PERK Activation Promotes Medulloblastoma Tumorigenesis by Attenuating Premalignant Granule Cell Precursor Apoptosis

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Evidence suggests that activation of pancreatic endoplasmic reticulum kinase (PERK) signaling in response to endoplasmic reticulum stress negatively or positively influences cell transformation by regulating apoptosis. Patched1 heterozygous deficient (Ptc11/−) mice reproduce human Gorlin’s syndrome and are regarded as the best animal model to study tumorigenesis of the sonic hedgehog subgroup of medulloblastomas. It is believed that medulloblastomas in Ptc11/− mice results from the transformation of granule cell precursors (GCPs) in the developing cerebellum. Here, we determined the role of PERK signaling on medulloblastoma tumorigenesis by assessing its effects on premalignant GCPs and tumor cells. We found that PERK signaling was activated in both premalignant GCPs in young Ptc11/− mice and medulloblastoma cells in adult mice. We demonstrated that PERK haploinsufficiency reduced the incidence of medulloblastomas in Ptc11/− mice. Interestingly, PERK haploinsufficiency enhanced apoptosis of premalignant GCPs in young Ptc11/− mice but had no significant effect on medulloblastoma cells in adult mice. Moreover, we showed that the PERK pathway was activated in medulloblastomas in humans. These results suggest that PERK signaling promotes medulloblastoma tumorigenesis by attenuating apoptosis of premalignant GCPs during the course of malignant transformation. (Am J Pathol 2016, 186: 1939–1951; http://dx.doi.org/10.1016/j.ajpath.2016.03.004)

Pancreatic endoplasmic reticulum kinase (PERK) activation in response to endoplasmic reticulum (ER) stress promotes cell survival under stressful conditions through inhibition of global protein biosynthesis and induction of certain stress-induced cytoprotective genes by phosphorylating translation initiation factor 2α (eIF2α). Nevertheless, PERK activation also controls an apoptotic program to eliminate ER-stressed cells. Studies found that PERK activation induced by hypoxia and nutritional deficiency, hallmarks of the solid tumor microenvironment, influences tumor development by regulating tumor cell viability, tumor invasion, and angiogenesis. However, because of the double-edged sword nature of PERK signaling, the data about its role, promoting or inhibiting, in tumor development are contradictory. It is well documented that apoptosis serves as a natural barrier to malignant transformation. Cell transformation requires concomitant activation of unrestricted cell proliferation and suppression of apoptosis. Interestingly, recent reports suggest that PERK signaling participates in cell transformation by regulating apoptosis. A report found that PERK activation promotes Myc-dependent malignant transformation by attenuating cell apoptosis. In contrast, another report found that PERK activation suppresses malignant transformation by enhancing cell apoptosis.

Medulloblastoma is the most common solid malignancy of childhood. From gene expression profiling, medulloblastoma

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can be divided into four discrete molecular subgroups: sonic hedgehog (SHH) subgroup, WNT subgroup, subgroup 3, and subgroup 4. 17,18 Although multiple genetic mutations that drive sustained cell proliferation are documented in medulloblastoma, 17-19 the mechanisms that regulate cell apoptosis during malignant transformation remain unclear. Mice heterozygous for Patched1 (Ptc11+/−), a SHH receptor, recapitulate human Gorlin’s syndrome and are regarded as the best animal model to study tumorigenesis of the SHH subgroup of medulloblastomas. 20,21 Medulloblastoma in Ptc11+/− mice arises from granule cell precursors (GCPs) in the external granular layer (EGL) of the developing cerebellum and shows distinct steps of progression. 22-24 These mice have hyperplastic lesions that contain premalignant GCPs in the EGL as late as 6 weeks of age. Most hyperplastic lesions regress; however, a few hyperplastic lesions undergo malignant transformation and progress to medulloblastoma.

Mutations of Perk (also known as Eif2ak3) were observed in human medulloblastoma. A report found that 7 of 35 human medulloblastomas exhibit Perk mutations. 25 Our previous in vitro study found that PERK activation facilitates human medulloblastoma cell migration and invasion through induction of vascular endothelial growth factor A (VEGF-A). 26 The report also found that PERK inactivation suppresses human medulloblastoma cell migration and invasion. 26 Moreover, a study found that ER stress responsive genes are up-regulated in premalignant GCPs in young Ptc11+/− mice. 22 Thus, we sought to elucidate the impact of the PERK branch of the ER stress response on the development of medulloblastoma. In this study, we confirmed PERK activation in human medulloblastoma and reported that PERK activation facilitated medulloblastoma development in Ptc11+/− mice by attenuating premalignant GCP apoptosis. PERK haploinsufficiency reduced the incidence of medulloblastomas in Ptc11+/− mice, which was associated with increased apoptosis of premalignant GCPs in young mice. Surprisingly, PERK haploinsufficiency had a minimal effect on medulloblastoma cells in adult Ptc11+/− mice.

Materials and Methods

Generation of Mice

Ptc11+/− mice 20 on a mixed C57BL/6 × 129Sv background were purchased from The Jackson Laboratory (Bar Harbor, ME). Ptc11+/− mice were crossed with Perk heterozygous deficient (Perk+/−) mice 21 on the C57BL/6 background to obtain Ptc11+/−; Perk+/− mice and Ptc11+/−; Perk−/− mice. Genotypes were determined by PCR from the DNA extracted from tail tips. The following primers were used for PCR amplification: PJ1 (5’-ACAGAGAAAAAGATGCCGGG-3’) and PJ2 (5’-GTATCGACGTTGAAACGAC-3’) for Ptc11+/− mice genotyping; 1740S (5’-AACCAACCT-ATCTTCCTGTCGTGC-3’), PGK.255R (5’-GCTACCGGT-GGATGGAATGTTG-3’), and i.6AS (5’-CGGAGACAG-TACAGCGCAGATGA-3’) for Perk+/− mice genotyping.

Mice were monitored daily to detect medulloblastoma phenotypes, including ataxia, decreased movement, poor grooming, and doming of the skull, until the age of 8 months. All animal procedures were conducted in complete compliance with the NIH’s Guide for the Care and Use of Laboratory Animals 26 and were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Histology, Immunohistochemistry, and TUNEL Staining

Adult mice with medulloblastoma phenotypes and all 8-month-old asymptomatic mice received an intraperitoneal injection of 100 mg/kg bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) 2 hours before perfusion. Six-week-old asymptomatic mice received a BrdU injection 8 hours before perfusion. Anesthetized mice were perfused through the left cardiac ventricle with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Brains were bisected in the sagittal plane, and one-half was postfixed for at least 48 hours in 4% paraformaldehyde in phosphate-buffered saline, dehydrated through graded alcohols, and embedded in paraffin. Serial sections of 5-μm thickness were cut, and every 10th section in the series was routinely stained with hematoxylin and eosin (H&E). The other half of the brain was postfixed for 1 hour in 4% paraformaldehyde in phosphate-buffered saline, cryopreserved in 30% sucrose for 48 hours, embedded in OCT compound, and frozen on dry ice. Frozen sections were cut in a cryostat at 10-μm thickness. Immunohistochemistry (IHC) for NeuN (dilution 1: 200; Millipore, Temecula, CA), proliferating cell nuclear antigen (PCNA; dilution 1:50,000; Sigma-Aldrich), phosphorylated PERK (p-PERK; dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), glut fibrillar acidic protein (dilution 1:1000; Covance, Princeton, NJ), synaptophysin (dilution 1:200; Millipore), VEGF-A (dilution 1:50; Santa Cruz Biotechnology), active-caspase 3 (dilution 1:200; Cell Signaling Technology, Danvers, MA), p53 (dilution 1:100; Santa Cruz Biotechnology), and p53 up-regulated modulator of apoptosis (PUMA; dilution 1:100; Santa Cruz Biotechnology) were performed on paraffin sections. IHC for CD31 (dilution 1:50; Santa Cruz Biotechnology) was performed on cryosections. All primary antibodies, with the exception of p53 and PUMA antibodies, were detected with the Vectastain ABC kits (Vector Laboratories, Burlingame, CA) and 3’5’-diaminobenzidine/H2O2 reagent (Vector Laboratories) as substrate. p53 and PUMA antibodies were detected with the fluorescein-labeled secondary antibody (Vector Laboratories). Fluorescent-stained sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories) and visualized with the Zeiss Axioskop 2 microscope (Carl Zeiss GmbH, Jena, Germany).

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed on paraffin sections with the use of the ApopTag kit (Millipore) according to the manufacturer’s instructions.
Western Blot Analysis

Cerebellar tissues were removed from adult control wild-type (WT) mice and adult symptomatic Pch1<sup>+/−</sup>; Perk<sup>+/−</sup> mice and Pch1<sup>+/−</sup>; Perk<sup>−/−</sup> mice, which displayed typical medulloblastoma clinical phenotypes and enlarged cerebellum. These tissues were homogenized in 5 volumes of Triton X-100 buffer with the use of a motorized homogenizer as previously described. Upon incubation on ice for 15 minutes, the extracts were cleared by centrifugation at 18,000 × g for 30 minutes twice. The protein concentration of each extract was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). The extracts (120 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were then incubated with primary antibodies to p-PERK (dilution 1:1000; Santa Cruz Biotechnology), eIF2α (dilution 1:1000; Cell Signaling Technology), p-eIF2α (dilution 1:1000; Santa Cruz Biotechnology), and actin (dilution 1:5000; Sigma-Aldrich), followed by the horseradish peroxidase-conjugated secondary antibody (Vector Laboratories). The chemiluminescent signal was detected by the ECL Detection Reagents (GE Healthcare Biosciences, Piscataway, NJ). The intensity of the recorded chemiluminescent signal was quantified with the ImageQuantTL LAS4000 software version 1.2 from GE Healthcare Life Sciences.

TaqMan Real-Time PCR

Cerebellar tissues were removed from adult control WT mice and adult symptomatic Pch1<sup>+/−</sup>; Perk<sup>+/−</sup> mice and Pch1<sup>+/−</sup>; Perk<sup>−/−</sup> mice, which displayed typical medulloblastoma clinical phenotypes and enlarged cerebellum. RNA was isolated from the cerebellum with the use of TRIzol reagent (Invitrogen, Carlsbad, CA) and was treated with DNase I (Invitrogen) to eliminate genomic DNA. Reverse transcription was performed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) on the LightCycler 480 System (Roche Diagnostics Corporation, Indianapolis, IN) as described previously.

Human Medulloblastoma Samples and IHC

Paraffin-embedded human medulloblastoma sections (5-μm thickness) and brain sections from individuals without brain tumor were provided by Brain Tumor Tissue Bank (London, ON, Canada), including six cases of pediatric medulloblastoma, three cases of adult medulloblastoma, and three nontumor brain tissue samples. IHC for p-PERK and CAATT enhancer binding protein homologous protein (CHOP; dilution 1:100; Thermo Scientific, Grand Island, NY) was performed as described in Histology, Immunohistochemistry, and TUNEL Staining. The degree of positive staining for p-PERK was determined by using a semiquantitative staining index = intensity × distribution. The intensity of cytoplasmic staining detected by IHC was scored on a scale of 0 to 4: 0 = negative staining, 1 = weak staining, 2 = modest staining, 3 = moderate staining, and 4 = strong staining. The distribution was scored on a scale of 1 to 4 from the percentage of cells with any level of staining: 1 = 1% to 25% positive cells, 2 = 26% to 50% positive cells, 3 = 51% to 75% positive cells, and 4 = 76% to 100% positive cells.

Statistical Analysis

Data are expressed as means ± SD. Comparisons between two groups were statistically evaluated by Student’s t-test with the use of GraphPad Prism software version 6 (GraphPad Software Inc., La Jolla, CA). The incidence of symptomatic medulloblastoma between Pch1<sup>+/−</sup>; Perk<sup>+/−</sup> mice and Pch1<sup>+/−</sup>; Perk<sup>−/−</sup> mice was statistically evaluated by Kaplan-Meier analysis and the incidence of total medulloblastoma between these two groups was statistically evaluated by χ² test with the use of Prism 6 (GraphPad). P < 0.05 was considered significant.

Results

PERK Activation in Human Medulloblastoma

During ER stress, PERK is activated through oligomerization and autophosphorylation, which coordinates an adaptive program by phosphorylating eIF2α at serine 51. To assess the potential relevance of previous observations of the enhanced expression of ER stress responsive genes in mouse models of medulloblastoma, we estimated the level of PERK activity in human medulloblastoma by p-PERK IHC. As expected, immunoreactivity of p-PERK was undetectable in nontumor brain tissues (Figure 1A). Interestingly, a number of tumor cells were positive for p-PERK in clinical samples of pediatric medulloblastoma (Figure 1B, Table 1), and the p-PERK IHC signal was also markedly increased in the tumor cells in adult medulloblastoma (Figure 1C, Table 1). Moreover, semiquantitative analysis confirmed that PERK was activated in medulloblastoma in all six cases of pediatric patients and three cases of adult patients (Table 1). However, the intensity and distribution of p-PERK staining in human medulloblastoma were variable, which likely reflects the heterogeneity of human tumor. In addition, IHC for CHOP, a PERK-responsive gene, found that immunoreactivity of CHOP was undetectable in nontumor brain tissues (Figure 1D), but it became detectable in a number of tumor cells in both pediatric and adult medulloblastomas (Figure 1E and F). Taken together, our data suggest that PERK signaling is enhanced in human medulloblastoma, prompting an enquiry into its potential pathophysiologic significance.
PERK Activation in Medulloblastoma Cells in Adult Ptch1+/− Mice and in Premalignant GCPs in Young Mice

Medulloblastomas in Ptch1+/− mice arises from the cerebellar GCPs in the EGL. During early postnatal development, GCPs proliferate, differentiate, and migrate to their final destination, the internal granule layer. In normal mice, by postnatal day 21, this process is complete, and there are no GCPs in the EGL. In contrast, Ptch1+/− mice have abnormal patches of premalignant GCPs in the EGL of the cerebellum at the age of 3 weeks. By the age of 6 weeks, these mice have focal hyperplastic lesions or diffuse hyperplastic lesions that contain premalignant GCPs in the cerebellum. These hyperplastic lesions either undergo malignant transformation and progress to medulloblastoma or regress over time. H&E staining revealed typical medulloblastoma clinical phenotypes (Figure 2B) and typical hyperplastic lesions in the EGL in the cerebellum of 6-week-old Ptch1+/− mice (Figure 2D) compared with the normal cerebellum of age-matched WT mice (Figure 2, A and C).

| Medulloblastoma sample | Patient age | Patient sex | Staining intensity | Staining distribution |
|-----------------------|-------------|-------------|--------------------|-----------------------|
| Pediatric 1           | 11 years    | Female      | 2                  | 2                     |
| Pediatric 2           | 8 years     | Male        | 2                  | 1                     |
| Pediatric 3           | 3 years     | Female      | 1                  | 1                     |
| Pediatric 4           | 12 years    | Male        | 1                  | 1                     |
| Pediatric 5           | 6 years     | Male        | 3                  | 3                     |
| Pediatric 6           | 7 months    | Male        | 1                  | 1                     |
| Adult 1               | 32 years    | Male        | 4                  | 4                     |
| Adult 2               | 30 years    | Male        | 3                  | 4                     |
| Adult 3               | 39 years    | Female      | 2                  | 2                     |

Table 1: Semiquantitative Analysis of p-PERK IHC

IHC, immunohistochemistry; p-PERK, phosphorylated pancreatic endoplasmic reticulum kinase.

To investigate the role of PERK signaling in medulloblastoma tumorigenesis, we determined the activity of PERK signaling in medulloblastoma cells and premalignant GCPs in Ptch1+/− mice. Similar to our finding in human medulloblastoma samples (see Perk Activation in Human Medulloblastoma), p-PERK IHC found that the immunoreactivity of p-PERK was undetectable in nontumor brain tissues. A representative image from pediatric medulloblastoma 5 in Table 1 shows that a number of tumor cells are positive for p-PERK in pediatric medulloblastoma. A representative image from adult medulloblastoma 1 in Table 1 shows that the level of p-PERK is markedly increased in the tumor cells in adult medulloblastoma. Immunoreactivity of CHOP was undetectable in nontumor brain tissues. Many tumor cells were positive for CHOP in pediatric medulloblastoma. Many tumor cells were positive for CHOP in adult medulloblastoma. n = 3 patients (A, C, D, F); n = 6 patients (B and E). Scale bar = 50 μm. CHOP, CAATT enhancer binding protein homologous protein; IHC, immunohistochemistry; p-, phosphorylated; PERK, pancreatic endoplasmic reticulum kinase.

PERK Haploinsufficiency Reduces the Incidence of Medulloblastomas in Ptch1+/− Mice

Ptch1+/− mice develop symptomatic medulloblastomas typically between the ages of 8 and 35 weeks.21,38 Perk
Figure 2  Activation of PERK signaling in both hyperplastic lesions and medulloblastomas in the cerebellum of $\text{Ptch1}^{+/+}/\text{C0}$ mice. A–D: H&E staining revealed a normal cerebellum in adult WT mice (A), a medulloblastoma in the cerebellum of adult $\text{Ptch1}^{+/+}/\text{C0}$ mice (B), a normal cerebellum in 6-week-old WT mice (C), and a hyperplastic lesion (arrow) in the cerebellar EGL of 6-week-old $\text{Ptch1}^{+/+}/\text{C0}$ mice (D). E and F: p-PERK IHC found that p-PERK is undetectable in cells in the cerebellum of adult WT mice but became detectable in a number of cells in medulloblastomas in adult $\text{Ptch1}^{+/+}/\text{C0}$ mice. G and H: p-PERK IHC found that p-PERK was undetectable in cells in the cerebellum of 6-week-old WT mice but became detectable in a number of cells in hyperplastic lesions in the cerebellar EGL of 6-week-old $\text{Ptch1}^{+/+}/\text{C0}$ mice. I and J: Western blot analysis found significantly increased levels of p-PERK and p-eIF2α in the cerebellum of adult $\text{Ptch1}^{+/+}/\text{C0}$ mice with medulloblastomas compared with adult WT mice. K: Real-time PCR analysis found that the mRNA level of PERK in the cerebellum of adult $\text{Ptch1}^{+/+}/\text{C0}$ mice with medulloblastomas was comparable with adult WT mice and that the mRNA levels of CHOP and GADD34 were significantly increased in the cerebellum of adult $\text{Ptch1}^{+/+}/\text{C0}$ mice with medulloblastomas compared with adult WT mice. Data are expressed as means ± SD, $n = 3$ animals. *$P < 0.05$. Scale bars: 1000 μm (A–D); 100 μm (E–H). CHOP, CAATT enhancer binding protein homologous protein; EGL, external granular layer; eIF2α, translation initiation factor 2α; GADD34, growth arrest and DNA damage 34; H&E, hematoxylin and eosin; IHC, immunohistochemistry; p-, phosphorylated; PERK, pancreatic endoplasmic reticulum kinase; WT, wild-type.
homzygous-deficient mice die prematurely because of progressive diabetes and exocrine pancreatic deficiency.27 Perk heterozygous-deficient (Perk+/−) mice appear healthy but display evidence of haploinsufficiency.32,35,36 Importantly, it was found that Perk+/− mice do not display any abnormalities in the central nervous system under normal conditions.39–41 Therefore, we used Perk+/− mice to determine the impact of PERK signaling on medulloblastoma tumorigenesis in Ptch1+/− mice. Ptch1+/− mice were crossed with Perk+/− mice to obtain Ptch1+/−; Perk+/− mice and Ptch1+/−; Perk+/− mice. These mice were monitored daily up to 8 months to detect medulloblastoma phenotypes, including ataxia, decreased movement, poor grooming, and doming of the skull. Medulloblastoma formation in symptomatic mice was verified by gross examination, namely assessing enlargement of the cerebellum, and by H&E staining. We found that the frequency of symptomatic medulloblastomas in Ptch1+/−; Perk+/− mice was significantly reduced compared with Ptch1+/−; Perk+/− mice (8 of 57 Ptch1+/−; Perk+/− mice versus 17 of 57 Ptch1+/−; Perk+/− mice; P = 0.0375) (Figure 3). Moreover, necropsy was performed on all 8-month-old asymptomatic Ptch1+/−; Perk+/− and Ptch1+/−; Perk+/− mice. Gross examination and H&E staining revealed that 3 of 49 asymptomatic Ptch1+/−; Perk+/− mice and 3 of 40 asymptomatic Ptch1+/−; Perk+/− mice had medulloblastomas, respectively. Consistent with the previous studies,22,24 we did not find any hyperplastic lesions in the cerebellar EGL of 8-month-old Ptch1+/−; Perk+/− mice or Ptch1+/−; Perk+/− mice. Thus, PERK heterozygous deficiency significantly decreased the incidence of medulloblastomas in Ptch1+/−; Perk+/− mice (11 of 57 Ptch1+/−; Perk+/− mice versus 20 of 57 Ptch1+/−; Perk+/− mice; P = 0.0457).

We further determined whether PERK heterozygous deficiency impaired the activity of PERK signaling in medulloblastoma cells in Ptch1+/− mice. p-PERK IHC found that the immunoreactivity of p-PERK was noticeably decreased in medulloblastomas in Ptch1+/−; Perk+/− mice compared with Ptch1+/−; Perk+/− mice (Figure 4, C and D). Moreover, Western blot analysis found that the levels of p-PERK and p-eIF2α were significantly decreased in the cerebellum of Ptch1+/−; Perk+/− mice with medulloblastomas compared with Ptch1+/−; Perk+/− mice (Figure 4, E and F). As expected, real-time PCR analysis found that the level of PERK mRNA was reduced by approximately one-half in the cerebellum of Ptch1+/−; Perk+/− mice with medulloblastomas compared with Ptch1+/−; Perk+/− mice (Figure 4G). Moreover, real-time PCR analysis found that PERK heterozygous deficiency significantly reduced the expression of CHOP and GADD34 in the cerebellum of Ptch1+/−; Perk+/− mice with medulloblastomas (Figure 4G). Taken together, these results indicate that PERK haploinsufficiency reduces the incidence of medulloblastomas in Ptch1+/− mice, likely through reduced PERK-mediated eIF2α phosphorylation.

Next, we determined the effects of PERK haploinsufficiency on cell proliferation, cell apoptosis, and angiogenesis in medulloblastomas in Ptch1+/− mice. H&E staining indicated that the structural characteristics of medulloblastomas in Ptch1+/−; Perk+/− mice was comparable with Ptch1+/−; Perk+/− mice (Figure 4, A and B). IHC for glial fibrillary acidic protein and synaptophysin found that the expression pattern of both glial fibrillary acidic protein and synaptophysin in medulloblastomas in Ptch1+/−; Perk+/− mice was comparable with Ptch1+/−; Perk+/− mice (Figure 5, A–D). BrdU labeling (Figure 5, E, F, and M) and PCNA IHC (data not shown) found that PERK heterozygous deficiency did not significantly change the number of proliferating cells in medulloblastomas in Ptch1+/− mice. Moreover, TUNEL staining found that PERK heterozygous deficiency had no significant effect on cell apoptosis in medulloblastomas in Ptch1+/− mice (Figure 5, G, H, and M). A number of studies suggest that activation of PERK signaling enhances angiogenesis in tumors, including medulloblastoma, by induction of VEGF-A.29,42 Surprisingly, VEGF-A IHC found that PERK heterozygous deficiency did not significantly alter VEGF-A expression in medulloblastomas in Ptch1+/− mice (Figure 5, I, J, and M), and CD31 IHC found that PERK heterozygous deficiency did not significantly change angiogenesis in this tumor (Figure 5, K, L, and N). Collectively, these data suggest that PERK haploinsufficiency has a minimal effect on cell proliferation, cell apoptosis, and angiogenesis in medulloblastomas in adult Ptch1+/− mice. Thus, it is unlikely that PERK haploinsufficiency impairs medulloblastoma formation in Ptch1+/− mice through its effects on tumor cells.

PERK Haploinsufficiency Increases Premalignant GCPs

Apoptosis in the Cerebellum of Young Ptch1+/− Mice

Evidence is emerging that PERK signaling influences tumorigenesis by regulating cell apoptosis during the course
of malignant transformation.\textsuperscript{1,3,15,43} Medulloblastoma tumorigenesis in the cerebellum of \textit{Ptch1}\textsuperscript{+/−} mice found distinct steps of progression; first, focal hyperplastic lesions develop in the EGL; second, diffuse hyperplastic lesions form in the EGL and molecular layer; finally, medulloblastoma arises.\textsuperscript{3,24,37} Previous reports found that both focal hyperplastic lesions and diffuse hyperplastic lesions can be observed in the cerebellum of \textit{Ptch1}\textsuperscript{+/−} mice as late as 6 weeks of age.\textsuperscript{22,24} The diffuse hyperplastic lesions are reversible and represent the final step in the process of malignant transformation, in which premalignant GCPs either gain additional genetic mutations or epigenetic changes to become irreversible tumor cells, or undergo regression.\textsuperscript{23,24,37} Therefore, we determined the effects of PERK haploinsufficiency on the proliferation and apoptosis of premalignant GCPs in diffuse hyperplastic lesions in the cerebellum of 6-week-old \textit{Ptch1}\textsuperscript{+/−} mice.

We serially sectioned whole paraffin-embedded half-cerebellum of 6-week-old mice, each half-cerebellum yielded approximately 210 sections 5-μm thick. Every 10th section in the series was either stained by H&E or immunostained by the NeuN antibody or PCNA antibody. Focal hyperplastic lesions, which contained >10 premalignant GCPs that were negative for NeuN (a marker of mature neurons) and positive for PCNA, were observed in the cerebellum of \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/+} mice (Supplemental Figure S1, A, C, and E) and \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{−/−} mice (Supplemental Figure S1, B, D, and F). Moreover, both \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} and \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{−/−} mice had diffuse hyperplastic lesions in the cerebellum (Supplemental Figure S1, G–L), which contained a thick layer (>15 cell layers) of NeuN-negative and PCNA-positive premalignant GCPs diffusing in the EGL and molecular layer. We found, however, that the number of total hyperplastic lesions in the cerebellum of 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{−/−} mice was comparable with \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/+} mice (Figure 6A). Similarly, we found that PERK heterozygous deficiency did not reduce the number of diffuse hyperplastic lesions in the cerebellum of 6-week-old \textit{Ptch1}\textsuperscript{+/−} mice (Figure 6B and Figure 7, A and B).

p-PERK IHC found that immunoreactivity of p-PERK was noticeably decreased in diffuse hyperplastic lesions in the cerebellum of \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{−/−} mice (Figure 6B and Figure 7, A and B).
6-week-old $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice compared with $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice (Figure 7, C and D). BrdU labeling (Figure 7, E, F, and K) and PCNA IHC (data not shown) found that PERK heterozygous deficiency did not significantly alter the rate of cell proliferation in diffuse hyperplastic lesions in 6-week-old $\text{Ptch1}^{+/+}$ mice. Importantly, TUNEL labeling found that PERK heterozygous deficiency significantly increased the number of apoptotic cells in diffuse hyperplastic lesions in 6-week-old $\text{Ptch1}^{+/+}$ mice (Figure 7, G, H, and L). Active-caspase 3 IHC also found that the number of apoptotic cells were significantly increased in diffuse hyperplastic lesions in 6-week-old $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice compared with $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice (Figure 7, I, J, and M). However, there were noticeably fewer active-caspase 3-positive cells than TUNEL-positive cells in corresponding lesions. This discrepant result likely reflects that TUNEL labeling is a more sensitive method to detect cell apoptosis in diffuse hyperplastic lesions than active-caspase 3 IHC. Collectively, these data suggest that PERK haploinsufficiency enhances premalignant GCP apoptosis in diffuse hyperplastic lesions in the cerebellum of $\text{Ptch1}^{+/+}$ mice during

**Figure 5**  PERK heterozygous deficiency did not affect cell proliferation, cell apoptosis, or angiogenesis in medulloblastomas in adult $\text{Ptch1}^{+/+}$ mice. A and B: GFAP IHC found that PERK heterozygous deficiency did not change the expression pattern of GFAP in medulloblastomas in adult $\text{Ptch1}^{+/+}$ mice. C and D: Synaptophysin IHC found that PERK heterozygous deficiency did not change the expression pattern of synaptophysin in medulloblastomas in adult $\text{Ptch1}^{+/+}$ mice. E, F, and M: BrdU labeling revealed comparable number of proliferating cells in medulloblastomas in adult $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice and $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice. G, H, and M: TUNEL staining revealed comparable number of apoptotic cells in medulloblastomas in adult $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice and $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice. I, J, and M: VEGF-A IHC revealed comparable number of VEGF-A-positive cells in medulloblastomas in adult $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice and $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice. K, L, and N: CD31 IHC revealed comparable number of blood vessels in medulloblastomas in adult $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice and $\text{Ptch1}^{+/+}$/Perk$^{+/+}$ mice. Data are expressed as means ± SD, $n = 4$ animals. Scale bars: 50 μm (A–J); 100 μm (K and L). BrdU, bromodeoxyuridine; CD31, cluster of differentiation 31; GFAP, glial fibrillary acidic protein; IHC, immunohistochemistry; PERK, pancreatic endoplasmic reticulum kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling; VEGF-A, vascular endothelial growth factor A.
malignant transformation of medulloblastoma. As described in PERK Haploinsufficiency Reduces the Incidence of Medulloblastomas in Ptch1+/− Mice, we found that PERK haploinsufficiency reduced the incidence of medulloblastoma but had a minimal effect on tumor cells in adult Ptch1+/− mice after malignant transformation. Taken together, our data indicate that PERK haploinsufficiency suppresses medulloblastoma development in Ptch1+/− mice by enhancing premalignant GCP apoptosis in diffuse hyperplastic lesions during the course of malignant transformation.

It was found that PERK activation attenuates the activity of the p53 pathway, the master regulator of apoptosis during malignant transformation. A previous report found that p53 deficiency increases the incidence and accelerates the development of medulloblastomas in Ptch1+/− mice. Therefore, we were interested in the potential role of p53 in the proapoptotic effects of PERK haploinsufficiency on premalignant GCPs. p53 Immunostaining found immunoreactivity of p53 was noticeably increased in diffuse hyperplastic lesions in 6-week-old Ptch1+/−; Perk+/− mice compared with Ptch1+/−; Perk+/+ mice (Figure 7, N and O). PUMA, a Bcl-2 homology 3-only Bcl-2 family member, is a p53-responsive gene and a critical mediator of p53-induced apoptosis. Interestingly, PUMA immunostaining found immunoreactivity of PUMA was markedly elevated in diffuse hyperplastic lesions and diffuse hyperplastic lesions in the cerebellum of young Ptch1+/− mice, in medulloblastoma cells in adult Ptch1+/− mice, and in medulloblastoma cells in human patients. We demonstrated that PERK haploinsufficiency had a minimal effect on medulloblastoma cells in adult Ptch1+/− mice after malignant transformation but significantly enhanced premalignant GCP apoptosis in diffuse hyperplastic lesions in young Ptch1+/− mice during malignant transformation.

Apoptosis is believed to serve as a natural barrier to malignancy. The transforming effects of oncogenes, which drive unrestrained cell proliferation, are countered by intrinsic apoptosis mechanisms, which are triggered in response to various physiologic stresses that cells experience during the course of malignant transformation. Recent studies report that ER stress is activated in cells during malignant transformation and that PERK activation in response to ER stress either facilitates the transformation by suppressing apoptosis or impairs the transformation by promoting apoptosis. In this study, we showed that PERK signaling was activated in premalignant GCPs in focal hyperplastic lesions and diffuse hyperplastic lesions in the cerebellum of young Ptch1+/− mice, in medulloblastoma cells in adult Ptch1+/− mice, and in medulloblastoma cells in human patients. Our data suggest that PERK signaling promotes medulloblastoma tumorigenesis by circumventing apoptosis during the transformation of GCPs. It is generally believed that activation of PERK signaling preserves cell viability under stressful conditions through inhibition of global protein biosynthesis and induction of certain stress-induced cytoprotective genes. Several studies report that PERK activation attenuates the activity of the p53 pathway, the master regulator of apoptosis during malignant transformation. Loss-of-function mutations of p53 contribute

![Figure 6](ajp.amjpathol.org)

**Figure 6** PERK heterozygous deficiency did not reduce the number of the cerebellar hyperplastic lesions in young Ptch1+/− mice. A: The scatter plot shows that PERK heterozygous deficiency did not significantly change the number of total hyperplastic lesions in the cerebellum of 6-week-old Ptch1+/− mice. B: The scatter plot shows that PERK heterozygous deficiency did not reduce the number of diffuse hyperplastic lesions in the cerebellum of 6-week-old Ptch1+/− mice. Data are expressed as means ± SD. n = 10 mice. PERK, pancreatic endoplasmic reticulum kinase.
significantly to the tumorigenesis of many tumor types, including medulloblastoma.\textsuperscript{11,12} Approximately 10% of human medulloblastomas have mutations in p53.\textsuperscript{17,52} Moreover, p53 deficiency dramatically increases the incidence and accelerates the development of medulloblastomas in \textit{Ptch1}\textsuperscript{+/−} mice.\textsuperscript{47} Interestingly, we showed here that the increased premalignant GCP apoptosis in diffuse hyperplastic lesions of \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice was accompanied with the

Figure 7  PERK heterozygous deficiency enhanced apoptosis of premalignant GCPs in diffuse hyperplastic lesions in the cerebellum of young \textit{Ptch1}\textsuperscript{+/−} mice. A: H&E staining revealed a diffuse hyperplastic lesion (arrow) in the cerebellum of 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice. B: H&E staining revealed a diffuse hyperplastic lesion (arrow) in the cerebellum of 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice. C and D: p-PERK IHC found noticeably decreased immunoreactivity of p-PERK in diffuse hyperplastic lesions in 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice compared with \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/+} mice. E, F, and K: BrdU labeling revealed comparable number of proliferating cells in diffuse hyperplastic lesions in 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice and \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/+} mice. G, H, and L: TUNEL staining revealed significantly increased number of apoptotic cells in diffuse hyperplastic lesions in 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice compared with \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/+} mice. I, J, and M: Active-caspase 3 IHC revealed significantly increased number of apoptotic cells in diffuse hyperplastic lesions in 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice compared with \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/+} mice. N and O: p53 Immunostaining revealed noticeably increased expression of p53 in diffuse hyperplastic lesions in 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice compared with \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/+} mice. P and Q: PUMA immunostaining revealed noticeably increased expression of PUMA in diffuse hyperplastic lesions in 6-week-old \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/−} mice compared with \textit{Ptch1}\textsuperscript{+/−}; \textit{Perk}\textsuperscript{+/+} mice. Data are expressed as means ± SD. *P < 0.05.

Scale bars: 1000 μm (A and B); 100 μm (C and D); 50 μm (E–J); 32 μm (N–Q). BrdU, bromodeoxyuridine; GCP, granule cell precursor; H&E, hematoxylin and eosin; IHC, immunohistochemistry; p-, phosphorylated; PERK, pancreatic endoplasmic reticulum kinase; PUMA, p53 up-regulated modulator of apoptosis; TUNEL, terminal deoxynucleotidyl transferase–mediated biotinylated UTP nick end labeling.
enhanced expression of p53 and PUMA. Therefore, there is a possibility that PERK signaling promotes medulloblastoma tumorigenesis through attenuation of premalignant GCP apoptosis by inhibiting the activity of the p53-PUMA pathway.

The PERK-eIF2α pathway is a major regulator of cell viability during ER stress under various physiologic and pathologic conditions. As expected, we found that PERK heterozygous deficiency significantly attenuated the activity of the PERK-eIF2α pathway in medulloblastoma cells in adult Ptch1+/− mice. Surprisingly, TUNEL labeling found that PERK heterozygous deficiency had no significant effect on cell apoptosis in the tumor. Moreover, BrdU labeling and PCNA immunostaining found that PERK heterozygous deficiency did not alter cell proliferation in the tumor. Multiple cooperative mutations of oncogenes and oncosuppressor genes are necessary for tumorigenesis. It is generally believed that additional mutations of oncogenes or oncosuppressor genes, besides Ptch1 heterozygous mutation, are required for medulloblastoma tumorigenesis in Ptch1+/− mice. The diminished impact of PERK haploinsufficiency on medulloblastoma cell viability, compared with its proapoptotic effect on premalignant GCPs in diffuse hyperplastic lesions, suggests that the tumor cells in Ptch1+/−; Perk−/+ mice gain additional mutations or epigenetic changes that compensate or overcome the deficiency of the PERK-eIF2α pathway.

Data from in vitro studies and xenograft models suggest that PERK activation enhances tumor angiogenesis by inducing VEGF-A expression. In vitro studies from our laboratory and other groups found that PERK activation enhances VEGF-A expression in human medulloblastoma cells. Our previous study also suggests that inactivation of GADD34, a regulatory subunit of a phosphatase complex that dephosphorylates eIF2α, enhances the activity of the PERK-eIF2α pathway and may facilitate medulloblastoma formation in the cerebellum of transgenic mice that express the immune cytokine interferon-γ in the central nervous system by promoting angiogenesis through induction of VEGF-A. Moreover, a report indicates that PERK deficiency attenuates tumor cell proliferation and angiogenesis in β-cell insulinomas in transgenic mice that express the SV40 large T-antigen specifically in β cells. In contrast, another report indicates that PERK deficiency delays tumor development but has no important effect on angiogenesis in mammary adenocarcinoma in transgenic mice that express oncogene Neu specifically in mammary epithelium. We showed here that PERK haploinsufficiency reduced the incidence of medulloblastomas in Ptch1+/− mice but did not affect VEGF-A expression or angiogenesis in the tumor. Collectively, these data likely suggest that the promoting effects of PERK signaling on VEGF-A expression and/or tumor angiogenesis are not ubiquitous, possibly determined by genetic mutations or epigenetic changes in individual molecular subtypes of tumors.

Conclusions

With the use of Ptch1+/− mice, the results presented in this study suggest that PERK activation contributes to medulloblastoma development through inhibition of premalignant GCP apoptosis during the course of malignant transformation. This work represents the first experimental demonstration of a link between PERK activation and malignant transformation in an animal model that faithfully resembles human tumor. Moreover, we demonstrated PERK activation in human medulloblastoma. Unfortunately, human samples do not afford an opportunity to study the early stages of tumor development, but the persistence of PERK signaling is also consistent with a pervasive role in medulloblastoma tumorigenesis.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2016.03.004.

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