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PDGF-BB and bFGF ameliorate radiation-induced intestinal progenitor/stem cell apoptosis via Akt/p53 signaling in mice

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Liu Z, Liu H, Jiang J, Tan S, Yang Y, Zhan Y, Wu B. PDGF-BB and bFGF ameliorate radiation-induced intestinal progenitor/stem cell apoptosis via Akt/p53 signaling in mice. Am J Physiol Gastrointest Liver Physiol 307: G1033–G1043, 2014. First published October 9, 2014; doi:10.1152/ajpgi.00151.2014.—Radiation-induced gastrointestinal (GI) syndrome currently has no effective prophylactic or therapeutic treatment. Previous studies and our data have demonstrated the important role of p53 in acute radiation-induced GI syndrome in mice. Many cytokines, such as tumor necrosis factor-α and fibroblast growth factor (bFGF), have been found to protect against radiation-induced intestinal injury, although the underlying mechanisms remain to be identified. Here, we report blockage of p53 through a protein kinase B (Akt) pathway in intestinal progenitor/stem cells or crypt cells as a novel molecular mechanism of growth factor-mediated intestinal radioprotection. Treatment with platelet-derived growth factor (PDGF-BB) or bFGF activated Akt phosphorylation in the intestinal crypt, lessened intestinal crypt p53 expression, decreased radiation-induced apoptosis in mouse intestinal progenitor/stem cell marker leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5)-positive cells by an average of 50%, and increased the survival rate of mice with abdominal radiation by 3 days in average. Conversely, the Akt inhibitor perifosine obstructed growth factor-simulated Akt phosphorylation while promoting radiation-induced p53 expression in intestinal crypts. Importantly, reduced Akt phosphorylation and elevated p53 expression due to the Akt inhibitor perifosine impaired intestinal progenitor/stem cells radioprotection provided by PDGF-BB and bFGF. Consistently, PDGF-BB and bFGF both upregulated Akt activation, suppressed radiation-induced p53 expression, and abrogated radiation-induced apoptosis in IEC-6 cells, although p53 overexpression in IEC-6 cells partially counteracted the radioprotection of PDGF-BB and bFGF. Our data suggest that intestinal crypt radioprotection by PDGF-BB and bFGF is dependent on regulation of Akt/p53 signaling.

PDGF-BB; bFGF; intestinal apoptosis; Akt; p53

Tissues with rapid cell turnover, such as the gut, bone marrow, and hair follicles, are hypersensitive to ionizing radiation (IR)-induced apoptosis. IR-induced gastrointestinal (GI) syndrome currently has no effective prophylactic or therapeutic treatment. Adult tissue stem cells are thought to be responsible for maintaining tissue homeostasis and regeneration following genotoxic or nongenotoxic stress. Several studies in mice have indicated that leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), CD133/prominin1-, and Bmi-1-expressing cells at or near the crypt base are putative intestinal stem cells (ISCs) (1, 35, 24). Cells in at least two positions exhibit the properties of ISCs: the columnar cells at the crypt base (CBCs) and the cells at position 4 from crypt bottom (+4 cells) immediately above the Paneth cells. Some studies have revealed that IR-induced ISC apoptosis is largely responsible for acute intestinal toxicity, leading to rapid initiation of GI syndrome and death (4, 22). Growth factors ameliorate radiation or chemotherapy-induced mucosal injury (2, 21, 30). For example, interleukin-11, keratinocyte growth factor, fibroblast growth factor-2 (bFGF-2), and insulin-like growth factor 1 (IGF-1) have been shown to protect +4 cells and prolong mouse survival following IR, but their molecular targets and the underlying mechanism of intestinal radioprotection remain to be established.

IR has been shown to activate p53 in the small intestinal epithelium and initiate p53-mediated intestinal progenitor/stem cell apoptosis (15, 22, 19, 28). Conversely, targeted deletion of p53 prevents IR-induced apoptosis of most intestinal epithelial cells (22). Thus p53-mediated apoptosis has been implicated in regulating GI syndrome.

p53 regulates IR-induced apoptosis in small intestinal crypts. phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling controls the antiapoptotic function of platelet-derived growth factor (PDGF) in various cell types (17, 26, 32) and is also activated by bFGF in some cells (14). The mutual interplay between p53 and the PDGF/Akt pathway has been outlined in mammals and less advanced animals (17, 26, 32). The PI3K/Akt pathway is activated by PDGF signaling, which causes phosphorylation and activation of murine double minute (MDM2), and by subsequent inactivation of p53 through the ubiquitin-proteasome system (17, 26, 32). Links between p53 and bFGF signaling have also been reported. For example, bFGF facilitates the synthesis of MDM2, leading to sharply decreased activation of p53 and protection from DNA-damage-induced cell death in fibroblasts (25). Additionally, FGF1 has been shown to block p53-dependent apoptosis in neuronal cells treated with etoposide (3), whereas bFGF prevents IR-induced apoptosis in cultured endothelial cells (9). However, whether intestinal radioprotection by growth factors is mediated by the Akt/p53 pathway remains to be elucidated.

Therefore, we hypothesize that preventing p53-mediated apoptosis in ISCs may confer partial growth factor-mediated intestinal radioprotection. In this study, we explored the role of p53 in PDGF-BB- and bFGF-mediated intestinal health following IR. We demonstrated that the repression of p53 heavily impacted PDGF and bFGF-mediated radioprotection in the GI system through Akt signaling.

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MATERIALS AND METHODS

Mice and treatment. The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Eight- to 10-wk-old mice (20–25 g) were used for all experiments. The mice were housed in microisolator cages with access to water and a standard laboratory diet ad libitum. Intestinal stem cell marker leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) knock-in mice (Jackson Laboratory, Bar Harbor, ME) were genotyped by PCR by using genomic DNA extracted from tail snips as described previously (1). Mice were irradiated at a dose of 15 Gy at a rate of 2 Gy/min with an RS-2000 X-ray biological irradiator (RAD Source Technologies, Suwanee, GA). The small intestines were obtained at 24 h for apoptosis assessments, crypt depth comparisons, p53 staining, cleaved caspase-3 staining, mitochondrial analysis, and Lgr5-positive cell counting, and at 3.5 days they were assessed by hematoxylin-eosin and Ki67 staining after lethal radiation.

For growth factor treatment, mice were injected intravenously with a mouse recombinant PDGF-BB (50 μg/kg, Pepro Tech, Rocky Hill, NJ) or mouse recombinant bFGF (50 μg/kg, Pepro Tech) administered three times: 30 min before, 5 min before, and 30 min after radiation. In some experiments, mice were treated 2 h before radiation with 2.2 mg/kg pifithrin-α (Sigma-Aldrich, St. Louis, MO) or with the first dose of growth factor with 40 mg/kg perifosine (Sigma-Aldrich) through intraperitoneal injections. Mice were euthanized to collect the small intestines for histology analysis, and Western blotting was performed at 4 and 24 h after radiation. Three independent experiments were repeated.

Intestinal crypt isolation. This has been described previously (23). Briefly, isolated small intestines were opened longitudinally and washed with cold PBS. The tissue was cut into ∼5-mm pieces and further washed with cold PBS. The tissue fragments were incubated in 2 mM EDTA with PBS for 30 min on ice. After removal of EDTA medium, the tissue fragments were vigorously suspended by using a 10-ml pipette with cold PBS for 30 min. The supernatant was the villous fraction and was discarded; the sediment was resuspended with PBS. After further vigorous suspension and centrifugation, the supernatant was enriched for crypts. This fraction was passed through a 70-μm cell strainer (BD Bioscience, Franklin Lakes, NJ) to remove residual villous material. Isolated crypts were centrifuged at 150–200 g for 3 min to separate crypts from single cells. The final fraction consisting of essentially pure crypts was used for protein extraction.

TUNEL staining and crypt microcolony assay. Both procedures have been described previously (22). Briefly, formalin-fixed tissues were embedded in paraffin and sectioned. The 4-μm sections were stained by hematoxylin and eosin (H&E). Terminal deoxynucleotidyl transferred-mediating dUTP-biotin nick-end labeling (TUNEL) fluorescent staining (green); magnifications ×200 and ×400. DAPI, 4,6-diamidino-2-phenylindole. B: quantification of the apoptotic index in the crypts measured by TUNEL staining. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.01 vs. ionizing radiation (IR). C: hematoxylin and eosin (H&E) staining of small intestine sections; magnification ×200. D: number of surviving crypts was analyzed by H&E staining at day 3.5 after 15-Gy radiation. The surviving crypts were defined as containing 5 or more adjacent chromophilic non-Paneth cells, at least 1 Paneth cell, and a lumen. Values are means ± SD; n = 4 in each group. *P < 0.01 vs. untreated; #P < 0.01 vs. IR. E: crypt depth was assessed at day 2 after 15-Gy radiation. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.05 vs. IR. F: survival curves of mice with or without growth factors subjected to 15-Gy radiation. G: Ki67-positive cells were enumerated based on Ki67 staining (brown) at day 3.5 after 15-Gy radiation. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.05 vs. IR.

Fig. 1. PDGF-BB and fibroblast growth factor-2 (bFGF) treatments reduced radiation-induced intestinal crypt cell apoptosis. A: degree of apoptosis in the intestinal crypts of wild-type C57BL/6 mice subjected to various treatments was measured 24 h after 15 Gy of radiation by terminal deoxynucleotidyl transferred-mediating dUTP-biotin nick-end labeling (TUNEL) fluorescent staining (green); magnifications ×200 and ×400. DAPI, 4,6-diamidino-2-phenylindole. B: quantification of the apoptotic index in the crypts measured by TUNEL staining. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.01 vs. ionizing radiation (IR). C: hematoxylin and eosin (H&E) staining of small intestine sections; magnification ×200. DAPI, 4,6-diamidino-2-phenylindole. B: quantification of the apoptotic index in the crypts measured by TUNEL staining. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.01 vs. ionizing radiation (IR). C: hematoxylin and eosin (H&E) staining of small intestine sections; magnification ×200. D: number of surviving crypts was analyzed by H&E staining at day 3.5 after 15-Gy radiation. The surviving crypts were defined as containing 5 or more adjacent chromophilic non-Paneth cells, at least 1 Paneth cell, and a lumen. Values are means ± SD; n = 4 in each group. *P < 0.01 vs. untreated; #P < 0.01 vs. IR. E: crypt depth was assessed at day 2 after 15-Gy radiation. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.05 vs. IR. F: survival curves of mice with or without growth factors subjected to 15-Gy radiation. G: Ki67-positive cells were enumerated based on Ki67 staining (brown) at day 3.5 after 15-Gy radiation. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.05 vs. IR.
transferred to a 6-well plate to 85 to 90% confluency. Then 2 μl X-tremeGENE HP reagent were used to transfect IEC-6 cells. Briefly, IEC-6 cells were cultured in a 1:100 dilution at 4°C overnight, followed by incubation with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution at 37°C for 2 h. The color development for signals was carried out with a DAB kit (DAKO, Copenhagen, Denmark) and counterstained with hematoxylin.

Immunofluorescent double staining. TUNEL staining was performed by using an in situ cell death detection kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. After TUNEL fluorescent staining, the number of TUNEL-positive cells in each intact crypt was determined in 100 intact crypts per section and reported as means ± SD. Four mice were used in each group. Microscopically, intact crypts are defined as those containing at least 28 cells, including Paneth cells, in a full longitudinal section. Surviving crypts were defined as those containing five or more adjacent chromophilic non-Paneth cells, at least one Paneth cell, and a lumen. The number of surviving crypts was counted in 5–10 circumferences per mouse, each ~1 cm apart. Four mice were used in each group.

Immunohistochemistry. Sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide. Antigen retrieval was performed by boiling the sections for 2 min in 0.1 M citrate buffer antigen retrieval solution (pH 6.0). Nonspecific antibody binding was blocked by use of a 3-serum-blocking agent for 30 min. The section was then incubated with rabbit anti-p53 or anti-cleaved caspase-3 or anti-Ki67 (Cell Signaling Technology, Danvers, MA) at a 1:100 dilution at 4°C overnight, followed by incubation with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution at 37°C for 2 h. The color development for signals was carried out with a DAB kit (DAKO, Copenhagen, Denmark) and counterstained with hematoxylin.

Immunofluorescent double staining. TUNEL staining was performed by using an in situ cell death detection kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Antigen retrieval was performed after TUNEL staining in 0.1 M citrate buffer antigen retrieval solution (pH 6.0). The sections were blocked with 20% rabbit or goat serum for 30 min at room temperature and then incubated with a goat anti-MMP-7 antibody (Abcam, Cambridge, UK) or rabbit anti-EGFP-Lgr5 antibody (Santa Cruz Biotechnology) at a 1:100 dilution overnight at 4°C prior to incubation with the secondary antibody (Santa Cruz Biotechnology) at a 1:200 dilution at 37°C for 2 h. Color development for signaling was carried out with a DAB kit (DAKO) and sections were then counterstained with hematoxylin.

Cell culture, treatment, transfection, and immunocytochemistry. IEC-6 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HCT116 p53+/+ and HCT116 p53−/− cells have been described previously (29) and were obtained from Dr. Bert Vogelstein (Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins). IEC-6 was cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and 0.1% insulin-transferrin-selenium in a humidified incubator at 37°C with 5% CO2. HCT116 p53+/+ and HCT116 p53−/− cells were cultured in complete medium consisting of DMEM/F-12 (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum at 37°C in a CO2 incubator. PDGF-BB (400 ng/ml, Pepro Tech) or bFGF (400 ng/ml, Pepro Tech) were added after these cells had grown to a density of 90% prior to IR treatment. In some experiments, perfosine (4 μM) was added before pretreatment with growth factors.

IEC-6 cells were transfected with murine p53 expression plasmid (Addgene, Cambridge, MA) by using the X-tremeGENE HP DNA transfection reagent (Roche). Brefly, IEC-6 cells were cultured in a six-well plate to 85 to 90% confluence. Then 2 μg p53 plasmid DNA mixed with 4 μl X-tremeGENE HP reagent were used to transfected each well. After 6 h, the medium was replaced. Then, 48 h after the beginning of the transfection, the cells expressing plasmid were assessed by Western blotting to determine whether they expressed p53 more highly than that did the cells with transfected with empty vector. The cells overexpressing p53 were used in further experiments.

For immunocytochemistry analysis, IEC-6 cells were seeded onto glass coverslips, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Following washing and blocking, cells were incubated with anti-p53 antibody (diluted 1:100 in 5% BSA) overnight at 4°C in a humidified chamber, followed by incubation with Alexa Fluor 594-labeled goat anti-rabbit secondary antibody (Cell Signaling Technology) for 90 min at 37°C in a humidified chamber. Coverslips were mounted onto glass slides in antifade mounting medium (Life Technologies, Carlsbad, CA) and observed via a fluorescence microscope.

Analysis of Bax and cytochrome c translocation. To analyze Bax and cytochrome c translocation, an aliquot of intestinal epithelial cells

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**Fig. 2.** PDGF-BB and bFGF ameliorate intestinal stem cell apoptosis. A: representative images of intestinal Lgr5-positive stem cells in Lgr5 knock-in mice 48 h after 15-Gy radiation; magnification ×400. B: intestinal Lgr5-positive stem cells were enumerated by quantifying Lgr5 staining (red) on day 1 after 15-Gy radiation. Values are means ± SD; n = 4 in each group. *P < 0.01 vs. untreated; #P < 0.01 vs. IR. C: left: representative image of radiation-induced apoptosis in the columnar cell at the crypt base (CBC), the nucleus of which was stained by TUNEL (brown). The brown nucleus (circled) did not overlap with the MMP-7 staining (purple). Sections were stained with TUNEL followed by MMP-7 immunohistochemistry; magnification ×400. C: right: representative image of radiation-induced intestinal Lgr5-positive stem cell apoptosis. Sections were subjected to TUNEL fluorescent staining followed by Lgr5 staining; magnification ×400. D: quantification of Lgr5-positive, TUNEL-positive cells in 200 intact crypts on day 1 following 15-Gy radiation. Values are means ± SD, n = 4 in each group. *P < 0.01 vs. untreated; #P < 0.05 vs. IR.
isolated from the irradiated mice was used to isolate mitochondrial and cytosolic fractions by the differential centrifugation method as described previously (31). Briefly, the samples were washed with ice-cold PBS and resuspended in homogenization buffer (0.25 M sucrose, 10 mM HEPES, pH 7.4, and 1 mM EGTA) and subjected to 40 strokes of homogenization in a Dounce homogenizer. The homogenate was subjected to centrifugation (1,000g, 15 min, 4°C) to pellet the nuclei and unbroken cells. The supernatant was centrifuged again (10,000g, 15 min, 4°C) to obtain the cytosolic (supernatant) and mitochondrial fractions (pellet). The mitochondrial fraction was resuspended in homogenization buffer following one wash. Aliquots of both fractions were mixed with equal volumes of 2X Laemmli sample buffer and analyzed by Western blotting for Bax and cytochrome c.

Western blotting and antibodies. Total cellular protein and the mitochondrial and cytosolic fractions were analyzed by Western blotting. Antibodies used for Western blotting included anti-p53, anti-cleaved caspase-3, anti-p-Akt, anti-Akt, anti-COX IV, anti-Hsp60 (all from Cell Signaling Technology); anti-β-actin (Sigma-Aldrich); anti-Bax (Abcam); and anti-cytochrome c (Santa Cruz Biotechnology). Appropriate horseradish peroxidase-conjugated secondary antibodies were used to detect the primary antibody/antigen complexes. The signal was detected by use of ECL Western...

Fig. 3. Inhibition of p53 expression alleviated radiation-induced apoptosis. A: representative images of intestinal cell apoptosis, p53, and cleaved caspase-3 expression by TUNEL (green) or immunohistochemistry (brown) 24 h after 15-Gy radiation. B: apoptotic index in the crypts measured by TUNEL staining in mice with or without pifithrin-α (p53 inhibitor) 24 h after 15-Gy radiation. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.01 vs. IR. C: p53 and cleaved caspase-3 expression levels were determined by Western blotting in intestinal crypts 24 h after 15-Gy radiation. Three independent experiments were repeated. D: survival curves of mice with or without pifithrin-α subjected to 15-Gy radiation. E: Bax and cytochrome c (Cyto c) were analyzed in the cytosolic and mitochondrial fractions of HCT116 p53+/− and HCT116 p53−/− 24 h after 15-Gy radiation. β-Actin and Hsp60 were used as the controls for loading and fractionation. F: densitometric ratio of p53/β-actin or cleaved caspase-3/β-actin is indicated. Values are expressed as means ± SD; n = 4 in each group. *P < 0.01 vs. untreated; #P < 0.05 vs. IR. G: densitometric ratio of Bax/Hsp60 or Cyto c/β-actin is represented. Values are expressed as means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.05 vs. IR.
ern blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Total RNA extraction and real-time PCR. Total RNA was separated from intestinal epithelial cells isolated from the irradiated mice by using the RNAgents Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer’s instructions. First-strand cDNA was synthesized by using Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Real-time PCR was performed on a Chromo 4 Detector System (MJ Research, Sierra Point, CA) with use of gene-specific primers and DyNAmo SYBR Green Master Mix (Finnzymes, Espoo, Finland). The gene encoding p53 was amplified by using the forward primer 5'-TCA CAG CGT CTG TTG ACA TTT-3' and the reverse primer 5'-ACC AAG CTC ATT ACC CTG ACA-3'. Lgr5 mice were genotyped by using primers including the common primer 5'-CTG CTG CTG TTC CCC AGT CT-3', wild-type reverse 5'-ATA CCC CAT CCC TTT TGA GC-3' and mutant reverse 5'-GAA CTT CAG GGT CAG CTT GC-3' to yield a 152-bp product. Regarding the internal control, the expression of β-actin in each sample was also quantified by using the sense primers 5'-CGG TTG GCC TTA GGG TTC AGG GGG G-3' (242 bp product). Four mice were used in each group.

Statistical analysis. The data are expressed as means ± SD and were evaluated by one-way ANOVAs in which multiple comparisons were performed by the method of least significant differences. Survival data were analyzed by a log-rank test with the Origin 8.0 software. Differences were considered statistically significant if the probability of the difference occurring by chance was <5 in 100 (P < 0.05).

RESULTS

PDGF-BB and bFGF reduced radiation-induced intestinal crypt cells apoptosis. Treatment with PDGF-BB or bFGF without IR did not alter small intestinal crypt apoptosis in mice, compared with vehicle-treated mice (data not shown). Using an IR model, we found that 15 Gy of radiation induced progenitor/stem cell apoptosis in the intestinal crypts of wild-type mice at 24 h, and it was inhibited by 60 and 40% in response to PDGF-BB and bFGF treatment, respectively (Fig. 1, A and B). Since crypt apoptosis and regeneration are closely linked, it is reasonable to speculate that crypt regeneration in mice with PDGF-BB and bFGF treatment may have increased regardless of the injection of growth factors. Surviving crypt number and crypt depth after IR are indicators of crypt regeneration. Surviving crypt numbers in longitudinal sections at day 3.5 after 15-Gy radiation decreased sharply, whereas PDGF-BB and bFGF treatment corresponded to a 20% increase in surviving crypts (Fig. 1D). Moreover, the growth factors markedly decreased shrinkage of crypt depth in mice by 30% (Fig. 1E). Meanwhile, cell proliferation in the crypt was highly diminished by 15-Gy radiation at day 3.5, while...
Fig. 5. PDGF-BB and bFGF curtailed IR-stimulated apoptosis via suppression of p53 through Akt. A: representative images of p53 and cleaved caspase-3 immunohistochemistry staining (brown) in the small intestines 24 h after 15-Gy radiation; magnification ×400. B: Akt activity, p53 expression, and cleaved caspase-3 expression were measured by Western blotting in isolated intestinal crypts in mice with or without perifosine (Akt inhibitor) treatment 24 h after 15-Gy radiation. Three independent experiments were repeated. β-Actin was used as the loading control. C: densitometric ratio of p-Akt/Akt, p53/β-actin or cleaved caspase-3/β-actin is presented. Values are expressed as means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.05 vs. IR + PDGF-BB; #P < 0.05 vs. IR + bFGF. D: intestinal apoptosis in mice with or without perifosine 24 h after 15-Gy radiation; magnification ×400. E: apoptotic index in the crypts measured by TUNEL staining. Values are means ± SD; n = 4 in each group. *P < 0.01 vs. untreated; #P < 0.001 vs. IR + PDGF-BB; #P < 0.005 vs. IR + bFGF. F: representative images of intestinal Lgr5-positive stem cells in Lgr5 knock-in mice 48 h after 15-Gy radiation; magnification ×400. G: intestinal Lgr5-positive stem cells were enumerated by counting Lgr5 staining (red) at day 1 after 15-Gy radiation. Values are means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.05 vs. IR + PDGF-BB; #P < 0.01 vs. IR + bFGF.
PDGF-BB and bFGF were associated with a 40% increase (Fig. 1G). This accounts for the fact that intestinal villi were progressively shortened and the lamina propria below the crypt bases was thickened in mice untreated with growth factors on day 3.5 after 15-Gy radiation (Fig. 1C), which explains why, at 8 days following 15 Gy, more than 80% of untreated mice had died while 100% of mice that had received growth factors survived (Fig. 1F). Lethally irradiated mice lived 7.8 days, on average, whereas PDGF-BB or bFGF treatment increased survival to an average of 11.5 or 10.2 days, respectively. These findings suggest that PDGF-BB and bFGF enable intestinal progenitor/stem cells or crypt cells to resist IR damage, thereby preventing radiation-induced gastrointestinal syndrome in mice.

**PDGF-BB and bFGF prevented radiation-induced apoptosis in ISCs.** By confirming radiation-induced apoptosis in ISCs and then using ISC markers in Lgr5 knock-in (Lgr5) mice, we demonstrated the benefit of these growth factors in terms of apoptosis of CBCs (Fig. 2, A and C), which are located between Paneth cells and were stained by the MMP-7 marker. At 24 h after 15-Gy radiation, PDGF-BB and bFGF treatments had reduced apoptotic intestinal Lgr5+ stem cells by almost 50% (Fig. 2D). Furthermore, the mice treated with growth factors had 40% more intact intestinal Lgr5+ stem cells than those that did not receive growth factor treatment (Fig. 2B). These results indicate that PDGF-BB and bFGF may decrease radiation-induced ISC apoptosis.

**Inhibition of p53 expression alleviated radiation-induced apoptosis.** Since p53 protein is generally believed to be closely related to apoptosis, we explored the effect of blocking p53 signaling on IR-induced intestinal apoptosis. p53 inhibitor pifithrin-α significantly prevented intestinal apoptosis and cleaved-3 activity following 15 Gy of radiation (Fig. 3, A–C and F). Lethally radiated mice lived an average of 7.4 days,

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**Fig. 6.** PDGF-BB and bFGF curtailed IR-stimulated apoptosis via suppression of p53 through Akt. A: TUNEL analysis and p53 immunofluorescence staining of IEC-6 cultured cells pretreated with or without perifosine followed by growth factors 24 h after 15-Gy radiation; magnification ×100. B: apoptotic index in vitro was calculated by counting a minimum of 20 randomly selected fields following TUNEL staining. The index was obtained by dividing the number of TUNEL-positive cells by the total number of cells. Values are means ± SD; n = 4 in each group. *P < 0.01 vs. untreated; #P < 0.05 vs. IR+PDGF-BB; #P < 0.01 vs. IR+bFGF. C: Akt activity, p53 expression, and cleaved caspase-3 expression were measured by Western blotting in cultured IEC-6 cells with or without perifosine treatment (Akt inhibitor) 24 h after 15-Gy radiation. Three independent experiments were performed. β-Actin was used as the loading control. D: densitometric ratio of p-Akt/Akt, p53/β-actin, or cleaved caspase-3/β-actin is represented. Values are expressed as means ± SD; n = 4 in each group. *P < 0.05 vs. untreated; #P < 0.05 vs. IR+PDGF-BB; #P < 0.05 vs. IR+bFGF.
whereas pifithrin-α treatment increased survival to 10.9 days, on average. The survival rate was significantly improved in mice with pifithrin-α compared with those who had not received pifithrin-α (Fig. 3D). Consistently, using a HCT116 p53−/− cell line, we found that 15-Gy radiation caused marked apoptosis in HCT116 cells after 24 h, which was significantly decreased in HCT116 p53−/− cells (data not shown), and p53 deficiency abrogated the IR-induced mitochondrial-mediated apoptosis (Fig. 3, E and G). These results indicate that p53 is crucial to IR-induced apoptosis.

**PDGF-BB and bFGF downregulated p53 expression in intestinal crypt cells in irradiated mice.** Next, we used real-time PCR and Western blotting to examine the effects of growth factor treatment on p53 expression in the intestinal crypts of irradiated mice. p53 mRNA was induced by ~10-fold 24 h after 15-Gy radiation (Fig. 4A). This induction was...
curtailed by at least 70% with PDGF-BB and bFGF treatment (Fig. 4A). An increase in p53 protein was observed 24 h after irradiation, which was significantly suppressed by PDGF-BB and bFGF treatment (Fig. 4, B–D). The p53 protein expression index revealed that p53 expression induced by IR was restricted to the 0–12 cell positions from the crypt base, which accounted for 90% of p53 induction (Fig. 4E). A lower dose (1.17 μg) of PDGF-BB and bFGF also greatly reduced IR-induced p53 expression in the intestinal crypts (data not shown). Notably, expression of the apoptotic indicator cleaved caspase-3 protein varied in a manner similar to p53 expression (Fig. 4, B–D). These results demonstrated that PDGF-BB and bFGF inhibit IR-induced p53 expression in intestinal crypt cells.

PDGF-BB and bFGF curtailed IR-stimulated apoptosis via suppression of p53 through Akt. It is well known that p53 is a target for Akt during cell proliferation. To investigate the mechanism by which growth factors regulate IR-induced intestinal p53 expression, we detected Akt expression with or without the Akt inhibitor perifosine in vivo and in vitro. In mice, 15 Gy of radiation did not induce p-Akt expression, which was markedly enhanced by PDGF-BB and bFGF treatment (Fig. 5B). Expression of p53 and cleaved caspase-3 was significantly increased by 15-Gy radiation and was reduced markedly by PDGF-BB or bFGF treatment. In contrast, the chemical agent perifosine blocked growth factor-induced p-Akt expression while facilitating expression of p53 and cleaved caspase-3 induced by 15-Gy radiation (Fig. 5, A–C). With TUNEL analysis, the benefit of growth factors on IR-induced intestinal apoptosis was counteracted by the Akt inhibitor perifosine (Fig. 5, D and E). Nevertheless, we found that perifosine decreased the survival of ISCs in Lgr5 mice that received PDGF-BB and bFGF treatment (Fig. 5, F and G). To obtain further data on this topic, we used cell line IEC-6 and again found that the Akt inhibitor perifosine reversed growth factor-induced radioprotection and aggravated IR-induced apoptosis in vitro (Fig. 6, A–D). These results indicate that PDGF-BB and bFGF perform as radiomitigators in the intestine by downregulating p53 expression via activation of Akt signaling.

p53 overexpression compromised the radioprotection of PDGF-BB or bFGF in IEC-6 cells. Our results indicated that PDGF-BB and bFGF reduced IR-induced apoptosis in intestinal crypt cells and IEC-6 cells via downregulation of p53 induction. To further show that p53 is important downstream of PDGF-BB and bFGF for radioprotection, we transfected IEC-6 cells with p53 plasmid. We found that p53 overexpression could partially counteract the radioprotection by PDGF-BB and bFGF in IEC-6 cells at 24 h after 15-Gy treatment, although PDGF-BB and bFGF still markedly improved IR-stimulated cell apoptosis in IEC-6 cells even under high p53 expression (Fig. 7, A and B). Enhanced Akt activity was inhibited partially, and downregulated p53 expression was also restored partially in IEC-6 cells under p53 overexpression after PDGF-BB and bFGF treatment at 24 h after 15-Gy (Fig. 7, C–F). These findings are consistent with the PDGF-BB and bFGF regulation of IR-induced apoptosis via the Akt/p53 pathway.

PDGF-BB and bFGF diminished intestinal crypt mitochondrial apoptosis in response to radiation via the Akt pathway. To examine the mechanisms of p53-mediated apoptosis following IR, we analyzed several mitochondria-related events in intestinal crypts. Radiation induced cytosolic release of cytochrome c and mitochondrial translocation, which had been virtually blocked by PDGF-BB and bFGF treatment (Fig. 8, A and B). However, growth factor-induced mitochondrial radioprotection was reversed by the Akt inhibitor perifosine (Fig. 8, A and B).

DISCUSSION

The fundamental molecular underpinnings of IR-induced gastrointestinal syndrome remain relatively unclear. Intestinal
stem cell insulted by IR is an important element in the process. Various researchers have suggested that the primary initiating factor of GI syndrome is IR-induced apoptosis in two types of ISCs that reside at position 4 from the bottom or the crypt base bottom, whereas others believe that IR first targets vascular endothelial cells in the crypt-villus axis, then switches to ISCs as the target (1, 4, 7, 12, 27). Earlier studies demonstrated that several growth factors decrease IR-induced ISC death in the intestinal epithelium (2). This study indicates that p53 is an important target of PDGF-BB- and bFGF-mediated radioprotection in intestinal progenitor/stem cells (Figs. 1 and 4). These findings suggest that suppression of p53 by PDGF-BB and bFGF, which confers protection from IR-induced intestinal injury, may be generalized to other growth factors. However, 40% of IR-induced intestinal apoptosis was not inhibited by the prohibitive effect of PDGF-BB or bFGF on p53, suggesting that, in addition to p53, other proteins are involved in apoptotic processing. Prior studies have shown that tumor necrosis factor-α (TNF-α) (20), prostaglandin E₂ (11), interleukin-1 (20), and lysophosphatidic acid (6) could ameliorate the sensitivity of intestinal progenitor/stem cells to IR-induced apoptosis via downregulation of caspase-9 or transforming growth factor-β. It seems plausible that combining two or more benefit-proven growth factors that target different apoptotic-related proteins may better prevent radiation-induced intestinal apoptosis. The analysis of radiomitigators combined with growth factors in intestinal apoptosis induced by IR is planned as the focus of our next study.

The Akt pathway has been identified as an antiapoptotic effector of PDGF signaling (18). Both the MAPK and Akt pathways are believed to interplay with bFGF in promoting survival, although their relative contributions are not well understood (14). Our results showed that the Akt pathway was activated by both PDGF-BB and bFGF in the GI system, suppressing the expression of a critical apoptotic initiator, p53, following radiation (Fig. 5). The degree of suppression of IR-induced intestinal p53 expression was fairly similar between the two growth factors. These observations are consistent with those previous studies in which bFGF promoted radiation-induced crypt reformation (10) and engaged p53 signaling to enhance the survival of fibroblasts (25), and fibroblast growth factor receptor FGFR3 was massively expressed in the bottom of the crypts (8). Our data suggest that the Akt/p53 axis may be a common mechanism of growth factor-mediated intestinal radioprotection. However, whether MAPK-related kinases such as JNK and p38 family members regulate Akt or p53 activities and PDGF-BB- or bFGF-mediated intestinal radioprotection remains unknown. Next, the effects on intestinal radioprotection on growth factors and the JNK and p38 pathways should be investigated in future work.

p53 was found to cause apoptosis following cytokine deprivation (33) and to regulate apoptotic responses to a wide range of genotoxic stresses through transcriptional activation of its downstream targets (34). Our results also indicate that p53, as a central calibrator in response to DNA damage, activates mitochondrial-mediated cell apoptosis (Figs. 7 and 8). In ISCs, p53 is activated by the ATM/Chk2 pathway following radiation (4), whereas growth factors activate Akt to reduce p53 expression (Fig. 4). The extent of p53 induction largely determines the survival of ISCs and subsequent crypt regeneration. On the other hand, other proteins including ataxia-telangiectasiasia mutant (ATM) (4), p53 upregulated modulator of apoptosis (PUMA) (22), p21 (16), p53BP1 (5), and polymerase-1 (PARP-1) (13), have been found to regulate IR-induced GI damage and crypt cell apoptosis. It is thus possible that proteins other than p53 are involved in growth factor-mediated intestinal radioprotection. Nevertheless, in our studies, administration of growth factors indicated that their antiapoptotic roles were more important for intestinal radioprotection. However, our findings do not exclude the involvement of other signaling pathways in the epithelial or endothelial compartment, particularly when exposed to different doses of radiation (30). Whether the other regulators significantly impact IR-induced GI syndrome independently of p53 remains to be determined.

Next, by utilizing ISC lineage tracing models, we aim to investigate the critical regulator of the survival and regeneration of ISCs damaged by IR.

In summary, we demonstrated that the underlying mechanism of growth factor-mediated intestinal radioprotection depends on manipulation of Akt/p53 signaling. This provides potential targets for radiomitigators that selectively protect ISCs against IR-induced apoptosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Z.L. and H.L. performed experiments; Z.L., H.L., J.J., S.T., Y.Y., Y.Z., and B.W. analyzed data; Z.L., H.L., and J.J. interpreted results of experiments; Z.L., H.L., J.J., and B.W. prepared figures; Z.L. and J.J. drafted manuscript; B.W. conception and design of research; B.W. edited and revised manuscript; B.W. approved final version of manuscript.

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