Inhibition of hippocampal neurogenesis is implicated in neurocognitive dysfunction after cranial irradiation for brain tumors. How irradiation results in impaired neuronal development remains poorly understood. The Trp53 (p53) gene is known to regulate cellular DNA damage response after irradiation. Whether it has a role in disruption of late neuronal development remains unknown. Here we characterized the effects of p53 on neuronal development in adult mouse hippocampus after irradiation. Different bromodeoxyuridine incorporation paradigms and a transplantation study were used for cell fate mapping. Compared with wild-type mice, we observed profound inhibition of hippocampal neurogenesis after irradiation in mice deficient in p53 despite the absence of acute apoptosis of neuroblasts. The putative neural stem cells were apoptosis resistant after irradiation regardless of p53 genotype. Cell fate mapping using different bromodeoxyuridine incorporation paradigms revealed enhanced activation of neural stem cells and their consequent exhaustion in the absence of p53 after irradiation. Both p53-knockout and wild-type mice demonstrated similar extent of microglial activation in the hippocampus after irradiation. Impairment of neuronal differentiation of neural progenitors transplanted in irradiated hippocampus was not altered by p53 genotype of the recipient mice. We conclude that by inhibiting neural progenitor activation, p53 serves to mitigate disruption of neuronal development after irradiation independent of apoptosis and perturbation of the neural stem cell niche. These findings suggest for the first time that p53 may have a key role in late effects in brain after irradiation.

Cell Death Discovery (2016) 2, e16072; doi:10.1038/cddiscovery.2016.72; published online 3 October 2016
those derived from p53+/+ mice. Dissociated neurosphere p53−/− cells cultured in non-differentiation medium also demonstrated a higher density compared with p53+/+ cells. These cells were positive (+) for nestin and sex-determining region Y-box 2 (SOX2), markers of early NPCs (Supplementary Figures 1a–f).

NPCs cultured from dissociated neurosphere showed only the occasional γH2AX nuclear foci. At 1 h after 5 Gy, there was a marked increase in nuclear foci (Supplementary Figures 1g–j). The number of foci per nucleus returned to non-irradiated level by 24 h. Compared with p53+/+ NPCs, there was delay in clearance of γH2AX nuclear foci in p53−/− NPCs at 3 h after irradiation, and the effect of p53 was independent of time after irradiation (number of foci per nucleus: time after irradiation, P < 0.0001; p53 genotype, P < 0.0001; interaction, P = 0.0001; % nuclei with foci; time after irradiation, P < 0.0001; p53 genotype, P < 0.01; interaction, P = 0.0001; two-way analysis of variance (ANOVA); Supplementary Figures 1k–l). These results were consistent with altered DNA damage response in NPCs in vitro after irradiation in the absence of p53.

Deficiency in p53 results in profound inhibition of neurogenesis after irradiation

Irradiation is known to inhibit hippocampal neurogenesis.8 At 9 weeks after irradiation, a very apparent change in dentate gyrus was the marked loss of cells immunoreactive for doublecortin (DCX) and calretinin, markers of neuroblasts and immature neurons, respectively (DCX+ cells, 315 ± 104, 17 Gy versus 9896 ± 483, 0 Gy, P < 0.0001, t-test (Figures 1a–d); calretinin+ cells, 423 ± 12, 17 Gy versus 910 ± 188, 0 Gy, P < 0.05 (Figures 1e–j)).

To determine directly the effects of irradiation on neurogenesis, mice were given bromodeoxyuridine (BrdU), 50 mg/kg daily x7 days, 4 weeks after irradiation. Animals were killed 9 weeks after irradiation for an analysis of the number of newborn neurons or BrdU+ cells immunoreactive for the neuronal marker, neuronal nuclei (NeuN) (Figures 1k–m). Irradiation resulted in a dose-dependent decrease in the number of BrdU+/NeuN+ cells. Consistent with the negative effect of p53 in cell proliferation,15,16 an increase in BrdU+/NeuN+ cells was associated with p53 deficiency. In contrast, the number of BrdU+/NeuN+ cells after irradiation demonstrated the opposite effect, highest in p53+/+ mice, intermediate in p53 heterozygous (+/−) mice and lowest in p53−/− mice (radiation dose, P < 0.0001; p53 genotype, P < 0.0005; interaction, P < 0.0001, two-way ANOVA; Figure 1n).

Results of pairwise comparisons are shown in Supplementary Table 1. The number of BrdU+/NeuN+ cells after 5 Gy in p53+/+, p53+/− and p53−/− mice decreased to 50.6%, 10.4% and 1.9%, respectively, compared with their respective genotype controls (Supplementary Figure 2). This profound inhibition of neurogenesis associated with p53 deficiency was also observed after a clinically relevant irradiation schedule of 20 Gy in 5 daily fractions (irradiation, P < 0.0001; p53 genotype, P < 0.005; interaction, P < 0.005; Figure 1o).

To determine whether an extra copy of p53 gene conferred protection, neurogenesis in super-p53 (p533) mice that have an extra copy of p53 gene17 was compared with their wild-type littermates after irradiation. The number of BrdU+/NeuN+ cells was significantly reduced in both p533 mice and wild-type controls after 5 Gy, but there was no evidence of a protective effect because of the extra copy of p53 gene (irradiation, P < 0.005; p533 genotype, P-value not significant; Supplementary Figure 3).

P53 regulates impairment of neurogenesis after irradiation independent of apoptosis of neuroblasts

NPCs in the SGZ of dentate gyrus are known to undergo apoptosis within hours of irradiation.18,19 It has been postulated that apoptosis of NPCs contributes to impaired neurogenesis after irradiation.18 In non-irradiated p53+/+ mice, apoptotic cells were rarely observed in the SGZ, a robust apoptotic response in the SGZ within hours after irradiation as shown previously.19,20 The peak response, 9849 ± 622, of apoptotic cells based on the morphologic criteria was observed at 8 h after irradiation, compared with 91 ± 27 in control (P < 0.001, t-test). The response returned to non-irradiated control level by 24 h. Similar results were observed using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and caspase-3 immunohistochemistry (data not shown).

Of the apoptotic cells that showed characteristic nuclear condensation and fragmentation, about a third expressed DCX. Among the TUNEL+ and caspase-3+ cells, about a third also expressed DCX (Figures 2a–h). None of the DCX+ apoptotic cells expressed nestin. Consistent with DCX-expressing cells or neuroblasts representing the apoptosis-susceptible population after irradiation, a marked clearance of DCX+ cells was observed at 24 h after irradiation (5553 ± 2126, 17 Gy versus 21773 ± 1598, 0 Gy, P < 0.005, t-test; Figures 2i–l).

Type-1 cells express glial fibrillary acidic protein (GFAP) and nestin, and have a characteristic long radial process that spans the entire granule cell layer and ramifies in the molecular layer.2,3 Although the occasional apoptotic cells expressed nestin, no GFAP+/nestin+ apoptotic cells were observed. At 24 h after irradiation, the number of GFAP+/nestin+ cells remained unchanged (1851 ± 179, 17 Gy versus 1743 ± 150, 0 Gy, t-test, P-value not significant). These results provide no evidence that type-1 cells undergo radiation-induced apoptosis.

Radiation-induced apoptosis of subgranular cells is known to be p53 dependent.31,27 It was extremely difficult to observe apoptotic cells in p53−/− mice after irradiation. Following irradiation, the number of TUNEL+/DCX+ cells at 8 h was dose and p53 genotype dependent (irradiation dose, P < 0.001; p53 genotype, P < 0.001, two-way ANOVA; Figure 2m). Abrogation of radiation-induced apoptosis in p53−/− mice supports the notion that p53 regulates inhibition of neurogenesis after irradiation independent of acute apoptosis of neuroblasts.

Irradiation results in p53-dependent late ablation of proliferating, newborn and total neural stem cells

We next asked if the profound late inhibition of neurogenesis in the absence of p53 after irradiation could be due to increased ablation of neural stem cells. We first characterized change in type-1 cell population (nestin+/GFAP+ or SOX2+/GFAP+ cells) in p53+/+ mice at 9 weeks after irradiation. Animals were given BrdU daily for 7 days at 4 weeks after irradiation for cell fate tracing. Among half of the nestin+/GFAP+ cells (361 ± 38, 17 Gy versus 693 ± 30, 0 Gy; P < 0.01, t-test) and SOX2+/GFAP+ cells (123 ± 10, 17 Gy versus 289 ± 530 Gy, 0 Gy; P < 0.01) disappeared at 9 weeks after 17 Gy. Newborn type-1 cells (BrdU+/nestin+/GFAP+ cells; Figures 3a–d) showed a dose-dependent ablation after irradiation (0 Gy, 70.0 ± 10.1; 5 Gy, 23.4 ± 11.6; 17 Gy, none observed, P < 0.005; one-way ANOVA).

We next performed a population analysis of type-1 cells in p53+/+ and p53−/− mice at 9 weeks after 0 and 5 Gy using the same BrdU-labeling paradigm. A 5-Gy dose was used as it resulted in the loss of approximately half of the number of newborn neurons at 9 weeks, and was considered optimal to discern the effect of p53 or the lack of it. In non-irradiated mice, p53 genotype had no effect on the total number of type-1 cells, BrdU+/ (newborn) type-1 cells and Ki67+ (proliferating) type-1 cells (Figures 3e–h). Increased ablation of total, newborn and proliferating type-1 cells was observed in p53−/− mice compared with p53+/+ mice after irradiation (total type-1 cells: irradiation, P < 0.001; p53 genotype, P < 0.05; BrdU+ type-1 cells: irradiation, P < 0.05; p53 genotype, P < 0.001; Ki67+ type-1 cells: irradiation, P < 0.001; p53 genotype, P < 0.005, two-way ANOVA; Figure 3i).
We did not observe any BrdU+/nestin+ cells that were non-type-1 cells in any control or irradiated p53+/+ and p53−/− mice. BrdU+ cells immunoreactive for Mash1, another marker of INPs, were also not observed. These results were consistent with culling and/or differentiation of INPs over the 5 weeks after they incorporated BrdU. Taken together, these results are consistent with increased neural stem cell exhaustion in p53−/− mice after irradiation.

Figure 1. Inhibition of hippocampal neurogenesis after irradiation is p53 dependent. There is loss of DCX+ (a and b, 0 Gy; c and d, 17 Gy; DCX, green; DAPI, blue) and calretinin+ cells (e–g, 0 Gy, h–j, 17 Gy; calretinin cells, arrow, green; NeuN, red; DAPI, blue) in SGZ at 9 weeks after irradiation. Arrowhead (e) denotes the normal band of calretinin+ nerve fibers at the inner molecular layer. Newborn neurons in dentate gyrus demonstrate BrdU (k, arrows, green) and NeuN immunostaining (l, red; m, merged). The p53 genotype has an independent effect on the number of BrdU+/NeuN+ cells at 9 weeks after single doses of cranial irradiation (n) or 20 Gy in 5 daily fractions (o). Mice were given BrdU daily for 7 consecutive days 4 weeks after irradiation. Data are expressed as mean ± S.E.M. and analyzed with two-way ANOVA with three to five mice per dose per genotype.

PS3 regulates neural stem cell and progenitor cell fate after irradiation
To determine if dysregulated neural stem cell and NPC fate underlies the increased inhibition of neurogenesis associated with p53 deficiency after radiation, a single dose of BrdU (150 mg/kg) was given at 4 weeks after 0 or 5 Gy, and the number of type-1, -2 and -3 cells in p53+/+ and p53−/− mice was determined at 2 h, 2 days, 1 week and 5 weeks after BrdU administration. Using these schemas, BrdU+ cells at 2 h represented proliferating cells, those at 2 days a blend of proliferating and newly divided cells and
those at 1 and 5 weeks were principally cells born during the 1-
and 5-week interval, respectively, after BrdU administration.

In non-irradiated mice, the number of BrdU+ type-1 (BrdU+/nestin+/GFAP+) cells declined over the 5 weeks after BrdU but p53 genotype had no effect on the cell numbers (time after BrdU, \( P < 0.0001 \); p53 genotype, \( P \)-value not significant; two-way ANOVA; Figure 4a). In contrast, the number of BrdU+ type-1 cells after 5 Gy was p53 genotype dependent (time after BrdU, \( P < 0.0001 \); p53 genotype, \( P < 0.05 \); interaction, \( P < 0.005 \); Figure 4b). Irradiation resulted in a spike of BrdU+ type-1 cells in p53\(^{-/-}\) mice at 2 days after BrdU compared with p53\(+/+\) mice (\( P < 0.001 \), Bonferroni post hoc analysis (Figure 4b), see Supplementary Table 1 for results of pairwise comparisons). Hence, neural stem cell fate was not altered by p53 genotype in the absence of irradiation, but there was enhanced activation in the absence of p53 after irradiation.

The number of BrdU+ type-2 cells (BrdU+/nestin+/GFAP\(^{-}\) cells) decreased rapidly by 2 days and 1 week after BrdU in both non-irradiated p53\(+/+\) and p53\(-/-\) mice (time after BrdU, \( P < 0.0001 \); p53 genotype, \( P \)-value not significant; Figure 4c). Irradiation resulted in an increase in BrdU+ type-2 cells at 2 days in p53\(-/-\) compared with p53\(+/+\) mice (\( P < 0.01 \); Figure 4d), and p53 genotype had a significant effect in the number of BrdU+ type-2 cells observed after irradiation (time after BrdU, \( P < 0.005 \); p53 genotype, \( P < 0.05 \); interaction, \( P < 0.05 \); Figure 4d). No BrdU+ type-2 cells were identified at 5 weeks after BrdU in control or irradiated mice irrespective of p53 genotype.

In non-irradiated mice, BrdU+/DCX+ cells declined over 5 weeks after BrdU and p53 genotype had no effect (time after BrdU, \( P < 0.0001 \); p53, \( P \)-value not significant; two-way ANOVA; Figure 4e). After irradiation, BrdU+/DCX+ cells also showed an increase at 2 days in p53\(-/-\) mice compared with p53\(+/+\) mice after 5 Gy (\( P < 0.001 \); Bonferroni post hoc analysis (Figure 4e), see Supplementary Table 1). After irradiation, BrdU+/DCX+ cells showed a marked loss at 24 h after irradiation (i and j, 0 Gy; k and l, 17 Gy; DCX, green; DAPI, blue). The number of DCX+/TUNEL+ apoptotic cells observed at 8 h is radiation dose and p53 genotype dependent. Data are expressed as mean ± S.E.M. and analyzed with a two-way ANOVA with three to five mice per experimental group.

**Figure 2.** Neuroblasts in SGZ undergo p53-dependent apoptosis after irradiation. DCX+ apoptotic cells are identified using TUNEL (a–d, arrows) and caspase-3 immunohistochemistry (e–h, arrows). There is a marked loss of DCX+ cells at 24 h after irradiation (i and j, 0 Gy; k and l, 17 Gy; DCX, green; DAPI, blue). The number of DCX+/TUNEL+ apoptotic cells observed at 8 h is radiation dose and p53 genotype dependent. Data are expressed as mean ± S.E.M. and analyzed with a two-way ANOVA with three to five mice per experimental group.
type-1 (nestin+/GFAP+) BrdU doublets (Figure 4g) at 2 days after BrdU. In the absence of irradiation, there was no difference in the number of BrdU doublets in p53+/+ mice compared with p53−/− mice. After 5 Gy, the number of BrdU doublets decreased in p53+/+ mice but increased in p53−/− mice (Figure 4h). Similar observations were noted for type-1 BrdU doublets (Figure 4i). Thus, p53 does not alter neural stem cell fate in non-irradiated hippocampus, but absence of p53 results in enhanced activation and renewal after irradiation.

PS3 deficiency does not alter neuroinflammation or neurovascular niche dysfunction after irradiation

The fate of neural stem cells and NPCs is regulated by neurovascular interactions.23 Damage to the neurogenic niche such as neuroinflammation is thought to contribute to the deficit in neurogenesis after irradiation.24-28 We thus asked whether the increased inhibition of neurogenesis after irradiation in p53-deficient mice could also be related to increased microglial activation after irradiation. Newborn microglia (BrdU+/CD68+ and BrdU+/Iba1+ cells; Figures 5a–i) have been extensively used as surrogates for activated microglia.29,30 Nine weeks after 5 Gy (BrdU given daily for 7 days at 4 weeks after irradiation), there was an increase in BrdU+/CD68+ and BrdU+/Iba1+ cells in dentate gyrus, independent of p53 genotype (BrdU+/CD68+ cells: irradiation, P < 0.0001; p53 genotype, P-value not significant; BrdU+/Iba1+ cells: irradiation, P < 0.0001; p53 genotype, P-value not significant; two-way ANOVA; Figures 5e and j).

To examine whether there was increased damage of the neurogenic niche after irradiation in the absence of p53, and hence its ability to support neurogenesis, we asked if there could be increased inhibition of neuronal differentiation of NPCs transplanted into irradiated p53−/− mouse hippocampus compared with irradiated p53+/+ mouse hippocampus. P53−/− and p53+/+ mice were given 0 or 5 Gy. After 3 weeks, NPCs cultured from the hippocampus of enhanced green fluorescent protein (eGFP) mice were stereotactically transplanted into the hippocampus.19 At 5 weeks after transplantation, eGFP cells immunoreactive for DCX or Prox1 could be seen in the hippocampus (Figures 5k and m). Only the rare NeuN+/eGFP+ cells were found. The proportion of eGFP cells that expressed DCX or Prox1 was decreased in irradiated hippocampus compared with control, but p53 genotype had no effect (DCX+ cells: irradiation, P < 0.01; p53 genotype, P-value not significant; Prox1+ cells: irradiation, P < 0.005; p53 genotype, P-value not significant; two-way ANOVA; Figures 5l and n). These results did not support the notion that the irradiated microenvironment in p53−/− hippocampus had further inhibitory effects on neuronal differentiation compared with wild-type mice. Taken together, the increase in disruption of neurogenesis in p53−/− mice after irradiation is unlikely to be due to increased microglial activation or increased injury in the irradiated p53−/− neurogenic niche.

DISCUSSION

The adult mammalian brain contains neural stem cells that have the ability to proliferate and generate multipotential NPCs that differentiate into neurons.31 Although neural stem cells are able to proliferate, their capacity for self-renewal is finite. Fate mapping studies revealed that a type-1 cell upon exiting its quiescent state undergoes only a few rounds of asymmetric divisions to produce mature neurons and self-renew.32 Division coupled production of new neurons is thought to result in age-related depletion of the neural stem cell pool.33,34 We observed depletion of total, proliferating and newborn type-1 cells after irradiation. Their ablation after irradiation was further enhanced in the absence of p53. There was an increase in the number of BrdU+ type-1 cells and type-1 BrdU doublets at 2 days...
Figure 4. Deficiency in p53 alters neural stem cell and progenitor cell fate after irradiation. In non-irradiated mice, p53 genotype does not alter the decline of BrdU+ type-1 cells over time after BrdU (a). After 5 Gy, the decrease in the number of BrdU+ type-1 cells over time is p53 dependent (b). The decline of BrdU+ type-2 cells over time is independent of p53 genotype in non-irradiated mice (c) and is p53 genotype dependent after 5 Gy (d). The number of BrdU+/DCX+cells over time after BrdU is independent of p53 genotype in non-irradiated mice (e) but p53 genotype dependent after 5 Gy (f). A type-1 BrdU-doublet is observed in SGZ of a p53+/- mouse after irradiation (g, arrow; BrdU, green; nestin, red; GFAP, white). The number of BrdU doublets and type-1 BrdU doublets in SGZ at 2 days after BrdU is p53 genotype dependent following 5 Gy (h and i). BrdU was given at 4 weeks after 0 or 5 Gy, and cell populations determined at 2 h, 2 days, 1 and 4 weeks after BrdU. Data are represented as mean ± S.E.M. and analyzed with two-way ANOVA and post hoc Bonferroni test, *P < 0.05, **P < 0.01, ***P < 0.001, p53−/− versus p53+/-; †P < 0.01, 5 Gy versus 0 Gy in p53−/− mice. There was a minimum of three to four mice per genotype per time point.
Figure 5. Deficiency in p53 does not alter microglial activation or inhibition of neuronal differentiation after irradiation. An activated microglia demonstrates nuclear BrdU incorporation and CD68+ (a–d, arrow) or Iba1+ (f–i, arrow). The increase in the number of BrdU+/CD68+ (e) and BrdU+/Iba1+ (j) cells in the dentate gyrus at 9 weeks after cranial irradiation is independent of p53 genotype. An eGFP+ neural progenitor cell transplanted in mouse hippocampus demonstrates immunoreactivity for DCX (k, arrow) and another one for Prox1 (m, arrow). The percentage of eGFP+ cells that expresses DCX or Prox1 is reduced in mice given cranial irradiation before transplantation, independent of p53 genotype of the recipient mice (l, DCX+/eGFP+ cells; n, Prox1+/eGFP+ cells). Data are expressed as mean ± S.E.M. and analyzed with two-way ANOVA and post hoc Bonferroni test, *P < 0.05, **P < 0.01, ***P < 0.001, 5 Gy versus 0 Gy; a minimum of three to five mice per experimental group (e and j) and four to seven mice per experimental group (l and n).
after BrdU given 4 weeks after 5 Gy, whereas the opposite effect was seen in p53+/+ mice. Hence, the absence of p53 resulted in enhanced neural stem cell activation after irradiation, whereas neural stem cell fate did not appear to be altered by p53 in non-irradiated mice.

During neurogenesis in adult dentate gyrus, only a few newborn cells become mature neurons. The majority of newborn die of apoptosis within a few days of birth before they transition into DCX+ neuroblasts. In non-irradiated mice, regardless of p53 genotype, we also observed a sharp decline in the number of BrdU+ type-2 and BrdU+/DCX+ cells between 2 and 7 days after BrdU.

A homeostasis of neural stem cell activation and quiescence allows for the continuous generation of new neurons throughout life. Disruption of signaling pathways that lead to excessive activation of neural stem cells resulted in their subsequent depletion and failure of neurogenesis. Certain brain pathologies such as seizures and trauma associated with activation of stem cell division also demonstrated their accelerated loss. P53 is known to negatively regulate NPC proliferation and activation of neural stem cells resulted in their subsequent depletion and failure of neurogenesis. P53 is known to negatively regulate NPC proliferation and activation of neural stem cells.
Primary culture of NPCs

Neurospheres were cultured from 8-week-old p53+/+, p53−/− and eGFP mouse hippocampus. After 10 days in culture, mechanically dissociated neurosphere cells were plated onto culture slips precoated with poly-l-ornithine (Sigma-Aldrich, St Louis, MO, USA) and fed with DMEM/F12 medium containing penicillin/streptomycin, B27 supplement, basic fibroblast growth factor and epidermal growth factor. The non-differentiation medium was changed every other day until cells grew to confluence on day 8. NPCs cultured from p53+/+, p53−/− and eGFP mice demonstrated multipotential properties as reported previously.

Transplantation of eGFP-NPCs

eGFP-NPCs after 8 days in culture were dissociated into single-cell suspensions in DMEM/F12 medium, and stored in ice before transplantation. Transplantation was carried out within 3 h following cell harvesting. eGFP-NPCs were transplanted into the hippocampus of p53+/+ and p53−/− mice, which had received 0 or 5 Gy of cranial irradiation 3 weeks previously. The cranium was fixed in a stereotactic frame (Kopf Small Animal Stereotactic 900) during transplantation with the animals under anesthesia using a cocktail of ketamine and xylazine. Two craniotomies were performed to allow cell transplantation into the right dentate gyrus in two locations: first location, 1.8 mm laterally to the right, 1.1 mm caudally and 3.3 mm ventrally; second location, 2.6 mm laterally to the right, 1.6 mm caudally and 3.6 mm ventrally, all with reference to the bregma. A suspension of 2.5 μL of eGFP cells (50 000 cells per μL) in DMEM/F12 medium was stereotaxically injected at 1 μL/min into each transplantation site, and for an additional 2 min to allow pressure equalization. The scalp was closed with synthetic suture monofilament after transplantation. Subcutaneous buprenorphin (0.05–0.1 mg/kg) was given as applicable. Antibiotics were not used.

Histopathology and immunohistochemistry

Under anesthesia with ketamine and xylazine, mice were perfused with 0.9% saline followed by 4% paraformaldehyde in PBS. Mouse brains were retrieved, postfixed for 2 days and cryoprotected in a 30% sucrose solution. Coronal sections between 1.3 and −3.5 mm caudal to the bregma were cut at 40-μm thickness, collected in tissue cryoprotectant solution in 96-well plates and stored at −20 °C before immunohistochemistry. As morphological characterization remains the gold standard for identification of apoptotic cells, cells that showed nuclear condensation and fragmentation upon 4′,6-diamidino-2-phenylindole (DAPI) staining were considered apoptotic cells. Apoptotic cells were further identified and quantified using TUNEL and caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA, USA) immunohistochemistry. NPCs, immature and mature neurons and microglia were identified by different phenotypic markers using antibodies listed in Supplementary Table 2. Secondary antibodies were conjugated to Cy2, Cy3 (1:200; Jackson ImmunoResearch, West Grove, PA, USA) or Alexa Fluor 647 (1:200; Jackson ImmunoResearch, West Grove, PA, USA) or Alexa Fluor 647 (1:200; Invitrogen, Waltham, MA, USA). Colocalization of BrdU (1:200; Abcam, Toronto, ON, Canada), Ki67 (1:1000; Novocastra, Newcastle upon Tyne, UK) and phenotypic markers in selected sections were evaluated using a confocal laser scanning microscope (Zeiss LSM700, Carl Zeiss AG, Corporate, Oberkochen, Germany). A BrdU-doublet was defined as two abutting DAPI-stained nuclei that demonstrated nuclear BrdU immunoreactivity.

Stereological analysis

Apoptotic cells and cells labeled using different phenotypic markers were counted within the dentate gyrus including a 50-μm hilar margin of the SGZ. Cell counting was performed using a Zeiss Imager M1 microscope (Carl Zeiss AG Corporate) with the Stereo Investigator software (MBF Bioscience, Williston, VT, USA). The observers were blinded to the experimental groups. Apoptotic cells were counted using a counting frame and a sampling grid of 75 × 75 μm². NPCs using counting frame of 20 × 20 μm² and sampling grid of 180 × 180 μm², and microglia, counting frame and sampling grid of 75 × 75 μm², all at a magnification of ×630. Every seventh section was used as the periodicity of sections sampled.

For the transplantation study, 10 coronal sections containing the hippocampus at 5-section intervals from each mouse were used for exhaustive cell counting of eGFP cells with a 100 × 100 μm² sampling grid. The coefficient of error for all the stereology data was between 0.03 and 0.06.

Assessment of DNA damage repair foci

NPCs from p53+/+ and p53−/− mice were cultured in non-differentiation medium for 8 days before they were given a single dose of 0 or 5 Gy. At various time intervals up to 24 h after irradiation, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After treatment with 0.5% nonylphenoxypolyethoxyethanol in PBS, sections were incubated with mouse anti-phospho-histone H2AX IgG1 antibody (1:200; Millipore, Billerica, MA, USA) at 4 °C overnight followed by donkey anti-mouse Cy3 for 45 min at room temperature, and counterstained with DAPI. A minimum of 50 nuclei from a minimum of five independent experiments per treatment group was used to determine the number of γH2AX foci per nucleus. As the occasional non-irradiated NPC nuclei contained up to six foci, the nuclei with ≥ 5 foci were considered foci+.

Statistical analysis

All cell population analysis represented data from three to five mice per genotype per dose per time point, except for the cell fate experiments where there were three to four mice per genotype per dose group. There were four to seven mice per experimental group in the transplantation experiment. All data were expressed as mean ± S.E. Comparison of cell numbers after irradiation to controls was performed using t-test. Dose–response analysis for cell numbers was performed by one-way ANOVA. The effect of variables, namely irradiation and p53 genotype, or p53 genotype and time after BrdU on cell numbers, was determined using two-way ANOVA. Pairwise comparisons were based on post hoc Bonferroni correction for multiple comparisons. Differences were considered significant for P < 0.05. Statistical analyses were performed with the GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

ABBREVIATIONS

ANOVA, analysis of variance; BrdU, bromodeoxyuridine; DAPI, 4′-6-diamidino-2-phenylindole; DCX, doublecortin; eGFP, enhanced green fluorescent protein; GFAP, glial fibrillary acidic protein; INP, intermediate neural progenitors; NeuN, neuronal nuclei; NPCs, neural progenitor cells; +, positive; SGZ, subgranular zone; SOX2, sex-determining region Y-box 2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

ACKNOWLEDGEMENTS

The work was supported by funding from the Canadian Cancer Society Research Institute (CSW) and Cancer Research Society (CSW).

AUTHOR CONTRIBUTIONS

Conception and design: ZC Cheng, Y Li, CS Wong; development of methodology: ZCC, YL, CSW; acquisition of data: YL, ZCC; analysis and interpretation of data: ZCC, YL, CSW; writing, review and/or revision of the manuscript: IA, ZCC, YL, SL, CSW; administrative, technical or material support: YL, CSW; study supervision: CSW; other (oversight of every aspect of the research): CSW.

COMPETING INTERESTS

The authors declare no conflict of interest.

REFERENCES

1. Greene-Schloesser D, Moore E, Robbins ME. Molecular pathways: radiation-induced cognitive impairment. Clin Cancer Res 2013; 19: 2294–2300.
2. Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell 2008; 132: 645–660.
3. Bonaguidi MA, Song J, Ming GL, Song H. A unifying hypothesis on mammalian neural stem cell properties in the adult hippocampus. Curr Opin Neurobiol 2012; 22: 754–761.
4. Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E. Neurogenesis in the adult is involved in the formation of trace memories. Nature 2001; 410: 372–376.
5. Feng R, Rampon C, Tang YP, Shrom D, Jin J, Kyn M et al. Deficient neurogenesis in forebrain-specific preslin−/− knockout mice is associated with reduced clearance of hippocampal memory traces. Neuron 2001; 32: 911–926.
6. Sahay A, Scobie KN, Hill AS, O’Carroll CM, Kheirbek MA, Burghardt NS et al. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature 2011; 472: 466–470.
PS3 regulates neuronal development after irradiation

Y-Q Li et al.

7 Alers KG, Martinez-Canelab A, Restivo L, You AP, De Cristofaro A, Hsiang HL et al. Hippocampal neurogenesis regulates forgetting during adulthood and infancy. Science 2014; 344: 598–602.

8 Monje ML, Mizumatsu S, Fike JR, Palmer TD. Irradiation induces neural precursor-cell dysfunction. Nat Med 2002; 8: 955–962.

9 Monje ML, Palmer T. Radiation injury and neurogenesis. Curr Opin Neurol 2003; 16: 129–134.

10 Gudkov AV, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. Nat Rev Cancer 2003; 3: 117–129.

11 Kirsch DG, Santiago PM, di Tomaso E, Sullivan JM, Hou WS, Dayton T et al. PS3 controls radiation-induced gastrointestinal syndrome in mice independent of apoptosis. Science 2010; 327: 593–596.

12 Lee SW, Haditsch U, Cord BJ, Guzman R, Kim SJ, Boettcher C et al. P53 controls radiation-induced gastrointestinal syndrome in mice independent of apoptosis. Cancer Res 2006; 66: 9356–9361.

13 Burdelya LG, Komarova EA, Hill JE, Browder T, Tararova ND, Mavrikis L et al. Inhibition of p53 response in tumor stroma improves efficacy of anticancer treatment by increasing antiangiogenic effects of chemotherapy and radiotherapy in mice. Cancer Res 2006; 66: 9356–9361.

14 Baneth JP, Macphall SH, Olive PL. Radiation sensitivity, H2AX phosphorylation, and kinetics of repair of DNA strand breaks in irradiated cerebral cancer cell lines. Cancer Res 2004; 64: 7144–7149.

15 Meletis K, Wirta V, Hede SM, Nister M, Lundeberg J, Frisen J. P53 suppresses the self-renewal of adult neural stem cells. Development 2006; 133: 363–369.

16 Armessila-Diaz A, Bragado P, Del Valle I, Cuevaes E, Lazoar I, Martin C et al. P53 regulates the self-renewal and differentiation of neural precursors. Neuroscience 2009; 158: 1378–1389.

17 Garcia-Cao I, Garcia-Cao M, Martin-Caballero J, Criado LM, Klatt P, Flores JM et al. ‘Super p53’ mice exhibit enhanced DNA damage response, are tumor resistant and age normally. EMBO J 2002; 21: 6225–6235.

18 Mizumatsu S, Monje ML, Morhardt DR, Rola R, Palmer TD, Fike JR. Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. Cancer Res 2003; 63: 4021–4027.

19 Lu F, Li YQ, Aubert I, Wong CS. Endothelial cells regulate p53-dependent apoptosis of neural progenitors after irradiation. Cell Death Dis 2012; 3: e324.

20 Sienna A, Encinas JM, Deudero JH, Chancey JH, Enikolopov G, Overstreet-Wadiche C. Absence of p53 response in tumor stroma improves efficacy of anticancer treatment by increasing antiangiogenic effects of chemotherapy and radiotherapy in mice. Cancer Res 2006; 66: 9356–9361.

21 Chow BM, Li YQ, Wong CS. Radiation-induced apoptosis in the adult central nervous system is p53-dependent. Cell Death Differ 2000; 7: 712–720.

22 Limoli CL, Giedzinski E, Rola R, Otuka S, Palmer TD, Fike JR. Radiation response of neural precursor cells: linking cellular sensitivity to cell cycle checkpoints, apoptosis and oxidative stress. Radiat Res 2004; 161: 17–27.

23 Shen Q, Goderer SK, Jin L, Karranth N, Sun Y, Abramova N et al. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 2004; 304: 1338–1340.

24 Pineda JR, Daynac M, Chicheportiche A, Cebrian-Silla A, Sii Felice K, Garcia-Verdujo JM et al. Vascular-derived TGF-beta increases in the stem cell niche and perturbs neurogenesis during aging and following irradiation in the adult mouse brain. EMBIO Mol Med 2013; 5: 548–562.

25 Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. Science 2003; 302: 1760–1765.

26 Lee SW, Haditsch U, Cord BJ, Guzman R, Kim SJ, Boettcher C et al. Absence of CCL2 is sufficient to restore hippocampal neurogenesis following cranial irradiation. Brain Behav Immun 2013; 30: 33–44.

27 Belarbi K, Jopson T, Arellano C, Fike JR, Rosi S. CCR2 deficiency prevents neuronal dysfunction and cognitive impairments induced by cranial irradiation. Cancer Res 2013; 73: 1201–1210.

28 Jemrow KA, Brown SL, Lapanowski K, Naei H, Kolozsvary A, Kim JH. Selective inhibition of microglia-mediated neuroinflammation mitigates radiation-induced cognitive impairment. Radiat Res 2013; 179: 549–556.

29 Song J, Zhong C, Bonaguidi MA, Sun GJ, Hou D, Gu Y et al. Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. Nature 2012; 489: 150–154.

30 Pereboeva L, Harkins L, Wong S, Lamb LS. The safety of allogeneic innate lymphocyte therapy for glioma patients with prior cranial irradiation. Cancer Immunol Immunother 2015; 64: 551–562.

31 Garcia AD, Doan NB, Imura T, Bush TG, Sofroniew MV. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat Neurosci 2004; 7: 1233–1241.

32 Bonaguidi MA, Wheeler MA, Shapiro JS, Stadel RP, Sun GJ, Ming GL et al. In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. Cell 2011; 145: 1142–1155.

33 Kippin TE, Martens DJ, van der Kooy D. P21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. Gene Dev 2005; 19: 756–767.

34 Encinas JM, Michurina TV, Peunova N, Park JH, Tordo J, Peterson DA et al. Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. Cell Stem Cell 2011; 8: 566–579.

35 Mira H, Andreu Z, Suh H, Lie DC, Jessberger S, Consiglio A et al. Signaling through BMP/NFAT regulates quiescence and long-term activity of neural stem cells in the adult hippocampus. Cell Stem Cell 2010; 7: 78–89.

36 Guo JU, Ma DK, Mo H, Ball MP, Jang MH, Bonaguidi MA et al. Neuronal activity modulates the DNA methyltransferase activity in the adult brain. Nat Neurosci 2011; 14: 1345–1351.

37 Seg-Nishida E, Warner-Schmidt JL, Duman RS. Electroconvulsive seizure and VEGF increase the proliferation of neural stem-like cells in rat hippocampus. Proc Natl Acad Sci USA 2008; 105: 11352–11357.

38 Gao X, Enikolopov G, Chen J. Moderate traumatic brain injury promotes proliferation of quiescent neural progenitors in the adult hippocampus. Exp Neurol. 2009; 219: 516–523.

39 Uberti D, Piccioni L, Cadei M, Grigolato P, Rotter V, Memò M. PS3 is dispensable for apoptosis but controls neurogenesis of mouse dentate gyrus cells following gamma-irradiation. Brain Res Mol Brain Res 2001; 93: 81–89.

40 Inzinga A, Cialese A, Faretta M, Gallo B, Albano L, Ronzoni S et al. DNA damage in stem cells activates p21, inhibits p53, and induces symmetric self-renewing divisions. Proc Natl Acad Sci USA 2013; 110: 3931–3936.

41 Blanpain C, Mohrin M, Sotiropoulou PA, Passegue E. DNA-damage response in tissue-specific and cancer stem cells. Cell Stem Cell 2011; 8: 16–29.

42 Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 2000; 425: 479–494.

43 Yazyovitskaya EM, Edwards E, Thotala D, Fu A, Ousky KL, Wethsell WO Jr et al. Lithium treatment prevents neurocognitive deficit resulting from cranial irradiation. Cancer Res 2006; 66: 11179–11186.

44 Zanni G, Di Martino E, Omelyanenko A, Andang M, Delle U, Elmroth K et al. Lithium increases proliferation of hippocampal neural stem/progenitor cells and rescues irradiation-induced cell cycle arrest in vitro. Oncotarget 2015; 6: 37083–37097.

45 Roughton K, Kalm M, Blomgren K. Sex-dependent differences in behavior and hippocampal neurogenesis after irradiation to the young mouse brain. Eur J Neurosci 2012; 36: 2763–2772.