PARP-1 cooperates with Ptc1 to suppress medulloblastoma and basal cell carcinoma

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The patched (Ptc1) protein is a negative regulator of sonic hedgehog signaling, a genetic pathway whose perturbation causes developmental defects and predisposition to specific malignant tumors. Humans and mice with mutated Ptc1 are prone to medulloblastoma and basal cell carcinoma (BCC), both tumors showing dependence on radiation damage for rapid onset and high penetrance. Poly(ADP-ribose) polymerase (PARP-1) is a nuclear enzyme that plays a multifunctional role in DNA damage signaling and repair. In healthy and fertile PARP-1-null mice, radiation exposure reveals an extreme sensitivity and a high genomic instability. To test for interactions between PARP-1 and sonic hedgehog signaling, PARP-1-null mice were crossed to Ptc1 heterozygous mice. PARP-1 deletion further accelerated medulloblastoma development in irradiated Ptc1−/+ mice, showing that PARP-1 inactivation sensitizes cerebellar cells to radiation tumorigenic effects. In addition to increased formation and slowed-down kinetics of disappearance of γ-H2AX foci, we observed increased apoptosis in PARP-1-deficient granule cell progenitors after irradiation. Double-mutant mice were also strikingly more susceptible to BCC, with >50% of animals developing multiple, large, infiltrative tumors within 30 weeks of age. The results provide genetic evidence that PARP-1 function suppresses sonic hedgehog pathway-associated tumors arising in response to environmental stress.

Introduction

Poly(ADP-ribose) polymerase (PARP-1) is an abundant nuclear protein component of the base excision repair complex required for DNA single-strand break (SSB) repair (1–3). PARP-1 not only facilitates repair of SSBs but also binds to more lethal radiation-induced double-strand breaks (DSBs), apparently protecting the lesions and signaling repair (4). PARP-1 activates several proteins involved in the homologous recombination and the non-homologous end-joining repair pathways (5–10).

A number of PARP-1 knockout mouse models were generated by different groups (11–13). Despite its important role in cellular response to genotoxic stress, PARP-1 is not required for viability, and mice lacking functional PARP-1 develop normally and are not predisposed to early-onset tumors. PARP-1-null mice, however, show hypersensitivity to ionizing radiation and alkylating agents, and PARP-1-null cells exhibit chromosomal instability, shown by increased frequency of spontaneous sister chromatid exchange and DNA damage-induced micronucleus formation (12,14,15).

Growing evidence shows that PARP-1 activation plays an important role in the pathogenesis of several human diseases, such as stroke, myocardial infarction, circulatory shock, diabetes and neurodegenerative disorders, including Parkinson and Alzheimer (16–18). There is also evidence to implicate the role of PARP-1 activation in inflammatory disorders, such as arthritis, allergy, colitis and others (19). Thus, PARP-1 has been gaining increasing interest as a therapeutic target as PARP-1 inhibitors have been shown to be greatly effective in experimental models for some of these conditions. Several classes of PARP-1 inhibitors have been developed, and some of these are moving toward clinical development, or have already entered clinical trials for treatment of acute cardiac ischemia, or as chemotherapysensitizing agents (20).

On the other hand, several studies indicating important protective functions of PARP-1 in genome surveillance and DNA repair raise concerns about the clinical use of PARP-1 inhibitors (21). Important issues are the potential side effects of long-term treatment and whether PARP-1 inhibition may increase the risk of mutagenesis or oncogenesis. While the therapeutic effects of PARP-1 inhibitors may exceed their potential risk for acute life-threatening diseases, development of PARP-1 inhibitors for inflammatory or neurodegenerative conditions may be more challenging because of the unknown potential long-term side effects of PARP inhibition.

Patch (Ptc1) heterozygous knockout mice (22,23), the mouse model for Gorlin syndrome, are cancer prone and hypersensitive to DNA damage caused by ionizing radiation that accelerates development of brain and skin malignancies, i.e. medulloblastoma and basal cell carcinoma (BCC) (24–27). Development of both tumor types occurs through well-defined preneoplastic stages whose switch to malignancy could be driven by loss of the normal remaining Ptc1 allele (28,29). This suggests that processing of radiation-induced DNA damage is crucial for tumorigenesis in the Ptc1−/+ model.

To investigate whether PARP-1 is an important genetic factor in tumor susceptibility, mice with a deletion in exon 2 of the PARP-1 gene (11) were intercrossed to Ptc1−/+ mice, and the progeny was exposed to the damaging effects of radiation. Control and irradiated groups of Ptc1−/+ mice with two, one or no intact PARP-1 alleles were placed on a lifetime study for development of brain and skin tumors or other neoplasms.

Materials and methods

Animal breeding

Mice lacking one Ptc1 allele (Ptc1−/+; named Ptc1−/− throughout the text) generated through disruption of exons 6 and 7 in 129/Sv embryonic stem cells (22) and maintained on CD1 background were crossed to PARP-1−/− mice in a mixed (129/Sv × C57BL/6) background (11). F1 mice of the desired genotypes (PARP-1−/−/Ptc1−/+ and PARP-1−/−/Ptc1−/−) were intercrossed to produce a large F2 population. Genotyping for PARP-1 and Ptc1 loci was performed as described (22,30).

Animal treatment and irradiation

Animals were housed in the animal facility at ENEA CR-Casaccia (Rome, Italy) under conventional conditions with food and water available ad libitum and a 12 h light cycle. Mice were irradiated at post-natal day 1 (P1) with 3 Gy X-rays from a Gilarondo CHF 320G X-ray Generator (Gilarondi S.p.A., Mandello del Lario, Italy) operated at 250 kVp, 15 mA, with filters of 2.0 mm Al and 0.5 mm Cu (half-value layer = 1.6 mm Cu). To minimize mortality for medulloblastoma, two additional groups of PARP-1−/−/Ptc1−/+ and PARP-1−/−/Ptc1−/− mice were irradiated at P1 with 3 mm thick lead shields positioned to protect mouse head during irradiation. Experimental protocols were reviewed by the Institutional Animal Care and Use Committee.

Abbreviations: BCC, basal cell carcinoma; CNS, central nervous system; CGH, comparative genomic hybridization; DSB, double-strand break; EGL, external granule layer; GCP, granule cell progenitor; P1, post-natal day 1; PARP, polyadenosine diphosphate-ribose) polymerase; Ptc, patched; SSB, single-strand break.

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Histological analysis and tumor quantification

Mice were observed daily for their life span. Upon health decline (i.e. severe weight loss, paralysis, ruffling of fur and inactivity), they were killed and autopsied. Normally appearing and tumor-bearing brains and tumors from any other tissue were fixed in 4% buffered formalin. Grossly normal dorsal skin (3 cm²) was also fixed. Samples were processed for histological analysis using standard methods. Incidence of cytotoxic structural alterations in cerebellum was determined on histological sections of 5-week-old asymptomatic mice of PARP-1+/+/Ptc1+/+, PARP-1−/−/Ptc1+/+, PARP-1−/−/Ptc1+/+, and PARP-1−/−/Ptc1−/− genotypes irradiated at P1 or left unirradiated. The incidence of preneoplastic cerebellar lesions was determined in PARP-1+/+/Ptc1+/+ and PARP-1−/−/Ptc1−/− mice. Six sections, recovered with intervals of 70 µm to ensure representative sampling, were examined for each cerebellum. The incidence and multiplicity of microscopically BCC-like tumors were retrospectively determined in PARP-1+/+/Ptc1+/+ and PARP-1−/−/Ptc1−/− genotypes by analyzing dorsal skin samples from a subset (n = 8) of autopsied Ptc1 heterozygous mice as described (25). Tumor variables were expressed as the percentage of mice bearing one or more macroscopic tumor (incidence) and the mean number of macroscopic BCCs in the total mouse population (multiplicity).

Radiosensitivity analysis

Brains (three/time point) of PARP-1+/−/Ptc1+/+ and PARP-1+/−/Ptc1−/− mice irradiated with 3 Gy at P1 were collected 3 and 6 h post-irradiation and fixed in 4% buffered formalin. Serial sections of cerebellar tissues were cut at 4 µm thickness and stained with hematoxylin and eosin. Digital images from mid-sagittal cerebellar sections were collected by IAS image-processing software (Delta Sistemi, Rome, Italy). Cells showing signs of nuclear chromatin condensation and morphologically normal cells in the external granule layer (EGL) were counted using a double-blind method. Cell death was calculated as the percentage of pyknotic nuclei relative to the total number of cells. The total number of cells examined ranged from 1.5 × 10⁴ to 2.4 × 10⁴ per time point.

Immunohistochemistry analysis

Immunohistochemical analysis of rabbit polyclonal antibody against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) on brain samples was performed as described (27). Anti-ß-actin antibody was used to control protein loading. Antibodies included rabbit polyclonal antibody against p53 (Novocastra Laboratories, Newcastle, UK) and phospho-p53 (Cell Signaling Technology) and monoclonal antibody against ß-actin (Sigma-Aldrich, St Louis, MO). 1:1000 dilution.

Loss of heterozygosity and comparative genomic hybridization

Genomic DNA extracted from PARP-1+/−/Ptc1+/+ (n = 6), PARP-1−/−/Ptc1−/− (n = 5), PARP-1−/−/Ptc1−/− (n = 7) medulloblastomas and normal spleen DNA was fragmented by Ahd and RsaI restriction digest. After purification with QIAquick PCR Purification Kit (Qiagen, Milan, Italy), 500 ng of digested DNA was used for each labeling reaction. Random priming was performed by Bioprime labeling kit (Invitrogen, San Diego, CA) according to the manufacturer’s instructions with a modified dNTP: 100 µM each of dATP, dGTP, dCTP, and dTTP; 65 µM biotin-16-dUTP and 35 µM biotin-16-dUTP (tumor DNA) or digoxigenin-11-dUTP (normal mouse DNA). Labeled probes were ethanol precipitated with 50 µg of mouse Cot-1 DNA (Invitrogen) and hybridized on normal metaphases obtained from C57Bl/6 mouse embryonic fibroblasts. Biotin and digoxigenin probes were revealed by Avidin–fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA) and anti-digoxigenin–rhodamine antibodies (Roche Diagnostics Corp., Indianapolis, IN), respectively. After DAPI staining, three-color images, from at least 15 well-hybridized metaphases, were captured using IPLab Spectrum software (BD Biosciences, Rockville, MD) from a cooled CCD camera mounted on a Microphot-FXA microscope (Nikon France S.A.S., Champigny sur Marne, FR). Classification of G-banded chromosomes and the green-to-red fluorescence ratios along individual chromosomes were calculated by QUIPS-CGH software (Vysis, Downers Grove, IL).

Results

Hypersensitivity of PARP-1−/− cerebellum to radiation-induced DNA damage

To detect whether PARP-1 and Ptc1 mutation act in concert during development of the nervous system, we preliminarily examined specific defects in the cerebellum, such as altered development and architecture, in crosses between PARP-1 and Ptc1 mutants. Cerebellar alterations were assessed in individual PARP-1+/− or Ptc1−/− mice and in PARP-1/Ptc1 mutants. We found no alterations of cerebellar morphology in PARP-1-null mice of 5 weeks compared with age-matching PARP-1+/+ mice (Figure 1A–D). Next, we have compared susceptibility of PARP-1 genotypes to a single dose of radiation given in early post-natal age, when rapid proliferation of neural GCPs may
result in dramatic susceptibility to environmental exposures. We found a modest reduction in size of the cerebellum and disorganization of the internal granule layer in Ptc1+/− mice with intact PARP-1 (Figure 1E and F). Structural alterations were detected in 33.3% (4/12) of PARP-1+/−/Ptc1+/− and in 28.6% (2/7) of PARP-1+/−/Ptc1−/− mice (Figure 1I). In contrast, cerebellar development was severely impaired in irradiated PARP-1−/− mice (Figure 1G and H). Regardless of Ptc1 genotype, we observed a drastic enhancement in incidence and severity of cerebellum disorganization and major cerebellar abnormalities in 75% (6/8) of PARP-1−/− mice (Figure 1I). Alterations included dramatic decrease in thickness of the internal granule layer and disorganization of Purkinje cells, which instead of their normal monolayer arrangement were scattered within the cortex (Figure 1H). Thus, PARP-1 deficiency strongly influenced the degree of radiation-induced damage in the developing cerebellum and impaired cell repopulation and normal growth.

Early preneoplastic lesions in cerebellum

During post-natal cerebellar development, differentiating GCPs complete their migration from the external to the internal granule layer by 3 weeks of age. Persistence of ectopic EGL areas after this time is indicative of impaired migration or differentiation ability of GCPs, resulting in microlesions arising in unirradiated (C) or irradiated (D) PARP-1+/−/Ptc1+/− mice (Figure 2B). A major effect of PARP-1 genetic inactivation was the drastic increase to 71.4% (5/7) of PARP-1+/−/Ptc1+/− mice (Figure 2B). Consistently, no differences in expression of the cell proliferation marker Ki-67 were detected in lesions arising in unirradiated and irradiated PARP-1+/−/Ptc1+/− cerebella (Figure 2C and D).

Survival and Ptc1-associated tumorigenesis in crosses between PARP-1- and Ptc1-mutant mice

The observation that PARP-1 plays a crucial role in protection from the damaging effects of radiation in cerebellum tissue, and in suppression of the initial steps of medulloblastoma growth in Ptc1+/− mice, prompted us to examine whether PARP-1 germ line inactivation might also influence long-term survival and tumorigenesis. Ptc1+/− and Ptc1−/− mice on either wild-type or null PARP-1 background were analyzed to determine survival and spontaneous tumor rate. While PARP-1 abrogation did not modify substantially survival and tumorigenesis of Ptc1+/− mice (Figure 3A and D), we observed a trend toward increased mortality and tumorigenesis was observed in PARP-1−/−/Ptc1+/− mice (Figure 3B and D), although the differences were not statistically significant. The incidence of Ptc1-associated tumors in PARP-1/Ptc1 double mutants is shown in Figure 3D (bold figures). Ptc1+−− mice of both PARP-1 genotypes developed medulloblastomas. Of note, the high frequency of early neoplastic lesions associated with complete PARP-1 inactivation was not paralleled by increased medulloblastoma incidence in PARP-1−/−/Ptc1−/− mice (Figure 3C). This frequency was actually lower compared with PARP-1+/−/Ptc1+/− mice, although not significantly. The lack of correlation between early preneoplastic lesions and medulloblastoma development in PARP-1+/−/Ptc1+/− mice suggests that PARP-1 inactivation causes formation of hyperplastic areas lacking potential to progress to full malignancy.

Fig. 2. Lack of PARP-1 increases the frequency of preneoplastic lesions in the cerebellum of young Ptc1+/− mice. (A) Representative early cerebellar lesion in 5-week-old Ptc1+/− mouse. (B) Percentage incidence of microscopic lesions: a drastic increase was observed in unirradiated PARP-1+/−/Ptc1+/− mice compared with PARP-1+/−/Ptc1+/− mice (P < 0.05). Irradiation greatly increased the frequency of early lesions in PARP-1+/−/Ptc1+/− mice but did not produce further increase in PARP-1+/−/Ptc1+/− double mutants. (C and D) Immunohistochemical analysis showing no difference in expression of the cell proliferation marker Ki-67 in microlesions arising in unirradiated (C) or irradiated (D) PARP-1−/−/Ptc1+/− mice.
malignancy. Interestingly, despite increased genomic instability caused by PARP-1 inactivation, delayed tumor initiation was also observed in crosses between PARP-1+/− and tumor-prone p53−/− mice (32). Ptc1−/− mice of both PARP-1 genotypes developed soft-tissue sarcomas with higher incidence in PARP-1−/− mice, a trend that, however, was not statistically significant.

Ptc1−/− mice are highly radiation sensitive, with central nervous system (CNS) and skin representing preferential targets for tumor induction (24–26). To evaluate the effects of PARP-1 deficiency on tumor response to genotoxic damage, we next irradiated neonatal mice with a single X-ray dose. Ptc1−/− and Ptc1+/− mice of the three PARP-1 genotypes were monitored for survival and tumor development. While irradiation did not modify survival and tumorigenesis in Ptc1+/− mice, drastic life shortening was observed in Ptc1−/− mice irrespective of PARP-1 status (Figure 3A and B). Consistently, tumorigenesis was significantly increased (Figure 3D). As regards CNS tumorigenesis, PARP-1 genetic abrogation further increased medulloblastoma in irradiated Ptc1−/− mice (Figure 3C and D), with 100% (16/16) of PARP-1−/− mice dying of aggressive disease compared with 91% (38/42) of PARP-1+/− mice and 78% (14/18) of PARP-1+/+/ mice. Although these differences were not statistically significant due to limited size of the experimental groups, these data suggest a role for PARP-1 in protection from genotoxic damage leading to medulloblastoma. The histology of tumors revealed no morphological differences with respect to PARP-1 genotype, indicating that PARP-1 inactivation modulates tumor incidence but not disease pathogenesis in the Ptc1−/− model.

PARP-1 genetic abrogation sensitizes GCPs to DNA damage

GCPs are considered the cells of origin of medulloblastoma (33). At P1, proliferating GCPs are clustered over the surface of the developing cerebellum to form the EGL. Unlike post-mitotic neurons, GCPs are extremely sensitive to genotoxic injury (34). Since PARP-1 primary function is DNA damage detection, characterization of early response to genotoxic damage in GCPs can help to clarify the role of PARP-1 in tumor response of the developing CNS. One of the very early events after DNA DSBs is phosphorylation of histone H2AX on serine 139 to form γ-H2AX foci, detectable in the nucleus using specific antibodies (35). By immunohistochemistry, we determined the influence of PARP-1 deficiency on formation and elimination of γ-H2AX foci (Figure 4A–C). The background level of cells with detectable γ-H2AX foci was very low (0.0018%). Interestingly, PARP−1 deficiency caused a 10-fold increase in spontaneous levels of γ-H2AX (data not shown), suggesting a physiological role of PARP-1 in protecting the brain from damage derived from endogenous oxidative stress. In addition, our data show peak induction of γ-H2AX foci in GCPs from Ptc1−/− mice at 0.5 h after irradiation, and a gradual decrease at 3 and 6 h (Figure 4C). In GCPs from PARP-1-null mice (0.5 h), there is a 1.2-fold increase of γ-H2AX phosphorylation compared with PARP-1-proficient cells. Differences progressively increased at 3 (2.6-fold) and 6 h after irradiation (3.1-fold), indicating that PARP-1 deficiency slowed down kinetics of disappearance of γ-H2AX foci (Figure 4C). Differences in γ-H2AX phosphorylation between GCPs from PARP-1-deficient and

![Fig. 3. Survival and tumorigenesis in control and irradiated crosses between PARP-1- and Ptc1-mutant mice. (A) Tumor-free survival of unirradiated (PARP-1+/+ Ptc1−/− and PARP-1+/−/Ptc1−/−) and irradiated (PARP-1+/+Ptc1−/−, PARP-1−/−/Ptc1−/− and PARP-1−/−/Ptc1−/−) mice. (B) Tumor-free survival of unirradiated (PARP-1+/+Ptc1−/−, PARP-1−/−/Ptc1−/− and PARP-1−/−/Ptc1−/−) and irradiated (PARP-1+/+Ptc1−/−, PARP-1−/−/Ptc1−/− and PARP-1−/−/Ptc1−/−) mice. (C) Medulloblastoma incidence in irradiated and control mice. (D) Tumor spectrum.](https://academic.oup.com/carcin/article-abstract/29/10/1911/2476475)
-proficient mice were highly statistically significant \( (P < 0.001) \) at all time points examined. Since kinetics of disappearance of \( \gamma \)-H2AX closely parallels the rate of DSB repair (36,37), longer retention of \( \gamma \)-H2AX foci in PARP-1-deficient cerebella indicates impaired repair ability of radiation damage.

Increased DNA damage response and apoptosis in PARP-1-deficient GCPs

To further define the requirement of PARP-1 activity in early signaling of DNA damage response, we examined p53, which plays a critical role in the response of CNS to genotoxic insult. Phosphorylation of murine p53 at Ser18 promotes both the accumulation and functional activation of p53 in response to damage by radiation, triggering p53-dependent apoptosis and cell cycle arrest (38). We used western blot analysis to determine total and Ser18-p53 protein levels in the irradiated cerebellum of PARP-1/Ptc1 double mutants at 3 and 6 h post-irradiation (Figure 4D and E). Densitometric immunoblot analysis at 3 h showed 1.8-fold increased phosphorylation of Ser18-p53 in the cerebellum of PARP-1\(^{-/-}\)/Ptc1\(^{-/-}\) compared with PARP-1\(^{-/-}\)/Ptc1\(^{+/+}\) mice. This difference decreased out to 1.1-fold at 6 h. Our results suggest that PARP-1 inactivation alters signaling repair after DNA damage in GCPs.

We next examined the effect of PARP-1 deficiency and persistent DNA damage on cell viability. By analysis of neonatally irradiated cerebella of PARP-1/Ptc1 mutants, widespread cell death was present in the EGL of both PARP-1\(^{+/+}\)/Ptc1\(^{+/+}\) and PARP-1\(^{-/-}\)/Ptc1\(^{-/-}\) mice at 6 h post-irradiation (Figure 4F and G). Nuclear pyknosis was increased by 1.5-fold (13.4 versus 8.8%; \( P < 0.001 \)) at 3 h and 1.3-fold (43.8 versus 33%; \( P < 0.001 \)) at 6 h in PARP-1\(^{-/-}\) compared with PARP-1\(^{+/+}\) cerebellum (Figure 4L and M). Immunostaining against cleaved caspase-3, a specific marker of apoptosis, showed clear expression in the EGL from PARP-1\(^{+/+}\)/Ptc1\(^{+/+}\) and PARP-1\(^{-/-}\)/Ptc1\(^{+/+}\) mice at 3 h post-irradiation (Figure 4H and I), confirming that radiation-induced cell death occurred via caspase-dependent apoptosis. Thus, our data show a clear role of PARP-1 in protecting GCPs from damaging effects of environmental agents during neural development.

Chromosomal imbalance analysis in medulloblastomas

Since the PARP-1 protein has genome-stabilizing functions, and aneuploidy and genomic instability characterize PARP-1-deficient cells, we assayed medulloblastomas arising in irradiated Ptc-1\(^{+/+}\) mice with different PARP-1 genotypes \( (n = 6, 5 \text{ and } 7 \text{ tumors for each PARP-1}\(^{+/+}\)/Ptc1\(^{+/+}\), PARP-1\(^{-/-}\)/Ptc1\(^{+/+}\) and PARP-1\(^{-/-}\)/Ptc1\(^{+/+}\) genotype) for chromosomal imbalance by array comparative genomic hybridization (CGH). CGH profiles showed complex non-balanced genomic rearrangements, involving regions of nearly all autosomal chromosomes (supplementary Figure 1 is available at Carcinogenesis Online). Despite increased tumor numbers in PARP-1/Ptc1 double mutants, no differences in CGH profiles were found in medulloblastomas from different PARP-1 genotypes. Notably, 94% (17/18) of medulloblastomas examined showed loss of chromosomal material.
PARP-1 inactivation increases BCC tumorigenesis in Ptc1+/− mice

We examined grossly visible BCCs of the skin, a Ptc1-related tumor. As with Ptc1+/− mice, no macroscopic BCCs were observed in unirradiated PARP-1+/−/Ptc1+/− and PARP-1−/−/Ptc1+/− mice (Figure 3C). After irradiation, increased frequency of macroscopic BCC was detected in PARP-1+/−/Ptc1+/− mice (3/16; 18.7%) compared with PARP-1−/−/Ptc1+/− mice (1/18; 5.5%). Although this 3.4-fold difference is not statistically significant due to small size of experimental groups, the result is remarkable due to the fact that 100% of mice died of medulloblastoma before 20 weeks of age. Notably, in irradiated PARP-1+/−/Ptc1+/− mice, 3 of 16 mice with medulloblastoma also developed BCC, a multitumor phenotype not observed previously in radiation-sensitive Ptc1−/− mice. We took this as a further strong indication that, by impairing DNA damage response, PARP-1 deficiency facilitates development of hedgehog-dependent tumors.

We then carried out microscopic examination of the skin of mice to look for basaloid hyperproliferation areas (Figure 5A). We evaluated the effects of PARP-1 inactivation on early development by microscopic analysis of the normally appearing skin—routinely excised at necropsy—in PARP-1+/Ptc1 mutants. In unirradiated Ptc1+/− mice, PARP-1 mutation did not significantly modify the incidence (62%) in PARP-1+/− versus 50% in PARP-1−/−) and multiplicity (1.50 ± 0.63 in PARP-1+/− versus 1.5 ± 0.65 in PARP-1−/−) of skin lesions (data not shown). Thus, unlike cerebellum, inactivation of PARP-1 is not sufficient to modify substantially the skin phenotype of Ptc1−/− mice without exogenous DNA damage. Because radiation exposure is known to increase the size and frequency of early skin lesions in Ptc1+/− mice (24), grossly normal irradiated mouse skin was analyzed for microscopic preneoplastic proliferations. Remarkably, PARP-1−/−/Ptc1+/− mice show enhanced development of skin lesions, as reflected by the 90% incidence in PARP-1−/−/Ptc1+/− mice compared with 60% in PARP-1+/−/Ptc1+/− genotype (Figure 5B). From the same analysis, we detected a 6.3-fold increased multiplicity of skin lesions in PARP-1−/− compared with PARP-1+/− mice (8.8 ± 2.11 versus 1.4 ± 0.5; P = 0.0031) on Ptc1Ptc1 background (Figure 5C and D).

Because all irradiated PARP-1+/−/Ptc1+/− mice did not survive beyond the minimum latency normally required for BCC development, to improve characterization of the skin, we designed a second ad hoc experiment in which mice of PARP-1+/−/Ptc1+/− and PARP-1−/−/Ptc1+/− genotypes were irradiated with lead shields positioned to protect their heads from radiation damage. This experimental setup allowed reducing early mortality for medulloblastoma, thus affording time for events required for progression of skin lesions. Strikingly, at 30 weeks, PARP-1−/−/Ptc1+/− mice developed a 54% (13/24) incidence of grossly visible BCCs compared with 13% (2/16) in PARP-1+/−/Ptc1+/− mice. This difference was highly statistically significant (P = 0.0095). Tumor multiplicity was also remarkably increased by 13-fold (0.13 ± 0.09 in PARP-1+/−/Ptc1+/− mice versus 1.71 ± 0.53 in PARP-1+/−/Ptc1+/− mice; P = 0.0199), with individual mice developing up to nine independent macroscopic tumors (Figure 6A–C). While this lifetime experiment is still in course, preliminary data are provided here to highlight the critical role of PARP-1 in suppression of Ptc1-associated BCC.

Discussion

In this report, we have introduced PARP-1 mutations onto a Ptc1+/− background to investigate potential genetic interactions between the DNA strand break-detecting PARP-1 enzyme and Ptc1 during development and tumorigenesis. We show that cooperation of DNA end processing and Ptc1 function is required to suppress tumors arising from perturbations of sonic hedgehog signaling. This was reflected as increased frequency of both early and fully malignant tumors, reduced tumor latency and as the occurrence of frequent multiple tumors in PARP-1/Ptc1 mutants compared with Ptc1+/− animals.

Ptc1 has an early role in CNS tumorigenesis as young Ptc1+/− mice show abnormal hyperplastic EGL regions, suggestive of a preneoplastic condition (29). PARP-1 inactivation increased substantially the incidence of early lesions in Ptc1+/− mice, suggesting an important role for PARP-1 in suppression of early cerebellar abnormalities. Because the brain is an organ constantly exposed to oxidative stress and damage, it is possible that lack of PARP-1 activity in GCPs may
lead by itself to genomic instability and hyperproliferation of neural progenitor cells in the developing cerebellum, significantly increasing formation of initial lesions. Consistent with this hypothesis, we detected a significant increase in spontaneous formation of γ-H2AX foci in GCPs from PARP-1−/−/Ptc1−/− mice. However, the lack of correlation between frequency of abnormal EGL proliferation areas and tumorigenesis in irradiated PARP-1−/−/Ptc1−/− mice suggests that, without additional genetic damage (e.g. exogenous damage by radiation), the vast majority of abnormal hyperplastic regions undergo regression, possibly by differentiation or apoptotic processes.

Mice lacking one Ptc1 allele develop a high incidence of medulloblastoma (up to 80%) (27) following radiation damage in neonatal cerebellum. We found that PARP-1 inactivation further increased tumorigenesis in a setting of Ptc1 heterozygosity. In fact, 100% of irradiated PARP−/−/Ptc1−/− mice in this study developed aggressive cerebellar tumors. This strongly suggests a cooperation of DNA DSB repair and deregulated sonic hedgehog signaling in neuronal cells.

In mice lacking one functional Ptc1 copy, the major pathway to medulloblastoma is thought to involve loss of the remaining normal Ptc1 allele. Actually, Ptc1 loss of heterozygosity can be detected in early preneoplastic cerebellar lesions, and time course studies suggest a steadily increased loss of heterozygosity rate during medulloblastoma tumorigenesis in Ptc1−/− mice (28). Concordantly, CGH profiles indicate that medulloblastomas from PARP/Ptc1 mutants show characteristic losses of genetic material around the Ptc1 locus, similar to medulloblastomas from Ptc1−/− mice. Mechanistically, this suggests that PARP-1 deficiency may promote tumorigenesis in irradiated Ptc1−/− mice by facilitating loss of the remaining normal Ptc1 allele, consistent with the observation that inefficient damage signaling and repair of DNA breaks leads to enhanced chromosomal rearrangements (39). Interestingly, medulloblastomas arising in a variety of mouse models with combinations of targeted deletions in DNA damage signaling and repair genes, such as PARP−/−/p53, XRCC1/p53, p53/p16)((52) and Brca2<sup>−/−</sup>Nestin-cre/p53, also show Ptc1 loss, suggesting that the major pathway to medulloblastoma involves loss of this gene (31, 40–42).

Our findings identify DNA break processing as a critical factor in cerebellum tumorigenesis. This view is supported by the observation that PARP-1 inactivation affects DNA damage recognition through phosphorylation signaling, by enhancing expression of the DNA DSB marker histone γ-H2AX and increasing phosphorylation levels of Ser18-p53. Consistently, we found longer persistence of γ-H2AX foci in PARP-1−/− cells in response to genotoxic stress, reflecting defects in DNA damage repair. Whether this is due to unresolved SSBs persisting into the S phase of the cell cycle and collapsing replication forks to form DSBs (43) or, as recent findings suggest, to a direct contribution of PARP-1 to DSB repair remains to be determined (44); however, PARP-1 is clearly important for maintenance of DNA integrity in proliferating GCPs after genotoxic stress.

The role of PARP-1 in the apoptotic process is complex and remains to be determined as conflicting data showing inhibition, lack of effect or increased apoptosis upon PARP-1 chemical or genetic abrogation have been reported in different cell types and tissues after genotoxic stress. Particularly, PARP-1-deficient neuronal cells were reported to be resistant to ischemia or neurotoxic cell death (45). In this study, we show significantly increased apoptotic response to radiation in PARP-1−/−/Ptc1−/− compared with PARP-1−/−/Ptc1−/− mice. This is consistent with increased phosphorylation levels of Ser18-p53 in irradiated PARP-1−/− cerebella, triggering p53-dependent apoptosis and cell cycle arrest. This is also in keeping with earlier studies showing that PARP-1 is an essential survival factor following genotoxic exposures, allowing cells to receive appropriate signals for efficient DNA repair and avoiding genomic rearrangement, prolonged cell cycle arrest and apoptosis (12).

Taken together, our data on the effect of PARP-1 loss in the cerebellum show that PARP-1 deficiency leads—through un repaired DNA damage—to increased cell death in GCPs, and subsequent apoptosis, with escaping cells being genetically altered. This may help to explain both the disorganization of the cerebellar cytoarchitecture and the high susceptibility to brain tumor development in irradiated PARP-1−/−/Ptc1−/− mice.

Although PARP-1-deficient mice have been reported to develop alopecia of the skin and epidermal hyperplasia (46), we did not observe a skin phenotype in PARP-1−/− mice of this study, with or without irradiation. Interestingly, we found that the skin phenotype seen in Ptc1−/− mice was exacerbated by PARP-1 abrogation. PARP-1−/−/Ptc1−/− mice, in fact, were strikingly more susceptible than Ptc1−/− mice to BCC induction, i.e. they developed significantly more tumors with highly reduced latency after X-ray exposure. It was found that up to 54% of mice had developed macroscopic BCCs.
often as multiple independent tumors, within 30 weeks of age. This is remarkable for BCC that, even in responsive Ptc1/+/− mice, shows low frequency, generally delayed onset and delayed development as a solitary tumor. Consequent to inactivation of PARP-1, we also observed highly frequent, generally delayed onset and development as a solitary tu-

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