PI3K/mTOR Signaling Pathways in Medulloblastoma

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Abstract. *Medulloblastoma is the most common malignant brain tumor in children. Recent studies have implicated sonic hedgehog (SHH) and insulin growth factor (IGF) as important mediators in deregulated pathways, which directly inactivate tuberous sclerosis complex, leading to activation of the serine/threonine kinase, mammalian target of rapamycin (mTOR). mTOR consists of two catalytic subunits of biochemically distinct complexes called mTORC1 and mTORC2. This study aims to further elucidate the role of the mTOR pathway, in the development of medulloblastoma, and assess the use of mTOR inhibitors as novel therapeutic agents. Medulloblastoma cells treated with mTORC1 inhibitor, rapamycin, down- regulated pERK expression initially; however ERK activation was evident upon prolonged treatment. Phosphorylation of mTORC1 substrate, p70S6K at thr389 was reduced by rapamycin and pretreatment with rapamycin abrogated platelet-derived growth factor (PDGF)-induced activation of S6K, as well as that of mTORC2 substrate pAKT^{Ser473}. Activation of AKT was decreased at 1, 3, and 6 h of treatment, but extended treatment with rapamycin increased expression of pAKT^{Ser473}. Expression of cyclic dependent kinase inhibitor, P27, decreased following PDGF and increased following rapamycin treatment, suggesting their respective impact on cell proliferation via cell cycle control. Cell proliferation was increased by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment of medulloblastoma cells, while it was suppressed following treatment with rapamycin or U0126 (MEK1/2 inhibitor). pp242, a novel combined mTORC1/2 inhibitor, and rapamycin limited proliferation by reducing the S-Phase entry as assessed by EdU incorporation, while PDGF increased EdU incorporation. pp242 reduced the number of cells entering the S-phase to a greater extent than did rapamycin. Migration of medulloblastoma cells towards fibronectin was suppressed in a time-dependent manner after rapamycin treatment. These results indicate that the mTOR pathway is involved in the pathogenesis of medulloblastoma, and that targeting this pathway may provide a strategy for therapy of medulloblastoma.*

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Medulloblastoma is the most common malignant brain tumor in children. It represents one of five embryonic tumors that can develop within the central nervous system. Several clinical prognostic factors have historically been used to determine clinical management of these tumors using a risk stratification system. Early age at diagnosis, recurrence after resection, and the presence of metastases, all represent poor prognostic indications (1). More recently, stratifications take into account the existence of five histopathological variations of medulloblastoma tumors in making prognostic and other therapeutic decisions (2). Depending on the risk stratifications, patients are treated usually with either surgery, chemotherapy, radiation, or a combination. Radiation is generally avoided in patients under age 3 years due to the immaturity of the brain and its propensity for radiation-related damage (3). Despite the growing efficacy of these treatment modalities, the resulting long-term physical, cognitive and behavioral sequelae are still a major concern (4).

Further developments in genomic approaches have led to the identification of four subtypes of medulloblastoma based on the presence of distinct genetic markers. These subtypes are; WNT, sonic hedgehog (SHH), group C (NPR3), and group D (KCNA1). It is now thought that the use of this molecular classification will provide the most accurate basis for risk stratification and therapeutic management (5).

The identification of molecular markers within medulloblastoma has also spurred interest in assessing the presence of aberrant signaling pathways within medulloblastoma tumors. The confirmation of the alteration of these pathways provides hope for the development of novel alternative forms of treatment. Recent studies have implicated SHH and insulin growth factor (IGF) as important mediators within deregulated pathways. More specifically, IGF has been shown to directly inactivate tuberous sclerosis complex (TSC), leading to activation of mammalian target of rapamycin

(mTOR) and an upregulation of transcription. Mice models with inactive TSC, as well as models with SHH-induced medulloblastoma have demonstrated an increase in mTOR activity, resulting in cascades implicated in oncogenesis (6). Additional studies have provided evidence that within cerebellar granule neuron progenitors (CGNP), SHH affects downstream effectors of the mTOR pathway, S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF4E). When mTOR is suppressed, S6K is inhibited and eIF4E is activated, thus resulting in increased cell proliferation. These studies have provided evidence that during cerebellar development, S6K and eIF4E are regulated by SHH in a discrete manner and suggest that inactivation of these targets may reduce cellular proliferation (7). mTOR kinase is present in two cellular multiprotein complexes, mTORC1 and mTORC2. Each complex has its own unique subunit composition, substrates, and mechanisms of action, thus targeting mTOR protein is a good approach for cancer treatment. It is important to consider that these complexes are not suppressed by conventional allosteric inhibitors, such as, rapamycin (RAPA), therefore recent studies suggest that ATP-competitive binding inhibitor pp242 may provide better inhibition of both complexes.

The role of P27 in cancer is generally viewed by its role in regulation of cell cycle. However, recent studies have suggested that P27 is also involved in invasion and DNA repair process. Activating mutation of the SHH pathway with loss of P27, confers in a poor prognosis in animal model of medulloblastoma (8). P27 controls Ras homolog gene family member A, (RhoA) which is involved in regulation of migration of medulloblastoma cells (8). P27 is a cyclin dependent kinase (CDK) inhibitor, which normally functions as a cell cycle regulator. Recent studies have suggested that P27 regulates genomic stability, since loss of P27 leads to abnormal double-strand break repair in G2 cells leading to genomic instability (9).

This study aims to further elucidate the role of the mTOR pathway, as it relates to proliferation and migration of medulloblastoma cells, and thus ascertain the use of mTOR inhibitors as novel therapeutic agents.

Materials and Methods

Cell lines. Daoy cells (ATCC, Manassas, VA, USA) were used to investigate the involvement of the PI3K/AKT/mTOR signaling pathway in medulloblastoma progression. Daoy is a hypertetraploid human cell line derived from cerebellum of a patient with desmoplastic cerebellar medulloblastoma. Cells display thirteen or more chromosomal markers of two to four copies per cell, including t(1q5q), t(13q;?), 15p+, 7q+, der(9)t(3;9)(p21;q34) and eight others. In most cells, there are three copies of 15p+and four copies of der(9). Some cells have del(1)(p11).

Cell culture. Cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin/amphotericin in a humidified incubator with 5% CO_2 at 37˚C. Cells were made quiescent by serum deprivation 24 h prior to treatment with various combinations of RAPA (mTOR inhibitor, 10 nM), platelet-derived growth factor (PDGF, 25 ng/ml) (EMD Chemicals, Gibbstown, NJ, USA), U0126 (MEK1/2 inhibitor, 10 μM) fibronectin (extra-cellular matrix, component 20 ng/ml Sigma-Aldrich, St. Louis, MO, USA) (see figure legends for detailed treatment strategies).

Isolation of protein. Protein extraction was performed with whole cell lysis buffer containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 5 mM EDTA containing phosphatase and protease inhibitors (Sigma-Aldrich). Protein concentrations were determined by the modified Lowry method (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. Extracted protein, 75 μg was resolved on a 10% SDS-PAGE gel and then electrotransferred onto nitrocellulose membrane. Membranes were processed according to the manufacturers' instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cell Signaling Technology, Danvers, MA, USA) using primary antibodies for activated extracellular signal-regulated kinase (pERK) and total ERK, activated protein kinase B (pAKT) and total AKT, activated (p-P27) and total P27, activated pS6K and total S6K, and bands were detected by chemiluminescence (Cell Signaling Technology). Blots were stripped with reagent (EMD Chemicals) and re-probed with actin or the respective total antibodies to ensure equal loading. Experiments were conducted at least three times.

Immunofluorescence. Cells were made quiescent by exposure to serum free media for 24 h then treated with one of the following: i) vehicle ii) RAPA, iii) pp242, iv) PDGF, for 8 h This was followed by fixation with 4% paraformaldehyde/0.3% Tween. Cells were blocked with 5% goat serum, incubated overnight with FOXO antibody (1:100, Cell Signaling) and subsequently incubated with rhodamine-conjugated antibody (1:200; Jackson ImmunoResearch, West Grove, PA, USA) according to the manufacturers' instructions. 4',6' diamino-2-phenylindole·2HCl (DAPI) counterstain was performed (Sigma-Aldrich).

Cell proliferation assays. Cell growth was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-ditetrazolium bromide (MTT) assay according to the manufacturer's protocol (Chemicon, Billerica, MA, USA). Cells (3,000/well) were seeded onto a 96-well plate and made quiescent for 24 h prior to treatment. After completion of treatment, fresh medium containing 10 μL of MTT reagent was added to cells and plates were incubated at 37˚C for 4 h, 100 μl of detergent reagent was then added and the absorbance was measured after 2 h.

Chemotactic migration. Directional migration was performed using a 48-well modified Boyden chamber kit (NeuroProbe, Gaithersburg, MD, USA). Quiescent cells were subjected to timed treatments (1, 3, 6, 12, 24 h). Vehicle treated cells served as controls. Cells were aliquoted (3000 cells/μl) in either serum-free medium or their respective RAPA treated media. Fibronectin (20 ng/ml, Sigma-Aldrich) was used as a chemoattractant and cells were allowed to migrate for 4 h through a PVC membrane (8 μm pore). The membrane was fixed in 70% ethanol, scraped along the non-migrated cell surface, and stained with DiffQuick (IMEB, San Marcos, CA, USA). Migrated cells were imaged at ×2.5 (Axiovert 100M) and analyzed as a percentage of the total microscopic field occupied by migrated cells (ImageJ, NIH, Bethesda, MD, USA).

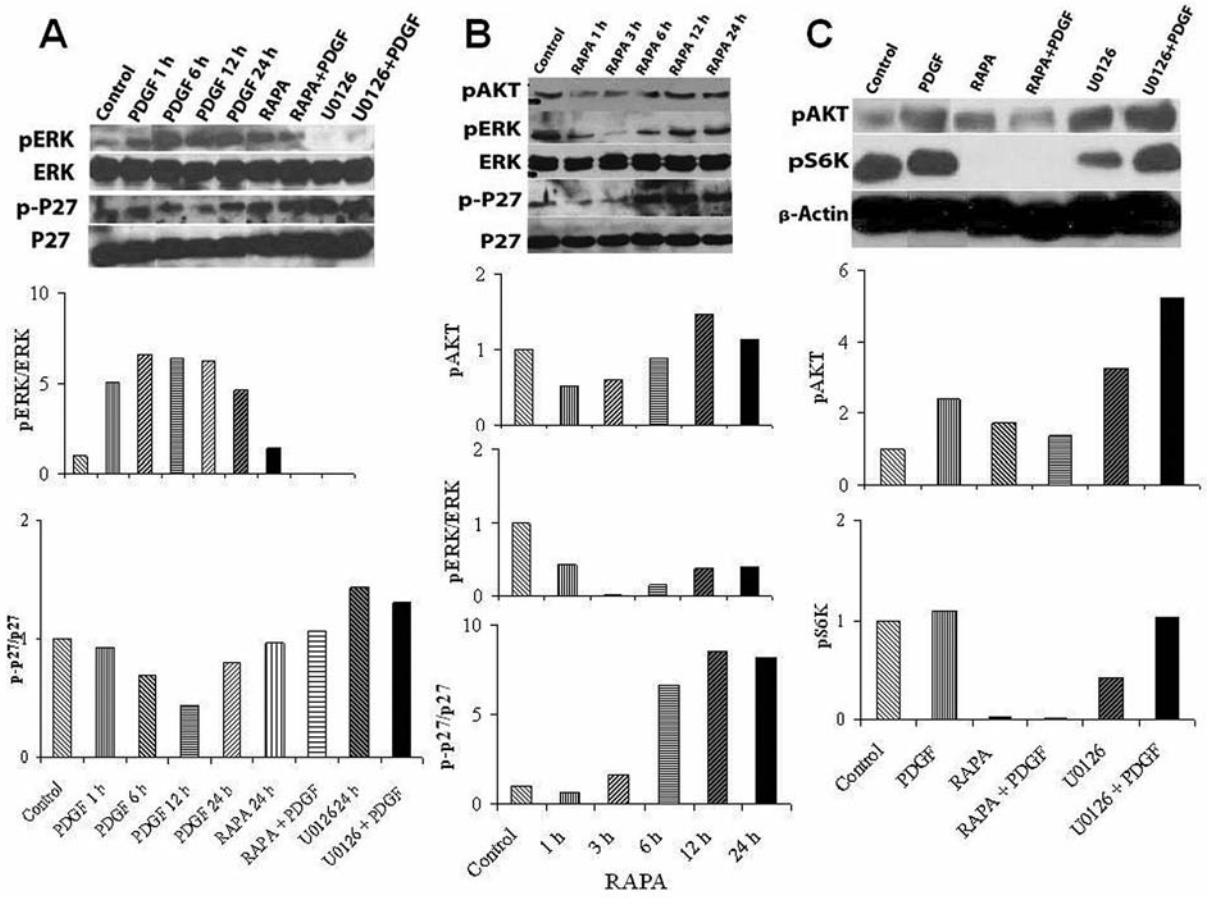

Figure 1. *A: (Left panel) Western blot with densitometric analysis shows time-dependent increase in expression of activated extracellular signal related kinase (ERK). pERK relative to total ERK following treatment with platelet derived growth factor (PDGF) treatments for 1, 6, 12, and 24 h. Treatment with rapamycin (RAPA) alone also caused an up regulation of expression, however cells treated with RAPA and PDGF were not significantly different from the control. Cells treated with U0126 alone and in combination with PDGF had complete downregulation of pERK. Densitometric analysis demonstrates a time-dependent decrease in the expression of activated p-P27 relative to total P27 following treatment with PDGF for 1, 6, and 12 h. There is a return to control levels of expression at 24 h of PDGF treatment. Treatment with RAPA and U0126, with or without PDGF caused an increase in p-P27 levels. B: (Middle panel) The effect of time-dependent treatment with RAPA on pAKT, pERK, and pS6K expression is shown by Western blot with densitometric analysis. pERK expression was down regulated completely at 3 h after treatment with RAPA. However the pERK expression resumed and increased in a time-dependent manner at 6, 12, and 24 h. p-P27 expression was up regulated, with an effect that plateaued at 12 h. pAKT expression was decreased at 1, 3, and 6 h of treatment, which was followed by a subsequent increase in pAKT expression at 12 and 24 h. C: (Right panel) Western blot with densitometric analysis illustrates PDGF-induced increase in activated AKT (pAKT) relative to control. Treatment with RAPA caused a slight increase in pAKT levels; however, RAPA in combination with PDGF suppressed the activation of PDGF-induced AKT expression. MEK inhibitor U0126 alone or in combination with PDGF caused an increase in pAKT levels. The expression of activated S6K (pS6k) relative to total S6K, as shown by densitometric analysis was suppressed totally by RAPA treatment for 24 h. Pretreatment with RAPA totally suppressed PDGF-induced activation of S6K.*

5-ethynyl-2-deoxyuridine (EdU) incorporation: proliferation assay. Proliferating cells were visualized by utilizing the Click-iT EdU Imaging Kit (Invitrogen). Control, RAPA, pp242 or PDGF-treated samples were incubated for 4 h in 10 μM EdU. They were subsequently fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized for 15 min in 0.1% Triton X-100 in phosphate buffered saline (PBS). EdU incorporation was detected by incubation in the Click-iT reaction cocktail (as prescribed by the kit) at room temperature. The samples were then washed for 5 min in PBS three times. Frequency maps of the cell proliferation were constructed from fluorescence images using a Zeiss microscope.

Results

mTOR/MAPK pathways in medulloblastoma. Medulloblastoma cells treated PDGF showed a significant up regulation in the expression of pERK, an activated form of mitogen activated protein kinase (MAPK), as determined by western blot analysis (Figure 1A). The cells were treated for different lengths of time 1, 6, 12, and 24 h. We present data as a ratio of activated ERK to total ERK. The increase in pERK plateaued after 6 h of exposure to PDGF and

remained activated to the same degree as in cells with treatment for 12 and 24 h.

The effect of PDGF on pERK mirror that of its effects on p-P27, the CDK inhibitor as shown by densitometry given as p-P27 relative to P27 (Figure 1A). As the time of PDGF exposure increased, the expression of P27 decreased, indicating enhanced cell cycle entry. p-P27/P27 levels reached a nadir at 12 h of exposure time. The contrary was observed when Daoy cells were treated with RAPA and U0126, with or without PDGF, that is, RAPA increases in p-P27 levels, suggestive of down regulation of cell cycle entry.

Medulloblastoma cells pretreated with mTOR inhibitor, RAPA, for 24 h followed by PDGF, showed an attenuated expression of pERK as compared to PDGF alone. U0126, treated cells given PDGF showed complete down regulation of ERK activity, indicating that the MAPK pathway is primarily activated in medulloblastoma cell growth.

mTOR inhibition causes alteration in pERK, pAKT and p-P27 activity. Inhibition of the mTOR pathway in medulloblastoma cells was evaluated by western blot analysis following timed treatment with RAPA (Figure 1B). pERK expression was down regulated with a maximal effect of complete down regulation at 3 h after treatment with RAPA. However the pERK expression resumed and increased in a time-dependent manner on treatment for 6, 12, and 24 h. On the contrary, p-P27 expression was up regulated, indicating a decrease in cell proliferation, with an effect that plateaued at 12 h. pAKT expression was decreased at 1, 3, and 6 h of treatment, which was followed by a subsequent increase in pAKT expression at 12 and 24 h.

mTORC1 phosphorylates S6K at Thr389 which subsequently phosphorylates downstream targets such as the ribosomal protein S6 and the mTORC2-mediated phosphorylation of the antiapoptotic proteins Akt/PKB at hydrophobic motif (Ser473). In order to investigate further the role of mTOR in medulloblastoma, we studied the phosphorylation of the mTORC1 substrate S6 kinase (S6K) at Thr389 and the mTORC2 substrate Akt at Ser473. (Figure 1C). PDGF caused an increase in $pAKT^{Ser473}$. However, pretreating cells with RAPA given PDGF, suppressed the PDGF-induced AKT activation. On the other hand, treatment with U0126 alone or in combination with PDGF caused an increase in pAKT levels. The expression of $pS6K^{Thr389}$ was suppressed totally by RAPA treatment for 24 h. Pretreatment with RAPA totally suppressed PDGF-induced activation of $S6K^{Thr389}$, indicating that PDGF was not able to overcome the inhibition from RAPA, suggesting that mTOR pathway inhibition can reduce growth factor induced activation.

mTOR inhibition suppresses cell viability. An analysis of cell viability was performed using MTT assay to elucidate the role of the mTOR pathway in the proliferation of medulloblastoma cells (Figure 2A). Cell cultures were treated with either RAPA alone, or in combination with PDGF or TPA. RAPA caused a significant suppression of cell viability. RAPA treatment attenuated PDGF-induced cell growth, whereas TPA-induced proliferation was only partially suppressed by pretreatment with RAPA. This implicates the importance of mTOR in sustainability of the medulloblastoma cells.

Inhibition of mTOR suppresses entry into S-phase. S-Phase analysis was done using EdU incorporation following treatment with pp242, RAPA, or PDGF (Figure 2B). After treatment with pp242, an inhibitor of mTORC1/2, there was a decrease in the number of cells entering the S phase. The cells treated with RAPA, which inhibits only mTORC1, exhibited a decrease in cells entering the S phase but to a lesser extent than pp242. Control and PDGF-treated cells, on the other hand, exhibited an increase in the amount of EdU incorporation.

mTOR pathways suppress migration of medulloblastoma cells. Chemotactic migration analysis was carried out to evaluate the role of mTOR pathway in cell migration of medulloblastoma cells. Quiescent cells were treated with RAPA for 3 or 24 h and allowed to migrate towards the serum-starve media or fibronectin. Results demonstrated that cells migrated toward serum free medium or medium with fibronectin (Figure 2C-D), however control cells migrated more toward fibronectin, as expected. Following treatment with RAPA for 3 or 24 h, cell migration was significantly suppressed. This effect was also time dependent, such that cells treated with RAPA for 24 h migrated less than the cells that were treated for only 3 h. The effect on cell migration toward serum free medium was similar, with the exception of the time dependent decrease due to the fact that the cells remained saturated with non-chemoattractants with use of serum free medium.

Discussion

In our studies here we have shown that medulloblastoma cells treated with PDGF exhibited a time-dependent up regulation of pERK expression. Cells treated with PDGF and RAPA exhibited a down regulation in the expression of pERK. In addition, medulloblastoma cells treated with PDGF had a decrease in p-P27, and then an increase with prolonged treatment. PDGF in combination with RAPA resulted in a slight increase in p-P27, and the combination of PDGF and U0126 caused an even greater up regulation of p-P27. Cells treated with PDGF and RAPA alone caused an increase in pAKT, however when treated in combination, there was a slight decrease in pAKT. Treatment of cells with RAPA caused complete suppression of pS6K, as well as in combination with PDGF. Time dependent treatment of cells with RAPA showed an initial decrease and then increase in pAKT and pERK expression with prolonged treatment. The viability of medulloblastoma cells was illustrated to be reduced by RAPA alone and in combination with PDGF. Migration of

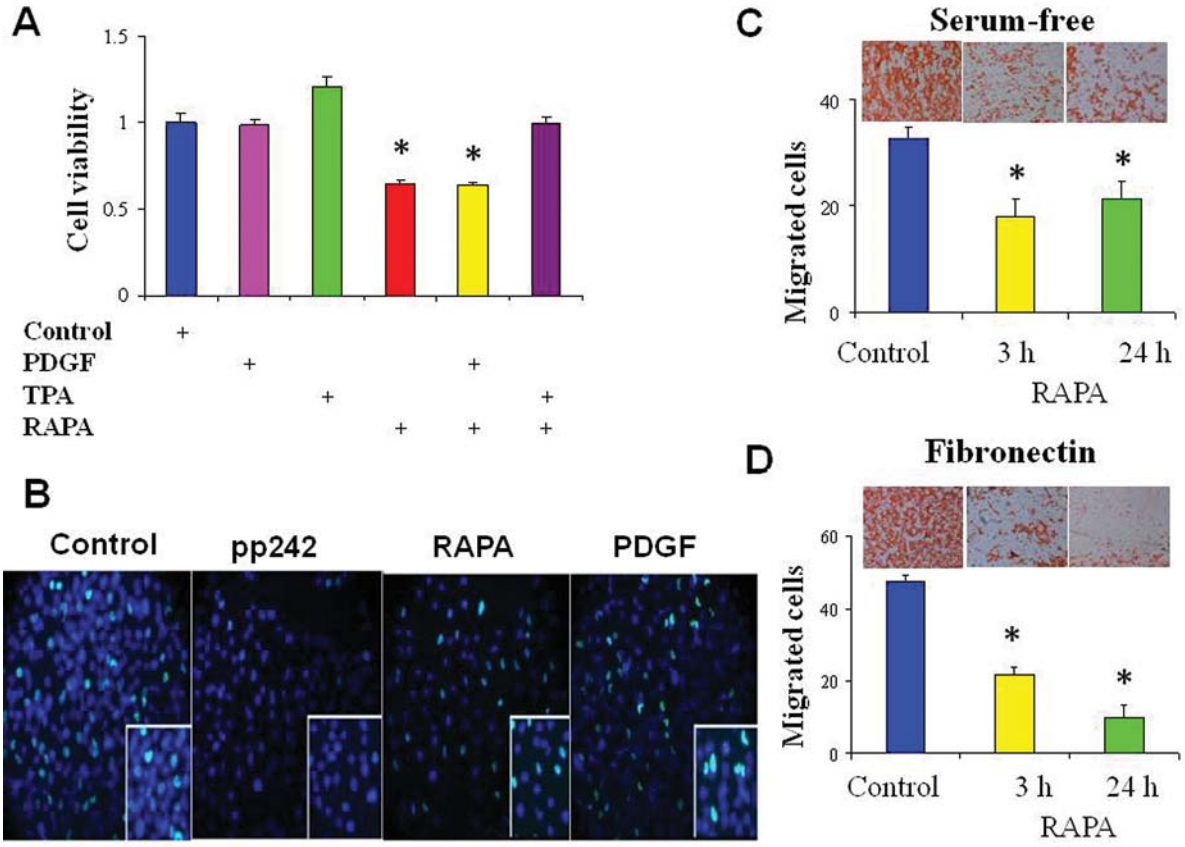

Figure 2. *A: MTT assay was used to assess cell viability following treatment with platelet derived growth factor (PDGF), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), or RAPA (RAPA), alone or in combination. RAPA treatment attenuated PDGF-induced cell growth, whereas TPA-induced proliferation was only partially suppressed by pretreatment with RAPA. B: S-Phase analysis was carried out using EdU incorporation following treatment with vehicle (control) PDGF or pp242 (an mTORC1/mTORC2 inhibitor) or RAPA. After treatment with pp242, there is a decrease in the number of cells entering S phase. The cells treated with RAPA also show a decrease in EdU incorporation, but the effect is not as significant as is seen with pp242. Control or PDGF-treated cells exhibited an increase in the amount of EdU incorporation. C-D: Chemotactic migration toward serum-free (C) or fibronectin containing (D) media was suppressed after treatment with RAPA. Migration toward fibronectin was suppressed in a time-dependent manner and to a greater degree than for that using serum-free media.*

medulloblastoma cells towards fibronectin decreased in a time dependent manner following treatment with RAPA. Moreover, EdU incorporation decreased more with pp242 than RAPA, but increased with PDGF. These studies demonstrated that the mTOR pathway can be targeted in medulloblastoma cells. The caveat of using a prolonged treatment of mTORC1 inhibitor, RAPA, alone is that it activates the MAPK pathway (Figure 1B). These findings corroborate the results of others who showed prolonged treatment with RAPA and its analog caused an activation of a potent mitogenic pathway involving ERK in brain tumor cells or other types of cancer cells (10). This could be a potential complication of clinical use of RAPA alone for the treatment of medulloblastoma, and thus warrants its combination with an MAPK inhibitor.

Various studies have already suggested targeting mTOR as a therapeutic intervention for medulloblastoma. As shown previously by Bhatia, mTOR is up regulated in SHH-mediated medulloblastoma in mouse models (7). In addition, aberrant IGF-signaling in medulloblastoma can cause decreases in mTOR. Other studies have demonstrated that phosphatidylinositol 3-kinase (PI3K)/AKT activation is crucial in the pathogenesis of medulloblastoma (11, 12). Importantly, certain targets for PI3K inhibition, such as P110 isoforms have already been considered as novel treatment options using RNA interference (13). Here we demonstrated that mTOR inhibition can suppress expression of both mTORC1/2 substrates. Another recent study also indicated the role of mTOR in the pathogenesis of medulloblastoma through the activation of S6K and eIF4E (7). Our results showed that activated S6K can be targeted through mTOR inhibition with RAPA, effectively suppressing S6K and more importantly, the PDGF-induced activation of S6K, suggesting that RAPA-induced changes in proliferation occur at the molecular level. Furthermore, expression of FOXO, a downstream target of AKT, has been

shown to play a significant role in the growth of medulloblastoma cells (14), and our results suggest that FOXO expression was suppressed more significantly by mTOR inhibition through pp242 as compared to RAPA (data not shown).

Studies have shown SHH and IGF to be important mediators within deregulated pathways. Furthermore, IGF has been shown to directly inactivate TSC and activate mTOR pathway. An enhanced mTOR activity was observed in animal models of SHH-induced medulloblastoma with inactive TSC, leading to oncogenesis (6). Also, CGNP and SHH affect downstream effectors of the mTOR pathway, S6K and eIF4E. Such studies have provided evidence that during cerebellar development, S6K and eIF4E are regulated by SHH in a discrete manner and suggest that inactivation of these targets may reduce cellular proliferation (7).

The CDK inhibitor $p27^{Kip1}$ (P27) is an important regulator of the mammalian cell cycle (15, 16). P27 negatively regulates G1 progression by binding to cyclin–CDK2 complexes and preventing their activity. The levels of P27 are high in quiescent cells and tend to decline upon mitogenic stimulation. Furthermore, both P27 and its homolog $p21^{Cip1}$ positively regulate cell cycle progression by promoting the assembly and activity of cyclin D–CDk4/6 complexes (17). Phosphorylation of p27 on Ser10 alters its localization from nucleus to cytoplasm. In fact, cytoplasmic localization may serve to reduce the abundance of P27 in the nucleus below a certain threshold required for activation of cyclin–CDK2 complexes (18). Results show that PDGF treatment suppresses P27 Ser10 levels in a time dependent manner, and RAPA, and the MAPK inhibitor, U0126, increases the levels of P27 Ser10. These results corroborate with our findings of EdU incorporation, which is noticeably increased in PDGF-treated cells and reduced in cells treated with RAPA or a combined inhibitor of mTORC1/2, pp242.

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References

1 Gilbertson RJ: Medulloblastoma: signaling a change in treatment. Lancet Oncol *5*: 209-218, 2004.

2 Sarkar C, Deb P, and Sharma MC: Medulloblastomas: New directions in risk stratification. Neurol India *54*: 16-23, 2006.

3 Rutkowski S, Bode U, Deinlein F, Ottensmeier H, Warmuth-Metz M, Soerensen N, Graf N, Emser A, Pietsch T, Wolff JE, Kortmann RD, and Kuehl J: Treatment of early childhood medulloblastoma by postoperative chemotherapy alone. N Engl J Med *352*: 978-986, 2005.

4 Edelstein K, Spiegler BJ, Fung S, Panzarella T, Mabbott DJ, Jewitt N, D'Agostino NM, Mason WP, Bouffet E, Tabori U, Laperriere N, and Hodgson DC: Early aging in adult survivors of childhood medulloblastoma: long-term neurocognitive, functional, and physical outcomes. Neuro Oncol *13*: 536-545, 2011.

5 Northcott PA, Korshunov A, Witt H, Hielscher T, Eberhart CG, Mack S, Bouffet E, Clifford SC, Hawkins CE, French P, Rutka JT, Pfister S and Taylor MD: Medulloblastoma comprises four distinct molecular variants. J Clin Oncol *29(11)*: 1408-1414, 2011.

6 Bhatia B, Northcott P, Hambardzumyan D, Govindarajan B, Brat DJ, Arbiser JL, Holland EC, Taylor MD and Kenney AM: Tuberous sclerosis complex suppression in cerebellar development and medulloblastoma: separate regulation of mammalian target of rapamycin activity and p27kip1 localization. Cancer Res *69*: 7224-7234, 2009.

7 Mainwaring LM and Kenney AM: Divergent functions for eIF4E and S6 kinase by sonic hedgehog mitogenic signaling in the developing cerebellum. Oncogene *30*: 1784-1797, 2011.

8 Bhatia B, Malik A, Fernandez-L A, and Kenney AM: $p27^{Kip1}$, a double-edged sword in Shh-mediated medulloblastoma. Cell Cycle *9*: 4307-4314, 2010.

9 See WL, Miller JP, Squatrito M, Holland E, Resh MD, and Koff A: Defective DNA double-strand break repair underlies enhanced tumorigenesis and chromosomal instability in p27-deficient mice with growth factor-induced oligodendrogliomas. Oncogene *29*: 1720-1731, 2010.

10 Albert L, Karsy M, Murali R, and Jhanwar-Uniyal M: Inhibition of mTOR activates the MAPK pathway in glioblastoma multiforme. Cancer Genomics Proteomics *6*: 255-261, 2009.

11 Hambardzumyan D, Becher OJ, Rosenblum MK, Pandolfi PP, Manova-Todorova K, and Holland EC: PI3K pathway regulates survival of cancer stem cells residing in the perivascular niche following radiation in medulloblastoma *in vivo*. Genes Dev *15*: 436-448, 2008.

12 Hartmann W, Digon-Söntgerath B, Koch A, Waha A, Endl E, Dani I, Denkhaus D, Goodyer CG, Sörensen N, Wiestler OD, and Pietsch T: Phosphatidylinositol 3'-kinase/AKT signaling is activated in medulloblastoma cell proliferation and is associated with reduced expression of PTEN. Clin Cancer Res *12*: 3019-3027, 2006.

13 Guerreiro AS, Fattet S, Fischer B, Shalaby T, Jackson SP, Schoenwaelder SM, Grotzer MA, Delattre O, and Arcaro A: Targeting the PI3K p110alpha isoform inhibits medulloblastoma proliferation, chemoresistance, and migration. Clin Cancer Res *14*: 6761-6769, 2008.

14 Srivastava VK, Yasruel Z, and Nalbantoglu J: Impaired medulloblastoma cell survival following activation of the FOXO1 transcription factor. Int J Oncol *35*: 1045-1051, 2009.

15 Sherr CJ and Roberts JM: Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev *9*: 1149-1163, 1995.

16 Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*: 1501-1512, 1999.

17 Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM, and Sherr CJ: The $p21^{Cip1}$ and $p27^{Kip1}$ CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J *18*: 1571-1583, 1999.

18 Rodier G, Montagnoli A, Di Marcotullio L, Coulombe P, Draetta GF, Pagano M, and Meloche S: p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis. EMBO J *20*: 6672-6682, 2001.

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