas from Patched heterozygote (Ptch+/-) and NeuroD2-smoothened mutant (SmoA1) mice were used for Western blot analyses of the indicated proteins. Data representative of four wild-type mice, four TSC2-RGΔ mice, three Ptch+/- medulloblastomas, and six SmoA1 medulloblastomas. B, medulloblastoma incidence in NeuroD2-SmoA1 and Ptch+/- mice in the presence or absence of TSC2-RGΔ allele. Increased tumor incidence suggests that inactivation of the TSC can synergize with oncogenic Shh signaling. C, Kaplan-Meier survival curve indicates survival latency in SmoA1 tumor-bearing mice in the presence or absence of TSC2-RGΔ allele. TSC2-RGΔ mice were crossed into the SmoA1 line. Tumors arising in the SmoA1/TSC2-RGΔ mice (red) have shorter latency than SmoA1 alone (blue). Survival curves are significantly different using Gehan-Breslow-Wilcoxon (P = 0.0084) and Mantel-Cox (P = 0.0083) tests. D, TSC2 inactivation in SmoA1 medulloblastoma enhances mTOR signaling, GSK-3α/β inactivation, and p27Kip1 phosphorylation. Protein lysates extracted from tumors of SmoA1 and SmoA1/TSC2-RGΔ mice were used for Western blot analyses of the indicated proteins. Data representative of three SmoA1 medulloblastomas and three SmoA1/TSC2-RGΔ medulloblastomas.
CGNPs. Interestingly, the TSC2-RGA cerebella also had increased levels of phosphorylated GSK-3α/β and Ser10 phosphorylated p27Kip1 (26, 34, 35). Likewise, we detected phosphorylated mTOR, rpS6, GSK-3α/β, and p27Kip1 (Ser10) in medulloblastomas from Ptc+/− mice and SmoA1 mice, which express an activated mutant allele of the Shh receptor component, Smoothened (37). Thus, the signaling abnormalities in TSC2-RGA cerebella are also present in mouse medulloblastomas. Taken together, our results indicate that

Figure 4. TSC inactivation and p27Kip1 dysregulation in medulloblastoma cells are associated with increased proliferation. A, under normal conditions, Pzp53med cells have phosphorylated ribosomal S6 protein. Cells treated with 10 nmol/L of phosphoinositide-3-kinase inhibitor wortmannin or 10 nmol/L of mTOR inhibitor rapamycin for 8 h shut down the levels of phosphorylated S6. Cells were immunostained for phosphorylated rpS6 (red) and costained for DAPI (blue). Magnification, ×40. B, under normal conditions, Pzp53med cells have barely detectable TSC2 and p27Kip1 (left). Akt inhibition with 10 nmol/L of wortmannin for 8 h rescued TSC2 and nuclear-localized p27Kip1 (middle). However, mTOR inhibition by 10 nmol/L of rapamycin for 8 h did not rescue either of these two proteins (right). TSC2 (red) and p27Kip1 (green) were costained. Magnification, ×40. C, ectopic TSC2 expression retains nuclear p27Kip1 in medulloblastoma cells. Nuclear p27Kip1 can be easily detected in cells transfected with exogenous TSC2 (left and middle). High-power image shows striking contrast in p27Kip1 localization between a TSC2-transfected cell and an untransfected neighboring cell (middle). Magnification, ×40 (left) and ×63 (right). TSC2 (red) and p27Kip1 (green) were costained. Right, automated quantification of TSC2 and p27Kip1 immunostaining. The vast majority of TSC2-positive cells have nuclear p27 (*, P < 0.0001). Two-tailed t tests were used to test for significance. D, TSC2-expressing cells have reduced immunostaining for BrdUrd incorporation (left). TSC2 (red) positive cells are BrdUrd-negative (green). Cells were costained with DAPI (blue). Right, results from an automated quantification of TSC2 and BrdUrd immunostaining. TSC2-positive cells have significantly reduced proliferation (*, P = 0.0003). Two-tailed t tests were used to test significance.
TSC inactivation in TSC2-RGΔ mice and Shh-induced mouse medulloblastomas could promote cell cycle progression as a result of increased mTOR-mediated protein synthesis, mislocalized p27Kip1, and inactivated GSK-3α/β, which is predicted to result in N-myc and D-type cyclin stabilization (2, 19).

We next introduced the TSC2-RGΔ transgene into mice with a heterozygous null mutation for Ptc and the NeuroD2-SmoA1 mice. Dominant negative TSC2 increased medulloblastoma incidence in both Ptc+/- (53.8% versus 25%) and SmoA1 (72% versus 52.8%) mice (Fig. 3B). Also, TSC2 inactivation in SmoA1 tumor-bearing mice

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**Figure 5.** TSC2 phosphorylation, p27Kip1 localization, and GSK-3α/β phosphorylation in Shh-mediated medulloblastoma. A, SmoA1 medulloblastoma tumor cells have robust Bmi 1 (DAB staining), phosphorylated S6 protein (red), and little GFAP (green). Only Purkinje neurons (PL) of wild-type adult cerebella have phosphorylated rpS6. Representative image from a wild-type mouse treated with mTOR inhibitor CCI-779 shows loss of phosphorylated rpS6 in Purkinje neurons, indicating that this drug crosses the blood-brain barrier. Magnification, ×40. B, SmoA1 medulloblastoma untreated (top row) or treated (bottom row) with CCI-779 for 10 d. Immunostaining for phosphorylated S6 (red; first column) serves as a read-out for mTOR activity. Phosphorylated GSK-3α/β (red; second column) is affected by mTOR inhibition. Inactivating Akt-mediated phosphorylation of TSC2 (red; third column) and complete cytoplasmic localization of p27Kip1 (green; fourth column) in murine SmoA1 medulloblastoma are not altered by mTOR inhibition. All cells were costained with DAPI (blue). Magnification, ×63 (first and second columns) and ×120 (third and fourth columns). C, quantification of staining for the mitotic marker phosphorylated histone H3 in SmoA1 medulloblastoma treated with or without CCI-779 for 10 d. mTOR inhibition reduced levels of phosphorylated histone H3 (*, P = 0.0052). Two-tailed t tests were used to test significance. D, CCI-779 treatment reduces the levels of cell cycle regulators in SmoA1 tumor cells. SmoA1 medulloblastomas lose N-myc, cyclin D2, and vascular endothelial growth factor (VEGF) protein upon mTOR inhibition for 10 d. Wild-type mice were used as controls. Bars, A and B (first and second columns), 16 μm; B (third and fourth columns), 8 μm.
decreases survival latency (Fig. 3C). Introducing the TSC2-RG1 transgene increased phosphorylation of rpS6, GSK-3α/β, and p27Kip1 (Fig. 3D) in the tumors. Taken together, these results suggest that Shh signaling and TSC inactivation could cooperate to enhance medulloblastoma formation.

**TSC rescues p27Kip1 localization and reduces the proliferation of Shh-mediated medulloblastoma cells.** In order to determine whether TSC inactivation and p27Kip1 dysregulation are related, we turned to a Shh-associated medulloblastoma cell line model, the Pzp53med cell line, derived from a Ptc+/P53−/− mouse medulloblastoma (38). Recently, the Scott group characterized Gli expression and sensitivity to sterol synthesis inhibitors in these cells (39). We found robust levels of phosphorylated rpS6 in Pzp53med cells (Fig. 4A, left). Consistent with mTOR activation lying downstream of Akt, which phosphorylates and inactivates TSC2 (13, 40), treatment of the Pzp53med cells with the phosphoinositide-3-kinase inhibitor wortmannin eliminated rpS6 phosphorylation (Fig. 4A, middle). Rapamycin treatment also eliminated rpS6 phosphorylation (Fig. 4A, right). Moreover, total TSC2 and p27Kip1 levels were low (Fig. 4B, left) in the Pzp53med cells. Wortmannin treatment increased TSC2 protein levels and returned p27Kip1 to the nucleus, whereas rapamycin did not, suggesting that TSC2 inactivation and p27Kip1 stabilization are regulated upstream of mTOR (Fig. 4B, middle and right) in the Pzp53med cells, as in CGNs. To determine whether p27Kip1 localization was dependent on TSC in these cells, we introduced exogenous TSC2 by transient transfection. Cells expressing TSC2 had strong nuclear p27Kip1 (Fig. 4C, left and middle). Indeed, nearly 90% of TSC2-positive cells had nuclear p27Kip1, as compared with only 10% of TSC2-negative cells (Fig. 4C, right). These results suggest that low levels of TSC2 may be associated with increased p27Kip1 turnover in the medulloblastoma cells. To determine whether reconstituting TSC

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**Figure 6.** Model for how TSC activity is integrated with Shh proliferative signaling during cerebellar development and in cancer. A, under normal conditions in the developing brain, CGNs are exposed to Shh and IGF. Normal levels of Shh signaling promote CGNP proliferation and cerebellar development, with appropriate levels of mTOR activity and p27Kip1 localization. B, when the TSC is inactivated, p27Kip1 moves to the cytoplasm where it can be phosphorylated. mTOR is released from inhibition, increasing mRNA translation. mTOR may also act through S6 kinase to further inhibit GSK-3α/β. C, in medulloblastomas, loss of Ptc function or aberrant activation of Smo drives oncogenic Shh activity, which cooperates with enhanced signaling by growth factors such as PDGF or IGF, leading to TSC inactivation. p27Kip1 moves to the cytoplasm where it may be inactivated or gain new oncogenic functions. mTOR activity is increased, contributing to the transformed phenotype. TSC inactivation by mutation/deletion also leads to mTOR activation, p27Kip1 misregulation, and enhanced activity of Shh pathway effectors.
activity impedes cell cycle progression in Pzp53med cells, we commumostained for TSC2 and BrdUrd. We observed an inverse correlation between TSC2-positive cells and BrdUrd-positive cells: overexpression of TSC2 reduced proliferation by ∼4-fold (Fig. 4D). Finally, TSC2 and p27Kip1 were rapidly degraded in the cytoplasm of Pzp53 medulloblastoma cells (Fig. 4B; Supplementary Fig. S2, left). TSC2 accumulates in Pzp53med cells treated with the proteasome inhibitor lactacystin (Supplementary Fig. S2, middle), and its localization to the nucleus is associated with increased nuclear p27Kip1 (Supplementary Fig. S2, right). Together, our results show that p27Kip1 localization depends on TSC2 expression and function.

p27 is mislocalized in Shh-mediated medulloblastoma. We wished to determine whether mTOR inhibition affects tumor growth and p27 localization in SmoA1 medulloblastoma in vivo. We treated wild-type adult mice with the mTOR inhibitor CCI-779 for 9 to 10 days (40 mg/kg daily) using i.p. injection of the drug. Data shown are representative of results from five treated mice and five untreated mice. Before treatment, phosphorylation of rpS6 was restricted to the Purkinje cells of normal adult cerebella. Following treatment, phosphorylated rpS6 was virtually undetectable (Fig. 5A). As shown in Fig. 3 and Fig. 5A and B, SmoA1 medulloblastomas possessed Bmi1 (41, 42), a progenitor cell marker, and phosphorylated rpS6, indicating mTOR pathway activation. The phosphorylated rpS6 signal was not found in GFAP-positive cells (i.e., astrocytes) present in the tumor (Fig. 5A). Both phosphorylated GSK-3α/β and Akt-mediated phosphorylation of TSC2 were found in tumor cells (Fig. 5B, top row). Because we observe that TSC2 is phosphorylated on inactivating sites in SmoA1 medulloblastomas, we asked whether p27Kip1 was mislocalized in these tumors. As shown in Fig. 5B (top row), p27Kip1 was found in the cytoplasm of the tumor cells. Indeed, no cells were observed with nuclear p27Kip1. We also detected only cytoplasmic p27Kip1 in Pcte−/− medulloblastomas (data not shown).

Recent reports (36, 43) have indicated that GSK-3α/β can be phosphorylated downstream of mTOR, and that p27Kip1 localization can be regulated downstream of mTOR. Following CCI-779 treatment, rpS6 phosphorylation was eliminated in NeuroD2-SmoA1 medulloblastomas (Fig. 5B, bottom row). GSK-3α/β phosphorylation was also down-regulated, suggesting that as in primary CGNPs (Figs. 2 and 3), GSK-3α/β phosphorylation is dependent on mTOR activity. This effect of mTOR on TSC2 inactivation is consistent with the observation that in vivo CCI-779 treatment (9 to 10 days) eliminated rpS6 phosphorylation in GFAP-positive cells (Supplementary Fig. S2, left). rpS6 phosphorylation was also down-regulated, suggesting that CCI-779 treatment inactivates mTOR, and that p27Kip1 localization in SmoA1 medulloblastomas is dependent on mTOR activity. The phosphorylated rpS6 signal was not found in GFAP-positive cells (data not shown). As shown in Fig. 5B (top row), p27Kip1 was found in the cytoplasm of the tumor cells. Indeed, no cells were observed with nuclear p27Kip1. We also detected only cytoplasmic p27Kip1 in Pcte−/− medulloblastomas (data not shown).

Discussion

Somatic mutations in TSC1 or TSC2 cause tuberous sclerosis (40, 45). Although patients with tuberous sclerosis themselves are not predisposed to develop medulloblastoma, the TSC can be inactivated by phosphorylation downstream of growth factors associated with medulloblastoma, such as PDGF and IGF, and a small study identified a point mutation in TSC2 in a subset of medulloblastomas (46). Here, we have asked whether loss of TSC activity may contribute to proliferation during normal cerebellar development and to the incidence or growth of medulloblastomas.

We found that mTOR-dependent proliferation of medulloblastomas had high levels of mTOR activity, and biochemically resembled CGNPs derived from mice constitutively expressing a dominant negative TSC2 allele (TSC2-RGA mice). When we analyzed cerebellar development in these mice, we found that their CGNPs had an extended proliferative capacity in vivo, and in vitro they exhibited Shh-independent proliferation. When the TSC2-RGA allele was introduced into mice with aberrantly activated Shh signaling, the incidence of medulloblastoma was markedly increased, with reduced latency to tumor formation. These studies suggest that inactivation of the TSC complex could contribute to medulloblastoma formation, perhaps as a secondary hit. Indeed, when we recently analyzed a human tumor collection for TSC-inactivating mutations, we identified TSC1 deletions in a significant number of tumors, and these tumors belonged to the Shh-associated subclass of medulloblastomas (Supplementary Fig. S3). The only known function of TSC1 is to stabilize TSC2 (47); therefore, loss of TSC1 in tumors results in the loss of TSC2 activity. We also explored the expression analysis of TSC2 and CDKN1B (p27Kip1) in human Shh-subgroup medulloblastomas and found moderate decreases compared with adult cerebellum (Supplementary Fig. S4). TSC2 and p27 are not classic tumor suppressors, as they are rarely mutated or deleted in cancer, but they are often deregulated in cancer by posttranslational modifications, as supported by our study and previous reports (48–50).

Interestingly, in the TSC2-RGA cerebellar EGL and NeuroD2-SmoA1 medulloblastomas, we observed p27Kip1 in the cytosol in all cells, associated with increased levels of phosphorylation on Ser10. Phosphorylation on p27Kip1 Ser10 leads to its nuclear export and increased stability in the cytosol (34). When we transplanted Pzp53med cells with exogenous TSC2, we found nuclear p27Kip1 in nearly 90% of TSC2-positive cells. A correlation between poor prognosis and cytoplasmic localization of p27Kip1 in human tumors has led to the hypothesis that p27Kip1 has an active tumor-promoting function in the cytoplasm (50). A recent study tested for CDK-independent functions of p27Kip1 by analyzing knock-in mice expressing a mutant p27 (p27ΔCK) that is unable to inhibit cyclin-CDK complexes (25). These mice developed various tumors, and the p27ΔCK mutant localized to the cytoplasm, hinting that a cytoplasmic function might mediate the oncogenic effects of p27Kip1. Taken together, our findings suggest the model shown in Fig. 6.

Under normal developmental conditions, TSC modulates mTOR signaling, and stabilizes p27Kip1 (Fig. 6A). Under conditions of TSC loss (Fig. 6B), mTOR activity is increased, p27Kip1 is localized to the cytoplasm where it may be degraded, inactivated, or gain abnormal functions, and GSK-3α/β is phosphorylated (43), thus increasing N-myct protein levels and CGNP proliferation. In the setting of Shh-induced medulloblastoma (Fig. 6C), signaling by growth factors such as PDGF (9) or IGF could lead to TSC inactivation via phosphorylation by Akt or Erk2; alternatively, the TSC can be disrupted by mutation. Signaling by mTOR is activated, leading to increased translation of growth-associated
proteins and potential inhibition of GSK-3α/β. These factors can all contribute to enhanced tumor growth or decreased latency.

Development of hedgehog pathway inhibitors such as cyclopamine and HHAntag (51) has raised the hope that targeting this pathway will be an effective antimedulloblastoma strategy (51, 52). However, it has recently been shown that systemic treatment of young animals with hedgehog pathway inhibitors results in devastating, irreversible effects on bone growth (53). Glit-transformed epithelial cells require mTOR activity for their survival (54). Our observation that the mTOR pathway is activated in mouse and human medulloblastomas suggests that inhibitors of this pathway may be useful. Moreover, our demonstration that modulation of mTOR is not the only tumor-suppressive function of the TSC in the developing brain and in brain tumors suggests that using drugs to block the activation of TSC2-inhibiting pathways, or that promote p27Kip1 nuclear localization, may present even more effective future medulloblastoma therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 4/7/09; revised 6/16/09; accepted 6/27/09; published OnlineFirst 9/8/09.

Grant support: The American Brain Tumor Association (B. Bhatia, in memory of Davis Ferguson), the National Institute of Neurological Disorders and Stroke (RO1NS061070, AM. Kenney), Children’s Brain Tumor Foundation (AM. Kenney), the Childhood Brain Tumor Foundation of Maryland (A.M. Kenney), the Pediatric Brain Tumor Foundation (AM. Kenney), the Sontag Foundation (AM. Kenney and M.D. Taylor), and the Brain Tumor Center of Memorial Sloan-Kettering Cancer Center (B. Bhatia).

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We thank Jeffrey Miller, Ph.D., Andy Koll, Ph.D., Andre Dlugosz, Ph.D., and Timothy Gershon, M.D., Ph.D. for their advice, helpful discussion, and expertise.

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