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ERK activation of p21 activated kinase-1 (Pak1) is critical for medulloblastoma cell migration

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Abstract

We previously identified that overexpression of the platelet-derived growth factor receptor (PDGFR) is associated with metastatic medulloblastoma (MB) and showed that PDGF treatment increases ERK activity and promotes MB cell migration. In this study, we investigated whether ERK regulates Rac1/Pak1 signaling and is critically linked to MB cell migration. Herein we demonstrate that PDGF-BB treatment of MB cells induces concomitant activation of PDGFR β , MEK1/ERK, Rac1 and Pak1, but suppresses Rho activity, which together significantly promotes cell migration. Conversely, cells transfected with either PDGFR β or Pak1 siRNA or treated with an inhibitor of Rac1 (NSC23766) or N-myristoyltransferase-1 (Tris-dipalladium) are unable to activate Rac1 or Pak1 in response to PDGF, and consequently, are unable to undergo PDGF-mediated cell migration. Furthermore, we also demonstrate that either chemical inhibition of MEK/ERK (U0126) or stable downregulation of PDGFR β by shRNA similarly results in the loss of PDGF-induced ERK phosphorylation and abolishes Rac1/Pak1 activation and cell migration in response to PDGF. However, specific depletion of Pak1 by siRNA has no effect on PDGF-induced ERK phosphorylation, indicating that in MB cells ERK signaling is Pak1-independent, but PDGF-induced migration is dependent on ERK-mediated activation of Pak1. Finally, using tissue microarrays, we detect phosphorylated Pak1 in 53% of medulloblastomas and show that immunopositivity is associated with unfavorable outcome. We conclude that Rac1/Pak1 signaling is critical to MB cell migration and is functionally dependent on PDGFR β /ERK activity.

Keywords

ERK; Medulloblastoma; Migration; Pak1; PDGF

Introduction

Medulloblastoma (MB), the most common malignant brain tumor of children, is characterized by a propensity to spread throughout the central nervous system (CNS), with approximately one-third of patients presenting with metastasis at diagnosis and up to two-thirds at the time of tumor relapse [1,2]. The presence of metastasis is the single most important prognostic indicator of poor outcome [3-5]. Understanding the signaling mechanisms regulating MB migration and invasion should aid in the development of novel therapeutic strategies aimed at preventing MB metastasis.

We previously identified overexpression of the *platelet-derived growth factor receptor* (PDGFR) in metastatic MB and showed that PDGF treatment of MB cells induces extracellular signal-regulated kinase (ERK) phosphorylation and promotes migration [6,7]. PDGFR, a membrane-bound receptor tyrosine kinase (RTK), was first identified as being essential for the regulation of neural cell proliferation, migration and survival in embryologic CNS development [8]. These findings implicate PDGFR/ERK signaling as an important effector of MB growth and metastasis.

Small GTPases of the Ras and Rho families play critical roles in coupling RTK signaling to the cytoskeleton and to other signaling molecules that are essential for cell motility [9-11]. Members of the Ras family generally regulate cell proliferation and differentiation, whereas Rho predominantly controls cytoskeleton rearrangement [12-14]. The most common members of the Rho family are RhoA, Rac1 and Cdc42. RhoA promotes focal adhesion and regulates contractility via actin stress fiber assembly and Rho-kinase inhibition affects cell morphology, motility and invasion through activation of Rac1 in some cell types [15,16]. Rac1 regulates lamellipodia formation to mediate cell migration [17,18]. In many cell types, RhoA-GTP level is negatively regulated by Rac1 activation via RTK-mediated signaling [16,19]. Thus, the balance between Rac1 and RhoA is critical to cell motility, cell-cell adhesion and cell morphology [20,21].

The Rac1 downstream effectors include a number of proteins, of which the best characterized is the p21 activated kinase (Pak) family [22,23]. Pak undergoes auto-phosphorylation on multiple sites and is activated upon binding to Rac1- or Cdc42-GTP [24,25]. Pak1 is a serine/threonine protein kinase that regulates cytoskeletal remodeling and cell motility through actin and microtubules [26-31]. Overexpression of constitutively active Pak1 enhanced cancer cell growth and invasion, whereas overexpression of dominant negative Pak1 suppressed invasion [27,28]. Pak1 has been reported to modulate activation of the Raf/MEK/ERK pathway by either directly activating Raf or priming MEK for activation of Raf in some cell types [32-37]. In HEK293 cells, Pak1 was shown to phosphorylate Raf-1 on serine 338 and MEK1 on serine 298 resulting in cross-activation of ERK [35]. Because of these reported interactions, we investigated whether PDGFR/ERK regulates Rac1/Pak1 signaling and is critically linked to PDGF-mediated MB cell migration and explored whether there is functionally associated cross-talk between PDGFR, ERK and Pak1 in MB cells.

Materials and methods

Cell culture and reagents

Daoy and D556 human medulloblastoma cells were cultured in EMEM with 10% fetal bovine serum (FBS). PDGF-BB was purchased from Sigma (St. Louis, MO). Rac1 inhibitor NSC23766 was purchased from Calbiochem (La Jolla, CA). Tris-dipalladium (Tris-DBA) was generously provided by Dr. Jack L. Arbiser (Emory University, Atlanta, GA).

Western blotting and antibodies

Western blot of whole cell lysates harvested in lysis buffer (Cell Signaling Technology, Danvers, MA) was performed with the following primary antibodies: RhoA and PDGFR β (Santa Cruz, CA); Rac1 (BD Biosciences, San Jose, CA); phospho-PDGFR β (Tyr751), phospho-MEK, phospho-ERK, phospho-Pak1 (Thr423)/Pak2 (Thr402) and Pak1 (Cell Signaling Technology); Ras (Upstate, Billerica, MA). Goat or rabbit anti-mouse horseradish peroxidase secondary antibodies (Santa Cruz) were used and the immunore-active bands were detected by ECL. Densitometric analysis of the visualized bands was used to quantitate and compare the relative changes in levels of target proteins between PDGF-treated and untreated cells.

siRNA transfection

Pak1 siRNA (L-003521-00 and J-003521-09), PDGFR β siRNA (L-003163-00) and negative control non-targeting siRNA (D-001810-01-05 and D-001810-02-05) were purchased from Dharmacon (Chicago, IL). For transfections, 1.5×10^5 cells were seeded in each well of a six-well plate and grown to 50–60% confluency prior to transfection. Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carisbad, CA) for 48 h according to the manufacturer's instruction. The final concentration of siRNA was 100 nmol/l.

GTPase pull-down assays

Rac and Rho GTPase activity was analyzed using respective GTPase assay kits (Millipore, Temecula, CA). Briefly, for each assay 200 μ g of freshly prepared cell extracts were reconstituted in 500 μ l of lysate buffer and added to 10–20 μ g of the respective GTPase assay reagent; Pak-1 RBD for Rac activity and Rhotekin RBD for Rho activity. The GTPases were detected by Western blot with anti-Rac1 for Rac-GTP and anti-RhoA for Rho-GTP. The significance of the experiments was determined by the Student's *t*-test.

Boyden chamber migration assay

Fibronectin-mediated cell adhesion and migration was assessed using the Chemicon QCM-FN Quantitative Cell Migration Assay (Chemicon International, Temecula, CA) as previously described [6]. Briefly, cells were cultured to 80% confluency and either treated with 5 μ M Tris-DBA in serum-free EMEM for 16 h or serum-starved for 24 h, harvested with trypsin/EDTA and resuspended in serum-free EMEM and treated with 100 μ M NSC23766 or 10 μ M U0126 for 1 h at 37°C. Following incubation, 1.5×10^5 cells in 200 μ l serum-free EMEM were seeded into the pre-coated (fibronectin or BSA) upper chambers. To determine whether Pak1 contributes to MB migration, aliquots of 4×10^4 cells (transfected with Pak1 or control siRNA) were placed into the upper chambers. The lower well contained 500 μ l serum-free EMEM, with or without 20 ng/ml PDGF-BB. The cells were incubated for 5 h at 37°C (3 h for Tris-DBA), stained with cell staining solution and then washed in distilled water. Cells were eluted with extraction solution and 100 μ l transferred to a 96-well microtiter plate for absorbance reading (570 nm). The commercial cell migration assay kits were used in accordance with the manufacturer's instructions for the accurate measurement of migrating cell number, including performing a pre-test cellular dye absorption nomogram calibration that correlates the OD

reading to a known quantity of serially diluted stained tumor cells and subtraction of background membrane absorbance of dye, performed with and without cells in the presence of control BSA. In all cases background membrane absorbance of dye was negligible. *P* value was calculated using Student's *t*-test.

Establishment of stable cell lines

To make stable medulloblastoma cell lines with continuous knockdown of PDGFR β expression, 2×10^4 Daoy and D556 cells were seeded in 24-well plates, 0.4 μ g PDGFR β shRNA (from SA Biosciences, Frederick, MD. Cat# KH00477 N) gently mixed with 4 μ l SureFECT transfection reagent (SA Biosciences, Cat# SA-01) in 50 μ l OPTI-MEM solution and then added into each well. 48 h later, cells were split into 60 mm dishes with G418 for further culture. Western Blots were used to verify whether expression of PDGFR β was downregulated by shRNA in the transfected cells, comparing to the cells transfected with negative control shRNA. After preliminary selection, 200 cells with knockdown of PDGFR β expression was seeded on each 100 mm dish with G418 for colonies formation. Single colony was picked up and Western Blots were performed at least three times to verify the stable knockdown cells. In this study, we defined A4 and A11 as Daoy PDGFR β -knockdown cells, B7 and B9 as D556 PDGFR β -knockdown cells, and NC1 and NC2 as negative control cells.

Tissue microarray

Construction of the tissue microarray (TMA) was performed at the National Institutes of Health (NIH) consisting of 144 previously untreated paraffin-embedded childhood medulloblastoma tumor tissue specimens, obtained from the pathology libraries of Children's National Medical Center (CNMC) and the Armed Forces Institute of Pathology (AFIP). IRB approval was obtained from each institution for this TMA construction and analysis. In brief, paraffin-embedded tissue samples were extracted using 0.6 μ m diameter needles and mechanically embedded in a paraffin block into an array. Each sample was spotted into the block twice, thus providing two identical spots for each tissue sample. A microtome was used to cut the paraffinized TMA blocks and 5 micron-thick sections were mounted onto positively charged slides for immunohistochemical analysis. A database relating each tumor specimen to its clinical and histologic characteristics was constructed and used for all correlations to the immunostainings. Of the 144 specimens, 94 were deemed evaluable for analysis.

Immunohistochemistry for phospho-Pak1

Standard immunohistochemistry (IHC) for phospho-Pak1^{Thr212} (#P3237, Sigma Chemical, St Louis, MO) was performed using the TMA described above. Immunostaining for phospho-Pak1 was first performed using normal brain and classic MB tissue samples to serve as negative control and for optimization of the staining procedure, respectively, prior to using the TMA for IHC. TMAs were treated with DAKO antigen retrieval solution and blocked with horse serum prior to application of the primary antibody. Incubation with anti-phospho-Pak1 (1:150 dilution) was performed overnight at 4°C. Immunodetection was performed using the Elite Vectastain ABC system (Vector Laboratories, Burlingame, CA). Color visualization was performed using 3,3'-diaminobenzide as the chromagen substrate (Innovex Biosciences, Pinole, CA). Haematoxylin was used as the counterstain for nuclear detail.

Each tissue sample in the array was independently scored for positivity by two neuropathologists (M.S. and E.J.R.). Scoring was performed blinded and the immunostaining results for each tumor specimen were defined as either negative or positive for phospho-Pak1. The positive or negative grading definitions used were established by the neuropathologists and were based on the relative diffuse cellular homogeneity observed with the specific target staining for each specimen tested. Of the 144 MB specimens, 50 were designated "not-amenable" for analysis to indicate that in the opinion of the grading neuropathologist, that

either the IHC staining or the tissue integrity on the TMA was not of suitable quality to confidently grade the staining. The significance was determined using two sample test of proportion (z test) in STATA software by statistician.

Results

PDGF concomitantly induces activation of PDGFR β /MEK1/ERK and Rac1, but suppresses RhoA activity in medulloblastoma (MB) cells

We hypothesize that in MB cells PDGFR signaling functions to increase ERK/Rac1 basal activity, which then promotes migration via activation of the downstream effector Pak1. Daoy cells, which we have previously shown have an mRNA expression profile that is similar to metastatic MB [6] and are metastatic in mouse xenografts [38], would thus be expected to express abundant Rac1 protein and to have significantly increased Rac1 and Pak1 activity over basal level in response to PDGF. To determine the effects of PDGF-BB on the temporal activation of PDGFR β /ERK/Rac1/RhoA in Daoy cells, we examined phosphorylation (activation) of PDGFR β and MEK1/ERK and the level of Rac1- or RhoA-GTP activity at different time points following PDGF-BB treatment. As shown in Fig. 1a, PDGF increases phosphorylation of PDGFR β at 8 and 15 min ($P = 0.006$ and $P = 0.028$, respectively, as measured by densitometry comparing PDGF treated and untreated cells). An increase of Ras-GTP also was detected at 8 and 15 min (data not shown). Similarly, PDGF induced increased phosphorylation of MEK1 and ERK at 8 and 15 min. As shown in Fig. 1b, Rac-GTP (indicated Rac1 activity) is marginally increased ($P = 0.027$, comparing the Rac-GTP/total Rac1 ratios at 0 and 15 min), while Rho-GTP is marginally decreased following PDGF treatment ($P = 0.003$, comparing the Rho-GTP/total RhoA ratios at 0 and 15 min). Together, these results demonstrate that PDGF activates the PDGFR/MEK1/ERK pathway and in turn concomitantly increases Rac1 activity, but suppresses RhoA activity.

Rac1 activity is essential for MB cell migration

Based on the finding that PDGF induces Rac1 activity in MB, we next sought to determine whether Rac1 activation is critical to PDGF-mediated MB cell migration. To address this question, we first determined whether the Rac1 inhibitor, NSC23766, specifically suppresses PDGF-mediated Rac1 activation in Daoy. As shown in Fig. 1c (upper panel), in comparison to untreated control cells, cells treated with NSC23766 showed marginally decreased levels of Rac-GTP, but maintained levels of Rho-GTP, in response to PDGF ($P = 0.03$). Importantly, no significant difference was observed on basal levels of Rac-GTP or Rho-GTP without PDGF treatment between NSC 23766 treated and untreated cells, indicating that the action of the inhibition is specifically growth factor-dependent.

Subsequently, we used fibronectin-coated Boyden chamber assays to determine whether Rac1 inhibition alters cell migration. As shown in Fig. 1c (lower panel), PDGF stimulation increased migration by 69% in comparison to unstimulated control cells ($P < 0.05$), while pretreatment with NSC23766 completely abolished PDGF-mediated migration without effecting basal level migration. We also performed scratch assays in the presence or absence of NSC23766 and found that PDGF-mediated cell motility is markedly blocked by treatment with the Rac1 inhibitor (data not shown), even in the presence of 10% serum (data not shown), indicating that Rac1 plays an important role overall in growth factor-mediated Daoy migration. To confirm that Rac1 activation is required for PDGF-mediated cell migration, we repeated the Rac/Rho-GTP pull down and Boyden chamber migration assays in the presence of Tris-DBA, an inhibitor of the enzyme N-myristoyltransferase-1 (NMT-1), which has been reported to act as an anti-neoplastic agent by inhibiting several Ras/Rac downstream signaling pathways, including Akt and ERK, which were correlated to Rac activation [39,40]. As shown in Fig. 1d, Tris-DBA prevented an increase in the level of Rac1-GTP and a decrease of Rho-GTP in

response to PDGF and completely abolished PDGF-mediated cell migration, but had no effect on the basal level growth factor-independent migration.

PDGF induces phosphorylation of Pak1 through PDGFR/Rac1 signaling and siRNA depletion of Pak1 suppresses PDGF-mediated MB cell migration

Our findings show that Rac1 is necessary for growth factor-induced MB cell motility. Pak1, a major common downstream effector for Rac1 in the brain [41], undergoes autophosphorylation on multiple sites and is activated upon binding to Rac-GTP [26,42,43]. Phosphorylation of Pak1 at the site of Thr²¹² is associated with neurite growth and motility of neurons [29,42] and phosphorylation of Thr⁴²³ has been directly associated with Pak1 activity in many cell types. To determine the role of Pak1 in PDGF-induced MB cell migration, we first examined Pak1 phosphorylation of Thr²¹² and Thr⁴²³ in Daoy cells at different time points following PDGF-BB treatment and then tested whether inhibition of PDGFR or Rac1 alters Pak1 phosphorylation in response to PDGF. As shown in Fig. 2a, there is an increase of Pak1 phosphorylation at both sites (Thr²¹² and Thr⁴²³) at various times following PDGF treatment, indicating that PDGF induces phosphorylation of Pak1 in Daoy. This is consistent with the temporal increase in Rac1 activity in response to PDGF. Conversely, as shown in Fig. 2b, depletion of PDGFR β by transient siRNA transfection abolished PDGF-induced phosphorylation of PDGFR β and activation of Rac1 and Pak1. Moreover, the expression level of a similar RTK-family protein, EphB1, was not altered by siRNA depletion of PDGFR β , indicating that the PDGFR β siRNA specifically targets PDGFR β expression. Likewise, inhibition of downstream Rac1 activity by NSC23766 or Ras/Rac activity by Tris-DBA similarly blocked PDGF-induced Pak1 activation (Fig. 2b), as well as blocked the activation of Pak1 in response to 10% serum (data not shown); thereby supporting a direct linkage between the PDGFR signaling pathway and Rac1 and Pak1 activation in MB cells.

We then tested whether Pak1 activity correlates with MB migration. To address this question we examined whether depletion of Pak1 by siRNA transient transfection alters PDGF-induced MB cell migration. As shown in Fig. 2c, depletion of Daoy Pak1 levels abrogated PDGF-induced phosphorylation of Pak1 at both sites (Thr²¹² and Thr⁴²³). In the Pak1-deficient cells, PDGF-induced cell migration was significantly attenuated compared to Daoy cells transfected with control siRNA (Fig. 2c). These results were validated using another MB cell type, D556, by again showing that PDGF treatment induces Pak1 phosphorylation and that Pak1 depletion and loss of PDGF-induced Pak1 phosphorylation concurrently and significantly attenuates MB cell migration (Supplementary Fig. 1).

PDGF-mediated activation of Rac1/Pak1 is ERK signaling dependent, but ERK activation is Pak1 independent in MB cells

Studies have shown that inhibition of ERK activation in colon carcinoma cells by treatment with the MEK inhibitor, U0126, results in an increase in cytoskeletal stress fibers and decreased cell motility, while the repression of stress fiber formation by ERK-MAP kinase signaling in Ras-transformed fibroblasts promotes cell motility [40,44]. One study also reported that PDGF-mediated Pak1 phosphorylation on Thr²¹² in smooth muscle cells was dependent in part on ERK signaling [36]. To test whether ERK is similarly involved in the regulation of PDGF-mediated activation of Rac1/Pak1 in MB cells, we examined the effect of pretreatment with the MEK/ERK specific inhibitor U0126 in MB cells stimulated with PDGF. We demonstrate that pretreatment of Daoy cells with U0126 completely inhibits PDGF-mediated activation of Rac1, without affecting the basal level of Rac-GTP, and abolishes PDGF-mediated Pak1 phosphorylation at Thr²¹² and Thr⁴²³ (Fig. 3a, $P = 0.016$ for Thr²¹², $P = 0.04$ for Thr⁴²³), comparing PDGF stimulated cells with or without U0126 pretreatment). Complete inhibition of PDGF-mediated Pak1 activation by U0126 was confirmed in D556 cells (data not shown), indicating that PDGF-mediated activation of Rac1/Pak1 in MB cells is dependent on the ERK

signaling pathway. However, other studies have shown that in a number of cell types specific downstream inhibition of Pak1 can result in the loss of ERK activation and diminished MEK/ERK pathway signaling [32-34,36]. To determine whether the cross-talk regulation between ERK and Pak1 in MB cells is bi-directional, we depleted Pak1 by siRNA and examined PDGF-mediated phosphorylation of ERK in Daoy and D556. Interestingly, in contrast to these reports in other cell types, downregulation of Pak1 does not affect the basal or PDGF-induced levels of phospho-ERK in MB cells (Fig. 3b), confirming that depletion of Pak1 alone is insufficient to alter activation of ERK and suggesting that cross-talk regulation between ERK/Pak1 is unidirectional in MB cells.

PDGF-mediated ERK activation is PDGFR β -dependent and is necessary for migration of MB cells

To determine the extent to which PDGFR signaling regulates ERK activity in MB cells, we used human PDGFR β shRNA to establish two MB cell lines with stable knockdown of PDGFR β expression and examined if sustained downregulation of PDGFR β affects the level of ERK phosphorylation. As shown in Fig. 4a, shRNA-induced knockdown of PDGFR β leads to significant loss of PDGF-mediated PDGFR β phosphorylation, and markedly decreases the level of PDGF-induced ERK phosphorylation in both MB cell types (*t*-tests of $P < 0.05$, as measured by densitometry comparing PDGF treated A4 and NC1 or B9 and NC1 for Daoy or D556, respectively). PDGF stimulation of control shRNA cells with intact PDGFR induces PDGFR phosphorylation, which can result in a concomitant decrease in PDGFR expression following PDGFR activation. This is a well described growth-factor mediated event in receptor-tyrosine kinases secondary to proteosomal degradation of the receptor following its activation as a regulatory mechanism to extinguish receptor activation. shRNA-induced knockdown of PDGFR β resulting in the loss of PDGF-mediated PDGFR β and ERK phosphorylation was validated in a separate set of shRNA PDGFR β positive and negative stable cell clones in both MB cell types (Supplementary Fig. 2). Loss of phosphorylation of ERK was also observed in Daoy transiently transfected with PDGFR β siRNA (data not shown), confirming that PDGFR β acts as an important upstream modulator of ERK signaling and is required for PDGF-mediated ERK activation in MB cells. To confirm the functional role of ERK in the regulation of MB cell migration, we treated cells with U0126 and performed Boyden chamber migration assays. The results show that U0126 completely blocks PDGF-mediated cell migration (Fig. 4b), indicating that ERK activity is necessary for growth factor-induced cell migration of MB cells.

Medulloblastoma expression levels of phosphorylated Pak1 correlate with poor clinical outcome

Aberrant Rac1 activity has recently been associated with the invasive and malignant phenotype in a variety of cancers [45,46] and an increased level of tumor-associated phosphorylated Pak1 has been associated with shorter survival time in patients with glioblastoma [29]. To explore the clinical and prognostic relevance of activated Pak1 in MB, we performed immunohistochemistry for phospho-Pak1 on 94 evaluable MB specimens. Of these MB specimens, we detected positive phosphorylated Pak1 immunostaining in 53% of all MB (Table 1). Positive immunostaining for phospho-Pak1, when present, was generally diffusely positive throughout the tumor sample (Fig. 5a). Furthermore, immunohistochemical analysis using the same TMA revealed that Rac1 is diffusely and ubiquitously expressed by nearly all 94 MB evaluated (data not shown). There was no association of immunopositivity with either a specific tumor histologic subtype or with clinical evidence of metastasis (Table 1). However, a highly significant positive rate for phospho-Pak1 was observed in the confirmed deceased MB patients (82%), compared to those who are known to have survived and are confirmed alive without disease recurrence or progression more than 5 years after the completion of the initial treatment for MB (22%; $P < 0.001$) (Table 1). In addition, Kaplan–Meier survival curve showed a

significant decrease of 5-year survival rate in the MB patients with positive phospho-Pak1 (36%, 5 out of 14), compared to the MB patients with negative phospho-Pak1 (90%, 18 out of 20, $P < 0.01$) (Fig. 5b). These results suggest that the presence of activated Pak1 is a poor prognostic indicator in MB.

Discussion

Aberrant PDGFR signaling disrupts neuronal migration and normal cerebellar development [47], while expression of PDGFR-related genes is associated with metastasis and somatic mutations of the Shh and Wnt developmental pathways in medulloblastoma [6,48]. Because Rho family GTPases have been implicated in the invasiveness of various cancer cells [49-52], we hypothesized that PDGFR promotes medulloblastoma (MB) cell migration via regulation of Rho GTPases. Here, we demonstrate that Pak1, a major effector of Rac1 RhoGTPase, is essential for PDGFR-mediated MB cell migration, based on our results that siRNA depletion of Pak1 completely suppresses PDGF-mediated cell migration. Moreover, ERK-dependent phosphorylation of Pak1 is regulated by PDGF-mediated ERK activation in MB cells while inhibition of ERK activation abolishes PDGF-mediated Pak1 activation and MB cell migration. Importantly, we also show that phosphorylation of Thr212 on Pak1 is associated with poor clinical outcome in medulloblastoma. Thus, taken together, we demonstrate for the first time a functional and clinical linkage between medulloblastoma and PDGFR-ERK-Pak1 signaling, which could have important therapeutic relevance.

We previously demonstrated that PDGF promotes ERK phosphorylation in MB cells [6] and here we show that PDGF-mediated activation of ERK is specifically PDGFR β -dependent. In keeping with reports in other cell types, PDGF-mediated activation of PDGFR β /Rac1/Pak1 signaling in medulloblastoma cells is rapid, as evidenced by significantly increased downstream effector phosphorylation as early as 8 min after PDGF treatment [16]. Early changes in receptor signaling activity has been linked to cell migration occurring at much later time points since RTK-mediated events responsible for cell migration, such as secretion of proteases and microtubule or intermediate filament dynamics, have been shown to be prolonged following the initiating RTK signal propagation [53]. For example, recent studies have similarly reported PDGF-mediated regulation of cell migration, as measured by Boyden chamber assay 4 h after PDGF stimulation, via transiently increased Rac-GTP and cellular cGMP levels at 5 min after PDGF stimulation [54].

In some cell types, Pak1 activity has been shown to be required for maximal activation of the canonical Raf/MEK/ERK signaling cascade by PDGF, likely because of Pak co-activation of Raf and MEK [35,36]. However, the convergence of RTK signaling and Pak modulation of MEK/ERK activation remains controversial, and in our study, specific downregulation of Pak1 in MB cells did not alter PDGF-mediated activation of ERK. Our result compares favorably to a study in osteoclast cells similarly showing that Pak1 does not modulate Raf-mediated MEK activation by M-CSF [37]. Thus, RTK-mediated interactions between Rac1/Pak1 and MEK/ERK is likely growth factor- and cell type-dependent, and possibly due to the underlying status of oncogenic Ras.

Although ERK dependency of Pak1 Thr212 phosphorylation by PDGF-BB has been demonstrated previously [36], a functional significance of Pak Thr212 phosphorylation by ERK was not described. Moreover, this earlier report primarily identified Pak1 as a facilitator of ERK signaling. In contrast, our results show that in MB cells Pak1 does not impact ERK activation and that ERK regulation of Pak1 is unidirectional rather than bi-directional. Furthermore, our results demonstrate phosphorylation of both Thr212 and Thr423 on Pak1 by ERK in response to PDGF. Phosphorylation of both Thr sites on Pak1 appears to correlate with Pak1 functional activity in MB cells since decreased phosphorylation at both sites was observed

with inhibition of ERK and Rac1, which correlated with ablation of cell migration, but the dependency of function on either specific phosphorylation site remains to be determined. This is significant in light of the finding that Pak1 Thr423 phosphorylation is required to maintain Pak1 in the open active conformation [55]. The absence of functional changes following ERK activation may thus suggest the inability of ERK to phosphorylate Pak1 Thr423 in certain cell types.

Overexpression of Pak1 has been shown in several cancers, and an association between phosphorylated Pak1^{Thr212} and decreased progression-free survival of patients with glioblastoma has been reported [29,56-58]. We show a highly significant association between poor clinical outcome and positive phosphorylated Pak1^{Thr212}. Since the number of metastatic tumors in our cohort is small, it is impossible to make a conclusion about the relation of Pak1 phosphorylation to metastasis. Confirmation of clinical correlations in this study was also limited by the lack of an available IHC antibody to reliably detect phosphorylation of tumor-associated Pak1^{Thr423}. Because Pak1 can also regulate survival and transcription, it is possible that the increased frequency of Pak1 phosphorylation observed in poor outcome tumors is related to these cellular functions. Thus, together these results provide crucial rationale for future expanded testing in both human MB and mouse MB models to validate that this pathway is mechanistically linked to in vivo metastasis and/or other oncogenic processes, and suggest that one novel therapeutic strategy for MB may be multi-targeted inactivation of PDGFR/ERK/Pak1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. PDGF concomitantly induces activity of PDGFR β /MEK1/ERK and Rac1, but suppresses RhoA activity, in medulloblastoma (MB) cells, and Rac activity is essential for MB cell migration. **a** Daoy MB cells were serum-deprived in medium containing 0.1% FBS for 24 h and stimulated with 10 ng/ml PDGF-BB for 8 and 15 min (0 min represents no PDGF treatment), and then Western blot was performed to detect changes in phosphorylation of PDGFR β or MEK1/ERK, respectively, at the time points indicated. **b** Rac-GTP and Rho-GTP levels were assessed by pull down assays using PAK-1 RBD and Rhotekin RBD, respectively. Data are representative of at least three separate experiments. **c, d** Daoy cells were pretreated with or without 100 μ M of the Rac1 inhibitor NSC23766 for 1 h or 5 μ M of Tris-DBA for 16 h, stimulated with or without PDGF-BB (10 ng/ml for 15 min), and then Rac- and Rho-GTP pull down assays were performed. Aliquots of 1.5×10^5 cells in serum-free EMEM were placed in the upper well of Boyden chambers and allowed to migrate toward a PDGF-BB (20 ng/ml) gradient in the lower well. NSC23766 and Tris-DBA treatments blocked the PDGF-induced increase in Rac-GTP and concomitant decrease in Rho-GTP (**c, d, upper panels**) and abolished PDGF-mediated cell migration, without affecting basal migration (**c, d, lower panels**). Each bar represents the S.E. of triplicate wells

Fig. 2. PDGF induces phosphorylation of Pak1 through PDGFR/Rac1 signaling and siRNA depletion of Pak1 suppresses PDGF-mediated MB cell migration. **a** Daoy cells were starved in serum-free medium overnight, stimulated with 10 ng/ml PDGF-BB, and Western blot for detection of changes in Pak1 phosphorylation using anti-phospho-Pak1^{Thr212} and anti-phospho-Pak1^{Thr423} was performed at the indicated time points following PDGF stimulation. **b Left panel**, Daoy cells were transfected with control or PDGFR β siRNA (lane 1 and 2, control siRNA; lane 3 and 4, PDGFR β siRNA), treated with or without PDGF-BB (10 ng/ml for 15 min), and assayed for changes in Rac-GTP and phosphorylation of PDGFR β and Pak1^{Thr212}. PDGFR β depletion results in loss of PDGF-induced activation of Rac1 and phosphorylation of Pak1. **Right panel**, treatment of Daoy cells with NSC23766 (100 μ M for 1 h) or Tris-DBA (5 μ M for 16 h), with or without 10 ng/ml PDGF-BB stimulation for 15 min, inhibits PDGF-induced Pak1 phosphorylation. Data are representative of at least two separate experiments. **c** Daoy cells transfected with control or Pak1 siRNA, stimulated with or without PDGF-BB (10 ng/ml for 15 min), and assayed for changes in Pak1 phosphorylation by Western blot. Pak1 siRNA knockdown cells show absence of PDGF-mediated phosphorylation of Pak1 at Thr²¹² and Thr⁴²³ (upper panel). Lower panel showed that control and Pak1 siRNA transfected Daoy cells were assessed for migration in the presence or absence of a PDGF-BB (20 ng/ml) chemoattractant gradient using the Boyden chamber migration assay. Pak1-depleted cells maintain basal migration, but demonstrate ablation of PDGF-induced migration. Each bar represents the S.E. of six transwells. Experiments were performed separately for three times

Fig. 3. PDGF-mediated activation of Rac1/Pak1 is ERK signaling dependent, but ERK activation is Pak1 independent in MB cells. **a** Daoy cells grown in serum-free medium were pretreated with or without 10 μ M of the ERK inhibitor U0126 for 1 h, stimulated with or without 10 ng/ml PDGF-BB for 15 min, and assayed for changes in phosphorylation of ERK and Pak1^{Thr212} and Pak1^{Thr423} by Western blot and Rac-GTP by pull down assay. U0126 treatment significantly suppresses PDGF-induced ERK phosphorylation and completely abolishes PDGF-mediated increased activity of Rac1 or Pak1, without affecting the Rac1-GTP basal level (unable to assess the effect on background Pak1 activity since basal level of phospho-Pak1 is undetectable). U0126 (-) represents vehicle control (DMSO) alone. **b** Daoy and D556 cells were transfected with Pak1 siRNA, stimulated with or without PDGF-BB (10 ng/ml for 15 min), and Western blot performed to detect resultant changes in ERK phosphorylation. Both MB cell types with downregulation of Pak1 by siRNA maintain basal and PDGF-mediated phosphorylation of ERK compared to control cells. Data in Fig. 3 are representative of at least three separate experiments

Fig. 4. PDGF-mediated ERK activation is PDGFR β -dependent and is necessary for migration of MB cells. **a** Daoy and D556 cells were transfected with control or shRNA PDGFR β and stable clones for each were established (NC1, Daoy or D556 cells with negative control shRNA; A4, Daoy with shRNA knockdown of PDGFR β ; B9, D556 with shRNA knockdown of PDGFR β), stimulated with or without PDGF-BB (10 ng/ml for 15 min), and Western blot was performed for the detection of changes in PDGFR β and ERK phosphorylation. MB cells with stable downregulation of PDGFR β show significant suppression of PDGF-mediated phosphorylation of PDGFR β and ERK ($P < 0.05$ for each). Data are representative of at least three separate experiments. **b** Boyden chamber migration assay performed, as previously described, shows that U0126 (10 μ M) pretreatment of Daoy cells for 1 h completely blocks PDGF-directed cell migration, without affecting basal migration. Each bar represents the S.E. of triplicate wells

Fig. 5.

Immunohistochemistry of MB tissue microarrays for phosphorylated Pak1 and Kaplan–Meier survival curve. **a** Representative immunostaining of tissue microarray containing 144 MB tumors, of which include 94 were evaluable for phospho-Pak1 analysis. (i) Negative control image for immunostaining for phosphorylated Pak1^{Thr212} performed on separate MB tumor tissue in order to validate the specificity of the antibody. (ii) Positive control image for immunostaining for phosphorylated Pak1^{Thr212} performed on separate MB tumor tissue in order for optimization for immunostaining of tissue microarray. Representative images of (iii) negative and (iv) positive phosphorylated Pak1^{Thr212} immunostaining of MB tumors on the tissue microarrays. **b** Kaplan–Meier survival curve showed a decrease of 5 year survival rate in the MB patients with positive phospho-Pak1 (36%), comparing to the MB patients with negative phospho-Pak1 (90%) ($P < 0.01$)

Table 1

Phospho-Pak1 immunostaining results in medulloblastoma (MB)

	Negative	Positive	Positive (%)
Histologic subtype			
Classic	11	14	56
Anaplastic	10	14	58
Desmoplastic	10	11	52
N/A ^a	13	11	46
Total	44	50	53
Non-metastatic	41	45	52
Metastatic	3	5	62
Deceased	2	9	82
Survived ^b	18	5	22 [*]

Histologic subtype of the tumors of deceased = classic (3), anaplastic (1), desmoplastic (2), N/A (5) and subtype of tumors of survived = classic (4), anaplastic (3), desmoplastic (6), N/A (10)

^aNot able to confirm subtype of MB

^bAlive without disease recurrence or progression >5 years from the end of initial treatment

^{*} $P < 0.001$, comparing positive % phospho-Pak1 tumors between deceased and survived