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Growth factors protect intestinal stem cells from radiation-induced apoptosis by suppressing PUMA through the PI3K/AKT/p53 axis

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Abstract

Gastrointestinal toxicity is the primary limiting factor in abdominal and pelvic radiotherapy, but has no effective treatment currently. We recently showed a critical role of the BH3-only protein PUMA in acute radiation-induced GI damage and GI syndrome in mice. Growth factors such as IGF-1 and bFGF have been shown to protect against radiation-induced intestinal injury, although the underlying mechanisms remain to be identified. We report here the suppression of PUMA through the PI3K/AKT/p53 axis in the intestinal stem cells (ISCs) as a novel molecular mechanism of growth factor-mediated intestinal radioprotection. IGF-I or bFGF impaired radiation-induced apoptosis and the expression of PUMA and p53 in the crypt cells and intestinal stem cells. Using colonic epithelial cells that undergo PUMA-dependent and radiation-induced apoptosis, we found that a PI3K inhibitor, dominant-negative PI3K, or Mdm2 antagonist restored the induction of PUMA, p53, and apoptosis in the presence of growth factors. In contrast, overexpression of AKT suppressed the induction of PUMA and p53 by radiation. Furthermore, inhibiting PI3K or activating p53 abrogated growth factor-mediated suppression of apoptosis and PUMA expression in the intestinal crypts and stem cells following radiation.

Keywords

PUMA; Growth factors; p53; apoptosis; intestinal stem cells; PI3K

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Introduction

Rapidly renewed tissues such as bone marrow, gut, and hair follicles are the most sensitive tissues in the human body to apoptosis induced by DNA damage. Gastrointestinal toxicity is the primary limiting factor in abdominal and pelvic radiotherapy, but has no effective treatment currently. Adult tissue stem cells are believed to be responsible for maintaining tissue homeostasis and regeneration following injury. Several elegant genetic studies in mice demonstrated that Lgr5 (Barker *et al.*, 2007), CD133/Prominin 1 (Zhu *et al.*, 2009), and Bmi-1 (Sangiorgi and Capecchi, 2008) expressing cells at or near the crypt base are intestinal stem cells (ISCs). The cells in at least two locations exhibit the properties of ISCs: the columnar cells at the crypt base (CBCs) and some +4 cells immediately above the Paneth cells. Work from us and others demonstrate that apoptosis in these cells is largely responsible for the acute intestinal damage and rapid onset of GI syndrome and death using a whole body radiation (WBR) model (Ch'ang *et al.*, 2005; Potten, 2004; Qiu *et al.*, 2008). Growth factors protect against radiation or chemotherapy-induced mucosal injury (Booth and Potten, 2001). For example, insulin-like growth factor 1 (IGF-1), interleukin 11, keratinocyte growth factor (KGF), and fibroblast growth factor-2 (FGF-2 or bFGF-2) have been shown to protect the +4 cells and increase animal survival following WBR, but their targets and the underlying mechanism of intestinal protection are not well understood (Booth and Potten, 2001; Paris *et al.*, 2001; Wilkins *et al.*, 2002).

The Bcl-2 family of proteins are evolutionarily conserved regulators of apoptosis, whose levels or activities are subjected to transcriptional or posttranslational regulation (Adams and Cory, 2007; Korsmeyer, 1999). The BH3-only subgroup of proteins appear to initiate and promote apoptosis in a cell type- and stimulus-specific manner (Labi *et al.*, 2006; Yu and Zhang, 2004). We and others identified PUMA as a BH3-only protein and a transcriptional target of p53 that plays an essential role in p53-dependent and -independent apoptosis through the mitochondrial pathway (Han *et al.*, 2001; Nakano and Vousden, 2001; Yu *et al.*, 2001). The potent proapoptotic function of PUMA compared to most other BH3-only members perhaps rests in its ability to effectively neutralize all five known antiapoptotic Bcl-2-like proteins (Labi *et al.*, 2006; Yu and Zhang, 2008). Our work suggests PUMA as a major mediator of apoptosis in the intestinal epithelium in response to various genotoxic and nongenotoxic stresses (Ming *et al.*, 2008; Qiu *et al.*, 2008; Wu *et al.*, 2007; Yu *et al.*, 2007; Yu *et al.*, 2003; Yu *et al.*, 2001). Our recent work indicated that *PUMA* deficiency protects the ISCs (both CBCs and +4 cells) and progenitors from radiation-induced apoptosis and improves crypt regeneration (Qiu *et al.*, 2008). The similar extent of apoptosis deficiency in the crypts of *PUMA* KO and *p53* KO mice suggests that PUMA is a mediator of the p53-dependent and radiation-induced apoptosis in the intestinal crypts and stem cells (Komarova *et al.*, 2004; Merritt *et al.*, 1994; Qiu *et al.*, 2008).

p53 regulates radiation-induced apoptosis in the crypts of the small intestine (Komarova *et al.*, 2004; Merritt *et al.*, 1994). The PI3K/AKT pathway confers the antiapoptotic function of IGF-1 in various cell types (Butt *et al.*, 1999), and is also activated by bFGF in some cells (Katoh, 2006). Connections between the p53 and IGF-I/AKT signaling pathways have been delineated in worms, flies, and mammals (Levine *et al.*, 2006). The PI3K/AKT pathway is activated by IGF-I signaling, which leads to phosphorylation and activation of MDM2, and

subsequent inactivation of p53 though the ubiquitin-proteasome system (Gottlieb *et al.*, 2002; Levine *et al.*, 2006). Connections between p53 and bFGF signaling have also been described. For example, bFGF induced the synthesis of MDM2, leading to reduced activation of p53 and protection from DNA-damage-induced cell death in fibroblasts (Shaulian *et al.*, 1997). FGF1 was found to inhibit p53-dependent apoptosis in neuronal cells treated with etoposide (Bouleau *et al.*, 2007), while bFGF protected cultured endothelial cells from radiation-induced apoptosis (Gu *et al.*, 2004). However, whether there is a common mechanism for IGF-I or bFGF and the PI3K/AKT/p53 pathway in radiation responses of the GI system remains to be elucidated.

Therefore, we hypothesize that inhibiting PUMA-mediated apoptosis in the intestinal stem cells may be a common mechanism of growth factor-mediated intestinal radioprotection. In this study, we investigated the role and mechanisms of PUMA in IGF-I and bFGF-mediated intestinal radioprotection. We demonstrated that the suppression of PUMA plays a critical role in IGF-I and bFGF-mediated radioprotection in the GI system through a PI3K/AKT/p53-dependent mechanism.

Materials and Methods

Mice and treatment

The procedures of all animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Eight to ten week-old *PUMA*$^{+/+}$ (WT), *PUMA*$^{-/-}$ (*PUMA* KO) and *p53*$^{-/-}$ (*p53* KO) littermates (F6 on C57BL/6 background) were generated from heterozygote breeding. The mice were housed in micro-isolator cages in a room illuminated from 7:00 AM to 7:00 PM (12:12-hr light-dark cycle), and allowed access to water and chow *ad libitum*. Genotyping of WT, *PUMA* KO, and *p53* KO alleles was performed as previously described (Wu *et al.*, 2007). Mice were irradiated at doses ranging from 0 to 18 Gy at a rate of 82 cGy/min using a ^{137}Cs irradiator (Mark I, J.L. Shepherd and Associates, San Fernando, CA).

Growth factor treatment: Mice were injected i.v. with 3.5 μg human recombinant IGF-I and 3.5 μg bFGF in three doses (1.17 μg/dose) delivered 30 min before, 5 min before, and 30 min after radiation. In some experiments, mice were treated additionally 1h after radiation with 10 mg/kg nutlin-3a (Sigma-Aldrich, St Louis, MO) through i.p. injection, or 1 hr before the first dose of growth factor with 40 mg/kg LY294002 (Cayman Chemical, Ann Arbor, MI) through i.p. injections. Mice were sacrificed to collect the small intestines for histology analysis and Western blotting at 4 hr and/or 24 hr after radiation. The experiment was repeated 3 times.

TUNEL staining and crypt microcolony assay

Both have been previously described using 5 μm sections (Qiu *et al.*, 2008). In brief, following TUNEL staining, the number of TUNEL positive cells in each crypt was scored in 100 crypts per section and reported as mean ± s.e.m. Three mice were used in each group. The survival of ISCs was quantified by counting regenerated crypts 4 days after irradiation in H&E stained cross sections. Surviving crypts were defined as containing 5 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, with each about 1 cm apart. Three mice were used in each group.

Immunohistochemistry (IHC) and immunoflourescence (IF)

p-AKT and Ki67 IHC

IHC was performed as previously described (Qiu *et al.*, 2008). In brief, sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide. Following antigen retrieval, sections were blocked with 10% goat serum for 30 minutes at RT. The sections were incubated with rabbit anti-p-AKT antibody (Cell Signaling, Beverly, MA) or rat anti-Ki67 antibody (DAKO, Carpinteria, CA) diluted 1:100 overnight at 4°C. The signal was detected with the Envision kit (DAKO, Carpinteria, CA). The sections were counter-stained with hematoxylin.

p-AKT/MMP-7 double staining

The p-AKT staining was performed as above without the counter stain followed by MMP-7 staining as described (Qiu *et al.*, 2008). The sections were then incubated with mouse anti-MMP-7 antibody (eBioscience, San Diego, CA) at 1:100 dilutions overnight at 4°C. The signals were detected with the biotinylated secondary antibodies (Pierce, Rockford, IL), and developed by alkaline phosphatase streptavidin (SA-AP) (Jackson ImmunoResearch Laboratories, West Grove, PA) and BCIP/NBT liquid substrate system (Sigma) following the manufacturer's instructions.

TUNEL/MMP-7/DAPI multicolor IF

TUNEL staining was performed using the ApopTag® Plus IFC In Situ Apoptosis Detection Kit (Millipore, Billerica, MA). Antigen retrieval was performed after TUNEL staining in 0.1 M Citrate Buffer Antigen Retrieval Solution (pH 6.0) as described above. The sections were blocked with 20% goat or rabbit serum for 30 min at RT and then incubated with mouse anti-MMP-7 antibody (eBioscience, San Diego, CA) at a 1:100 dilution overnight at 4°C. The signals were detected with the biotinylated secondary antibodies (Pierce, Rockford, IL), and developed by Alexa 594 Streptavidin (Invitrogen) following the manufacturer's instructions. The sections were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories).

Cell Culture, treatment and expression constructs

The human colorectal cancer cell line DLD1, HCT116 (ATCC, Manassas, VA), the *p21* targeted HCT116 *p21* KO (Waldman *et al.*, 1995), and *p53* targeted HCT116 *p53* KO cells (Bunz *et al.*, 1998) were cultured in McCoy's 5A modified medium (Invitrogen, Carlsbad, CA) supplemented with 10% defined FBS (HyClone, Logan, UT), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were maintained at 37°C with 5% CO_2. Cells were plated in 12-well plates at 20–30% density 24 hr before treatment with 1% serum. IGF-I (100 ng/ml) and bFGF (400 ng/ml) (Pepro Tech, Rocky Hill, NJ) were added into medium 1 hr before 15 Gy irradiation. The medium was changed with freshly added IGF-I and bFGF 1 hr after IR. Cells were irradiated at a rate of 82 cGy/min using a ^{137}Cs

irradiator (Mark I, J.L. Shepherd and Associates, San Fernando, CA). In some experiments, 50 μM LY294002 was added to cells 1 hr prior to IGF-I or bFGF treatment, or 10 μM nutlin-3a was added 8 hr after IR. The cells were collected and analyzed for protein expression and apoptosis at 24 and 48 hr after IR, respectively. The AKT dominant-negative PI3K (p85) expression plasmids have been previously described (Sun *et al.*, 2009). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions and subjected to radiation 24 hr after transfection.

Knockdown of Foxo3a by small interference RNA

HCT116 *p21* KO cells were transfected with ON-TARGETplus Duplex siRNA specific for *Foxo3a* (J-003007-10; Dharmacon, Lafayette, CO) or the control scrambled siRNA by Lipofectamine 2000 (Invitrogen). The cells were irradiated with 15 Gy and harvested 24 hr later for analysis.

Nuclear Fragmentation Assay

Apoptosis of the cultured cells was quantified by nuclear fragmentation assay. Both attached and floating cells were harvested at various time points after the treatment. Apoptosis was analyzed by counting cells with condensed chromatin and micronucleation after nuclear staining with Hoechst 33258 as described (Kohli *et al.*, 2004; Yu *et al.*, 1999). A minimum of 300 cells were analyzed in triplicate.

Total RNA extraction and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA and cDNA were prepared from freshly scraped intestinal mucosa as described (Qiu *et al.*, 2008). The primers used for *PUMA* and *β-actin* have been described (Qiu *et al.*, 2008). Real-time PCR was performed on a Mini Opticon Real-time PCR System (BioRad, USA) with SYBR Green (Invitrogen, Carlsbad, CA). Three mice were used in each group. Melting curve and agarose gel electrophoresis of the PCR product were used to verify the specificity of PCR amplification.

Western Blotting and Antibodies

Cell lysates were collected and Western blotting was performed as described (Yu *et al.*, 2003). The antibodies used for Western blotting included antibodies against human PUMA (Yu *et al.*, 2003), p53 (DO1) (Santa Cruz Biotechnologies, Santa Cruz, CA), p21, α-tubulin (EMD Biosciences), human and mouse phospho-AKT (Ser473, p-AKT), total-AKT, p38, p-p38 (Thr180/Tyr182), p44/42, p-p44/42 (Thr202/Tyr204), p-MDM2 (Ser166), c-Myc, p-Foxo3a (Thr32) (Cell Signaling, Beverly, MA), Foxo3a (Millipore), MDM2 (Santa Cruz), and antibodies against mouse PUMA (Abcam, Cambridge, MA), p53, p21 (Santa Cruz), and β-actin (Sigma).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism IV software. p-values were calculated by the student's t-test. p-values less than 0.05, indicating the probability of the

difference occurring by chance was less than 5 in 100, were considered significant. The means ± one standard deviation (SD) were displayed in the figures where applicable.

Results

Growth factors protected against radiation-induced apoptosis in the intestinal crypts and stem cells

Using a whole body radiation (WBR) model, we found that 15 Gy induced marked apoptosis in the intestinal crypts of WT mice at 24 hr, which was blocked by 45% and 66% by three doses of IGF-I and bFGF treatment, respectively (Figs.1A and 1B, and S1A). Using multicolor immunofluorescence, we determined the effects of these two growth factors specifically on the apoptosis of the CBCs, which reside at the bottom of the crypts in between Paneth cells that were stained positive for MMP-7 (Qiu *et al.*, 2008). Notably, IGF-I or bFGF blocked the apoptosis in the CBCs by over 60% 24 hr after radiation (Fig. 1C). A microcolony assay was used to measure the regeneration capacity of intestinal stem cells 4 days after 15 Gy radiation, which gives an indirect yet functional measurement of the ISC survival. We found that IGF-I or bFGF (3.5 μg/mouse) led to a nearly 2-fold (200%) increase in crypt regeneration (Figs. 1D and S1B). A lower dose of IGF-I treatment (1.8 μg/mouse) also significantly protected against radiation-induced apoptosis in the crypts and led to enhanced crypt regeneration (data not shown). To determine the effect of these factors on non-epithelial compartments, we examined the extent of apoptosis in the villus, which was similar 4 or 24 hr after 15 Gy radiation regardless of IGF-I or bFGF treatment (Fig. S1C and S1D, and data not shown).

Growth factors such as bFGF protect endothelial cells from radiation-induced apoptosis (Paris *et al.*, 2001; Qiu *et al.*, 2008). Using double staining of the endothelial cell-specific marker CD105 and TUNEL, we confirmed that IGF-1 or bFGF inhibited endothelial cell apoptosis by 34% or 63%, respectively, at 4 hr after 15 Gy radiation (Fig. S2A and B). This correlated with slightly increased $CD105^+$ cell counts in the villi (Fig. S2C). However, neither growth factor affected proliferation in the crypt at 4 or 24 hr after radiation in WT mice or *PUMA* KO mice based on Ki67 staining (Fig. S3). These results indicate that IGF-I and bFGF impaired radiation-induced and PUMA-dependent apoptosis in the stem cells and progenitors in the small intestine.

Growth factors reduced radiation-induced PUMA and p53 expression in the intestinal crypts

We then examined the effects of growth factor treatment on PUMA expression by real-time RT-PCR and western blotting in the intestinal mucosa of wild-type (WT) mice. *PUMA* mRNA was induced by approximately 9-fold 4 hr after 15 Gy WBR, consistent with our earlier study (Qiu *et al.*, 2008). This induction was decreased by at least 60% with IGF-I or bFGF treatment, respectively (Fig. 2A). The elevation of PUMA protein was evident 24 hr after irradiation, which was significantly blocked by IGF-I or bFGF treatment (Fig. 2B). A lower dose of IGF-I treatment (1.8 μg/mouse) also significantly suppressed PUMA induction by radiation in the intestinal mucosa (data not shown). We have previously shown that radiation induces *PUMA* in the CBCs and +4-+9 cells, which is p53-dependent (Qiu *et*

al., 2008). We therefore analyzed the levels of p53 and its target p21. We found that the induction of p53 and p21 was reduced by IGF-I or bFGF treatment. Importantly, IGF-1 or bFGF did not further suppress crypt apoptosis or enhance crypt proliferation and regeneration in *PUMA* KO mice (Figs. S3 and S4). The protection on ISCs against apoptosis provided by *PUMA* deficiency exceeds that by growth factors (Figs. S4 and 1) (Qiu *et al.*, 2008), perhaps reflecting the residual p53 activity and resulting PUMA induction in the presence of either growth factor (Fig. 2). These results demonstrate that IGF-I or bFGF reduced radiation-induced PUMA and p53 expression in the intestinal crypts.

Growth factors protected against radiation-induced apoptosis through the PI3K/AKT pathway *in vitro*

To further elucidate the mechanisms of PUMA regulation by growth factors following radiation, we tested whether IGF-I and bFGF are radioprotective in the colonic epithelial HCT116 cells that are deficient in *p21*. Our previous studies showed that radiation induces PUMA-dependent apoptosis in these cells (Wang *et al.*, 2007; Yu *et al.*, 2003). Radiation (15 Gy) induced about 60% apoptosis in HCT116 *p21* KO cells 48 hr after radiation, which was significantly blocked by IGF-I and bFGF (Fig. 3A). *PUMA* mRNA was induced by about 7-fold 24 hr after radiation. However, the induction was blocked by over 70% by IGF-I or bFGF treatment (Fig. 3B). Consistently, the levels of PUMA protein were significantly inhibited (Fig. 3C). Given an antiapoptotic role of the PI3K/AKT pathway downstream of IGF-I or bFGF signaling, we examined AKT activation. The levels of phosphorylated AKT (p-AKT) were significantly elevated by IGF-I or bFGF treatment (Fig. 3C). We also examined a possible involvement of p38 and p44/42 MAPKs. No change in p38 levels or phosphorylation, or only a small increase in phosphorylated p44/42 was detected in response to growth factors (Fig. S5). However, their inhibitors SB203580 and PD98059 did not significantly affect either PUMA induction or apoptosis induced by radiation (data not shown). In contrast, the pretreatment of PI3K inhibitor LY294002 reversed the inhibition of PUMA by either growth factor (Fig. 3D). The patterns of p53 expression changes were similar to those of PUMA in these experiments (Fig. 3C and 3D). Furthermore, PUMA was found to be induced by radiation in HCT116 cells but not in *p53* deficient (HCT116 *p53* KO) or *p53* mutant (DLD1) cells (Fig. 3E). These data suggest that the regulation of PUMA by p53 through the PI3K/AKT pathway might be involved in growth factor-mediated radioprotection.

Growth factors inhibited PUMA and apoptosis induction by radiation through a p53-dependent mechanism *in vitro*

To further probe this pathway, we overexpressed the dominant-negative PI3K (p85), which was found to abrogate AKT phosphorylation and restore the expression of PUMA and p53 with IGF-I or bFGF treatment (Fig. 4A). In contrast, a constitutive active form of AKT suppressed PUMA and p53 induction by radiation (Fig. 4B). To further investigate the role of p53 in this process, we treated the HCT116 *p21* KO cells with the Mdm2 antagonist nutlin-3a, which prevents ubiquitination-mediated degradation of p53 by disrupting the interaction of p53 from MDM2 (Gottlieb *et al.*, 2002; Shangary and Wang, 2009). Nutlin-3a treatment led to stabilization of p53 and restored the induction of PUMA in the presence of either growth factor (Fig. 4C). Furthermore, growth factor-mediated suppression of

radiation-induced apoptosis was significantly blocked by either LY294002 or nutlin-3a in these cells (Fig. 4D). Foxo3a can mediate PUMA induction in some immune cells in response to cytokine/growth factor withdrawal (You *et al.*, 2006). Interestingly, radiation was found to activate Foxo3a as suggested by decreased phosphorylation (Fig. 4A) (Yang *et al.*, 2006). However, knockdown of *Foxo3a* by siRNA had no effect on PUMA induction after radiation (Fig. 4E). These results suggest that activation of the PI3K/AKT pathway by growth factors blocks PUMA induction by radiation in colonic epithelial cells through p53, but not Foxo3a.

Growth factors protected the intestinal stem cells from radiation-induced apoptosis through the PI3K-AKT-p53-PUMA axis

To validate whether the mechanism of PUMA regulation by IGF-I and bFGF described above operates *in vivo*, we examined the phosphorylated-AKT expression in the intestinal crypts by immunohistochemistry. Phosphorylated AKT was almost completely absent in the crypts in either the unirradiated or irradiated mice, while cytoplasmic p-AKT was greatly induced in the bottom of crypts and in the CBC area following IGF-I or bFGF treatment (Fig. 5A). Using dual color immnunohistochemistry, we found that the phosphorylated-AKT was induced to high levels in the CBCs stained negative for MMP-7 (Fig. 5B). Western blotting results also confirmed the induction of phosphorylated-AKT in the intestinal mucosa (Fig. 5C). The treatment of PI3K inhibitor LY294002 or the MDM2 antagonist nutlin-3a abrogated the protection against apoptosis in the CBCs and crypt cells afforded by growth factors (Figs. 5C, 5D, S6 and S7). Consistently, both LY294002 and nutlin-3a restored the induction of PUMA and p53 (Fig. 5C and 5D). In addition, we also found that apoptosis in the CBCs was reduced more in *PUMA* KO mice than in *p53* KO mice (Fig. 6). These data demonstrate that the suppression of PUMA induction through the PI3K/AKT/p53 axis in the intestinal stem cells is an important mechanism of growth factor-mediated intestinal radioprotection.

Discussion

A number of growth factors protect against radiation injury in the gut of mice, though the underlying molecular mechanisms are unclear (Booth and Potten, 2001; Wilkins *et al.*, 2002). A better understanding of such mechanisms is critical for developing strategies to preserve the normal functions of the GI tract upon radiation injury. Earlier studies suggested that the protective effects of growth factors in both the endothelial and epithelial compartments might be important (Booth and Potten, 2001; Paris *et al.*, 2001; Wilkins *et al.*, 2002). Our current study demonstrates PUMA as a critical target of IGF-I- and bFGF-mediated radioprotection in the intestinal stem cells and crypts. Both IGF-I and bFGF reduced radiation-induced apoptosis and PUMA expression in the small intestinal crypts and stem cells, while they provided no additional protection in *PUMA*-deficient mice. Furthermore, our mechanistic data indicate the PI3K/AKT/p53 axis mediates PUMA suppression in response to growth factors *in vitro* and *in vivo*. These observations suggest that downregulation of PUMA is a common mechanism underlying intestinal radioprotection by growth factors.

The PI3K/AKT pathway is a well established antiapoptotic effector of the IGF-1 signaling (Levine *et al.*, 2006). Both the MAPK and PI3K/AKT pathways have been documented as downstream effectors of bFGF in promoting survival, though their relative contributions are not well understood (Katoh, 2006). Our results showed that the PI3K/AKT pathway is activated by both IGF-I and bFGF in the GI system to suppress the expression of a key apoptotic initiator, PUMA, following radiation. A slightly better protection against apoptosis was provided by bFGF *in vitro* and *in vivo*, which is correlated with a more efficient suppression of PUMA and p53. These observations are consistent with earlier ones in which bFGF enhanced radiation-induced crypt regeneration (Houchen *et al.*, 1999), and engaged the MDM2-p53 axis to promote the survival of fibroblasts (Shaulian *et al.*, 1997), and the enriched expression of FGFR3 in the bottom of the crypts (Gulati *et al.*, 2008). Several MAPK-related kinases such as JNK (Fuchs *et al.*, 1998) and p38 (Sanchez-Prieto *et al.*, 2000) family members have been shown to regulate p53 activities and genotoxic stress-induced apoptosis. However, we did not find definitive evidence for the involvement of either p38 or p44/42 in IGF-1 or bFGF-mediated protection against radiation-induced apoptosis or PUMA expression in HCT116 *p21* KO cells. Our results suggest that the PI3K/AKT/p53 axis is likely to be a common mechanism of growth factor-mediated intestinal radioprotection, while PUMA-independent mechanisms in the protection of endothelial cells against apoptosis remain to be defined (Paris *et al.*, 2001; Qiu *et al.*, 2008) (Fig. S2).

PUMA has been reported to be regulated by survival and growth factors in cancer cells independently of p53 (Han *et al.*, 2001; Ming *et al.*, 2008; You *et al.*, 2006). This study together with our previous results demonstrates that PUMA induction by radiation is completely p53-dependent (Qiu *et al.*, 2008; Wang *et al.*, 2007; Yu *et al.*, 2003). Despite its regulation by radiation and growth factors, Foxo3a does not appear to be a main regulator of PUMA expression following radiation. p53 status is clearly important in the responses to genotoxic stresses in many types of normal cells in several ways (Vousden and Lu, 2002; Yu and Zhang, 2005), and our data provide an explanation in the intestinal epithelium. p53 was firstly and serendipitously found to induce apoptosis following cytokine deprivation (Yonish-Rouach *et al.*, 1991), and later found to regulate apoptotic responses to a wide range of genotoxic and oncogenic stresses through transcriptional activation of its downstream targets (Vousden and Lu, 2002; Yu and Zhang, 2005). Our data suggest that p53, as a central regulator of DNA damage response, cleverly integrates signals from a variety of sources upon DNA damage to determine cell fate. In intestinal stem cells, p53 is activated by the ATM/Chk2 pathway following radiation (Ch'ang *et al.*, 2005), while growth factors activate the PI3K/AKT pathway to suppress p53 through MDM2 (this study). The extent of p53-dependent PUMA induction largely determines the survival of intestinal stem cells and subsequent crypt regeneration. Similar mechanisms might operate in radiosensitive tissues such as thymocytes, which undergo p53 and PUMA-dependent apoptosis (Jeffers *et al.*, 2003; Villunger *et al.*, 2003). It is important to point out that crypt apoptosis is blocked at a similar extent in mice deficient in either *p53* or *PUMA*, while crypt regeneration only increased in *PUMA* KO mice (Komarova *et al.*, 2004; Qiu *et al.*, 2008). This would suggest mechanisms that are dependent on p53, but not PUMA, are also required for crypt

regeneration. One possibility is that *PUMA* deficiency enhances the survival of the CBCs better than *p53* deficiency (Fig. 6).

Emerging evidence suggests that DNA damage signaling and apoptosis in the stem cell compartment play key roles in intestinal injury and regeneration. A number of genes and pathways have been identified as regulator of radiation-induced GI damage and crypt cell apoptosis, including p53 (Komarova *et al.*, 2004; Merritt *et al.*, 1994), ATM (Ch'ang *et al.*, 2005), PUMA (Qiu *et al.*, 2008), p21 (George *et al.*, 2009; Komarova *et al.*, 2004), p53BP1 (Clarke *et al.*, 2007), and Poly (ADP-ribose) polymerase-1 (PARP-1) (Ishizuka *et al.*, 2003). It is certainly possible that players other than p53 and PUMA are involved in growth factor-mediated intestinal radioprotection. Despite their well-documented growth promoting activities, the administrations of growth factors shortly before and after radiation in our studies would suggest that their antiapoptotic activities are perhaps more critical for intestinal radioprotection. However, our studies can not rule out the involvement of their other activities in the epithelial or endothelial compartment particularly when dosed differently (Booth and Potten, 2001; Paris *et al.*, 2001; Wilkins *et al.*, 2002). Expression or activities of several other BH3-only proteins can be regulated by growth factors independently of p53 (Labi *et al.*, 2006); whether any of them play a role in radiation-induced intestinal damage remains to be elucidated. In addition, the use of recently developed ISC lineage tracing models (Barker *et al.*, 2007; Sangiorgi and Capecchi, 2008) will help better elucidate the critical regulators of the survival and regeneration of ISCs following injury.

In summary, we identified suppression of PUMA by the PI3K/AKT/p53 axis as a common mechanism underlying growth factor-mediated intestinal radioprotection. This provides potential targets for developing radiation mitigators or protectors that can selectively preserve intestinal stem cells to facilitate tissue regeneration. Targeting this pathway by small molecule PUMA inhibitors might provide benefits to cancer patients or victims of radiation poisoning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene. 2007; 26:1324–37. [PubMed: 17322918]

Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007; 449:1003–7. [PubMed: 17934449]

Booth D, Potten CS. Protection against mucosal injury by growth factors and cytokines. J Natl Cancer Inst Monogr. 2001:16–20. [PubMed: 11694560]

Bouleau S, Parvu-Ferecatu I, Rodriguez-Enfedaque A, Rincheval V, Grimal H, Mignotte B, et al. Fibroblast Growth Factor 1 inhibits p53-dependent apoptosis in PC12 cells. Apoptosis. 2007; 12:1377–87. [PubMed: 17473910]

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science. 1998; 282:1497–501. [PubMed: 9822382]

Butt AJ, Firth SM, Baxter RC. The IGF axis and programmed cell death. Immunol Cell Biol. 1999; 77:256–62. [PubMed: 10361258]

Ch'ang HJ, Maj JG, Paris F, Xing HR, Zhang J, Truman JP, et al. ATM regulates target switching to escalating doses of radiation in the intestines. Nat Med. 2005; 11:484–490. [PubMed: 15864314]

Clarke AR, Jones N, Pryde F, Adachi Y, Sansom OJ. 53BP1 deficiency in intestinal enterocytes does not alter the immediate response to ionizing radiation, but leads to increased nuclear area consistent with polyploidy. Oncogene. 2007; 26:6349–55. [PubMed: 17452983]

Fuchs SY, Adler V, Pincus MR, Ronai Z. MEKK1/JNK signaling stabilizes and activates p53. Proc Natl Acad Sci U S A. 1998; 95:10541–6. [PubMed: 9724739]

George RJ, Sturmoski MA, May R, Sureban SM, Dieckgraefe BK, Anant S, et al. Loss of p21Waf1/Cip1/Sdi1 enhances intestinal stem cell survival following radiation injury. Am J Physiol Gastrointest Liver Physiol. 2009; 296:G245–54. [PubMed: 19056768]

Gottlieb TM, Leal JF, Seger R, Taya Y, Oren M. Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. Oncogene. 2002; 21:1299–303. [PubMed: 11850850]

Gu Q, Wang D, Wang X, Peng R, Liu J, Jiang T, et al. Basic fibroblast growth factor inhibits radiation-induced apoptosis of HUVECs. I. The PI3K/AKT pathway and induction of phosphorylation of BAD. Radiat Res. 2004; 161:692–702. [PubMed: 15161350]

Gulati AS, Ochsner SA, Henning SJ. Molecular properties of side population-sorted cells from mouse small intestine. Am J Physiol Gastrointest Liver Physiol. 2008; 294:G286–94. [PubMed: 18006601]

Han J, Flemington C, Houghton AB, Gu Z, Zambetti GP, Lutz RJ, et al. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc Natl Acad Sci U S A. 2001; 98:11318–23. [PubMed: 11572983]

Houchen CW, George RJ, Sturmoski MA, Cohn SM. FGF-2 enhances intestinal stem cell survival and its expression is induced after radiation injury. Am J Physiol. 1999; 276:G249–58. [PubMed: 9887002]

Ishizuka S, Martin K, Booth C, Potten CS, de Murcia G, Burkle A, et al. Poly(ADP-ribose) polymerase-1 is a survival factor for radiation-exposed intestinal epithelial stem cells in vivo. Nucleic Acids Res. 2003; 31:6198–205. [PubMed: 14576306]

Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell. 2003; 4:321–8. [PubMed: 14585359]

Katoh M. FGF signaling network in the gastrointestinal tract (review). Int J Oncol. 2006; 29:163–8. [PubMed: 16773196]

Kohli M, Yu J, Seaman C, Bardelli A, Kinzler KW, Vogelstein B, et al. SMAC/Diablo-dependent apoptosis induced by nonsteroidal antiinflammatory drugs (NSAIDs) in colon cancer cells. Proc Natl Acad Sci U S A. 2004; 101:16897–902. [PubMed: 15557007]

Komarova EA, Kondratov RV, Wang K, Christov K, Golovkina TV, Goldblum JR, et al. Dual effect of p53 on radiation sensitivity in vivo: p53 promotes hematopoietic injury, but protects from gastro-intestinal syndrome in mice. Oncogene. 2004; 23:3265–3271. [PubMed: 15064735]

Korsmeyer SJ. BCL-2 gene family and the regulation of programmed cell death. Cancer Res. 1999; 59:1693s–1700s. [PubMed: 10197582]

Labi V, Erlacher M, Kiessling S, Villunger A. BH3-only proteins in cell death initiation, malignant disease and anticancer therapy. Cell Death Differ. 2006; 13:1325–38. [PubMed: 16645634]

Levine AJ, Feng Z, Mak TW, You H, Jin S. Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways. Genes Dev. 2006; 20:267–75. [PubMed: 16452501]

Merritt AJ, Potten CS, Kemp CJ, Hickman JA, Balmain A, Lane DP, et al. The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. Cancer Res. 1994; 54:614–617. [PubMed: 8306319]

Ming L, Sakaida T, Yue W, Jha A, Zhang L, Yu J. Sp1 and p73 Activate PUMA Following Serum Starvation. Carcinogenesis. 2008; 29:1878–84. [PubMed: 18579560]

Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell. 2001; 7:683–94. [PubMed: 11463392]

Paris F, Fuks Z, Kang A, Capodieci P, Juan G, Ehleiter D, et al. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science. 2001; 293:293–7. [PubMed: 11452123]

Potten CS. Radiation, the ideal cytotoxic agent for studying the cell biology of tissues such as the small intestine. Radiat Res. 2004; 161:123–36. [PubMed: 14731078]

Qiu W, Carson-Walter EB, Liu H, Epperly M, Greenberger JS, Zambetti GP, et al. PUMA regulates intestinal progenitor cell radiosensitivity and gastrointestinal syndrome. Cell Stem Cell. 2008; 2:576–83. [PubMed: 18522850]

Sanchez-Prieto R, Rojas JM, Taya Y, Gutkind JS. A role for the p38 mitogen-acitvated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. Cancer Res. 2000; 60:2464–72. [PubMed: 10811125]

Sangiorgi E, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet. 2008; 40:915–20. [PubMed: 18536716]

Shangary S, Wang S. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction to reactivate p53 function: a novel approach for cancer therapy. Annu Rev Pharmacol Toxicol. 2009; 49:223–41. [PubMed: 18834305]

Shaulian E, Resnitzky D, Shifman O, Blandino G, Amsterdam A, Yayon A, et al. Induction of Mdm2 and enhancement of cell survival by bFGF. Oncogene. 1997; 15:2717–25. [PubMed: 9400998]

Sun Q, Ming L, Thomas SM, Wang Y, Chen ZG, Ferris RL, et al. PUMA mediates EGFR tyrosine kinase inhibitor-induced apoptosis in head and neck cancer cells. Oncogene. 2009; 18:2348–57. [PubMed: 19421143]

Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, et al. p53- and Drug-Induced Apoptotic Responses Mediated by BH3-Only Proteins Puma and Noxa. Science. 2003; 302:1036–8. [PubMed: 14500851]

Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer. 2002; 2:594–604. [PubMed: 12154352]

Waldman T, Kinzler KW, Vogelstein B. p21 Is Necessary For the p53-Mediated G(1) Arrest In Human Cancer Cells. Cancer Res. 1995; 55:5187–5190. [PubMed: 7585571]

Wang P, Yu J, Zhang L. The nuclear function of p53 is required for PUMA-mediated apoptosis induced by DNA damage. Proc Natl Acad Sci U S A. 2007; 104:4054–9. [PubMed: 17360476]

Wilkins HR, Ohneda K, Keku TO, D'Ercole AJ, Fuller CR, Williams KL, et al. Reduction of spontaneous and irradiation-induced apoptosis in small intestine of IGF-I transgenic mice. Am J Physiol Gastrointest Liver Physiol. 2002; 283:G457–64. [PubMed: 12121894]

Wu B, Qiu W, Wang P, Yu H, Cheng T, Zambetti GP, et al. p53 independent induction of PUMA mediates intestinal apoptosis in response to ischaemia-reperfusion. Gut. 2007; 56:645–54. [PubMed: 17127703]

Yang JY, Xia W, Hu MC. Ionizing radiation activates expression of FOXO3a, Fas ligand, and Bim, and induces cell apoptosis. Int J Oncol. 2006; 29:643–8. [PubMed: 16865280]

Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature. 1991; 352:345–7. [PubMed: 1852210]

You H, Pellegrini M, Tsuchihara K, Yamamoto K, Hacker G, Erlacher M, et al. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. J Exp Med. 2006; 203:1657–63. [PubMed: 16801400]

Yu J, Wang P, Ming L, Wood MA, Zhang L. SMAC/Diablo mediates the proapoptotic function of PUMA by regulating PUMA-induced mitochondrial events. Oncogene. 2007; 26:4189–98. [PubMed: 17237824]

Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. Proc Natl Acad Sci U S A. 2003; 100:1931–1936. [PubMed: 12574499]

Yu J, Zhang L. Apoptosis in human cancer cells. Curr Opin Oncol. 2004; 16:19–24. [PubMed: 14685088]

Yu J, Zhang L. The transcriptional targets of p53 in apoptosis control. Biochem Biophys Res Commun. 2005; 331:851–8. [PubMed: 15865941]

Yu J, Zhang L. PUMA, a potent killer with or without p53. Oncogene. 2008; 27(1):S71–83. [PubMed: 19641508]

Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell. 2001; 7:673–82. [PubMed: 11463391]

Yu J, Zhang L, Hwang PM, Rago C, Kinzler KW, Vogelstein B. Identification and classification of p53-regulated genes. Proc Natl Acad Sci U S A. 1999; 96:14517–22. [PubMed: 10588737]

Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, et al. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature. 2009; 457:603–7. [PubMed: 19092805]

Abbreviations

PUMA	p53 upregulated modulator of apoptosis
TUNEL	terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end labeling
PBS	phosphate buffered saline
WT	wildtype
KO	knockout
PI3K	Phosphoinositide 3-kinase
AKT	*Protein Kinase B*
IGF-1	Insulin-like growth factor 1
bFGF	basic fibroblast growth factor
ISCs	intestinal stem cells
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase

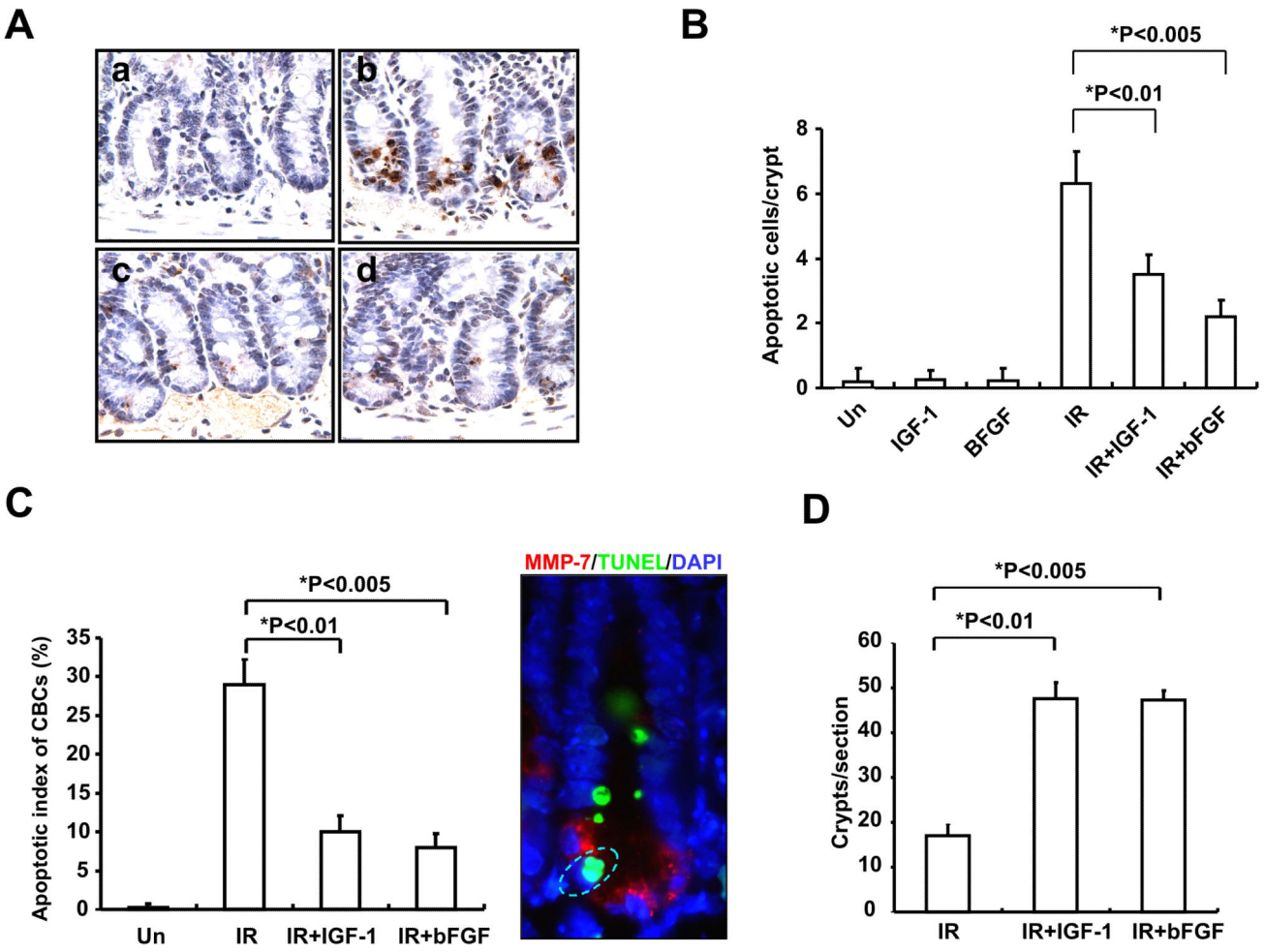

Figure 1. Growth factors protected against apoptosis induced by radiation in the intestinal crypts and stem cells in mice

(A) Apoptosis in the intestinal crypts of WT C57/BL6 mice subjected to various treatments 4 hr after 15 Gy WBR was assessed by TUNEL staining (brown), magnification × 400. a: untreated mice (UN). b: mice irradiated by 15 Gy (IR). c: mice injected i.v. with 3.5 μg human recombinant IGF-1 followed by 15 Gy radiation (IR+IGF-1). d: mice injected i.v. with 3.5 μg human recombinant bFGF followed by 15 Gy radiation (IR+bFGF). **(B)** Apoptotic index measured by TUNEL staining as in **(A)** was quantitated. Values are means ± SD; n=3 in each group. *, P<0.05. **(C) Left,** The fractions of crypts containing at least one TUNEL-positive CBC were calculated by counting 100 crypts with well preserved Paneth cell areas. Values are means ± SD; n = 3 in each group. **Right,** an example of MMP-7/ TUNEL double staining in the crypts 4 hr after 15 Gy with a CBC, magnification × 600. The nuclei were counterstained with DAPI. The CBCs are in between the MMP-7$^+$ Paneth cells at the bottom of the crypt. **(D)** Regenerated crypts at 96 hours after 15 Gy WBR were analyzed by the micro colony assay and quantitated. Values are means ±SD; n=3 in each group.

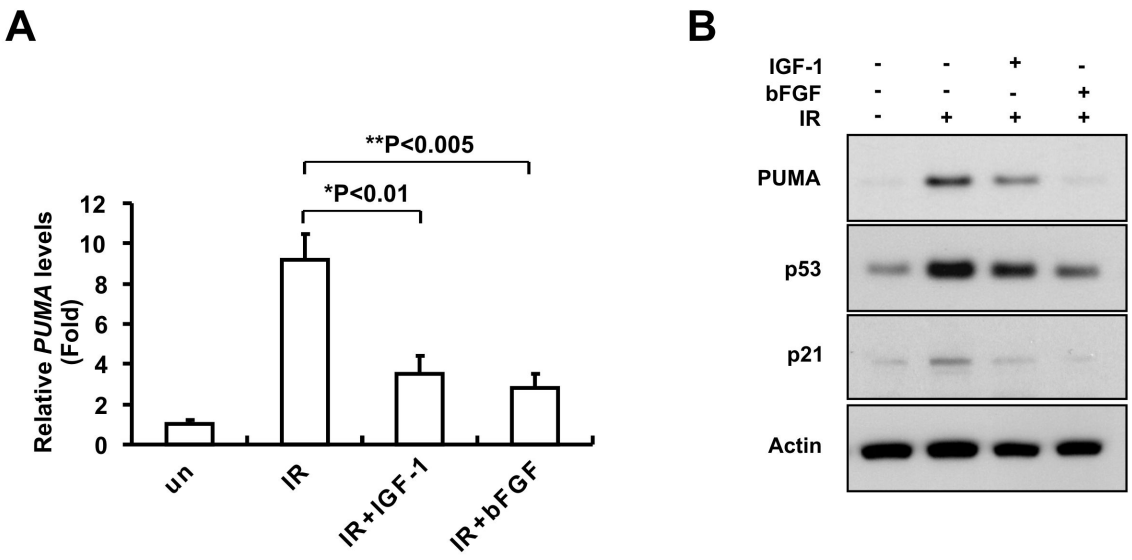

Figure 2. Growth factors blocked radiation-induced PUMA expression in the intestinal mucosa

(A) *PUMA* mRNA expression in the jejunal mucosa of mice following 15 Gy WBR with or without treatment of growth factors was evaluated by quantitative real-time RT-PCR. Values are means ± SD; n = 3 in each group. **(B)** The expression of PUMA, p53, and p21 in the jejunal mucosa of WT mice with the indicated treatments was determined by Western blotting. β-actin (Actin) was used as the control for loading.

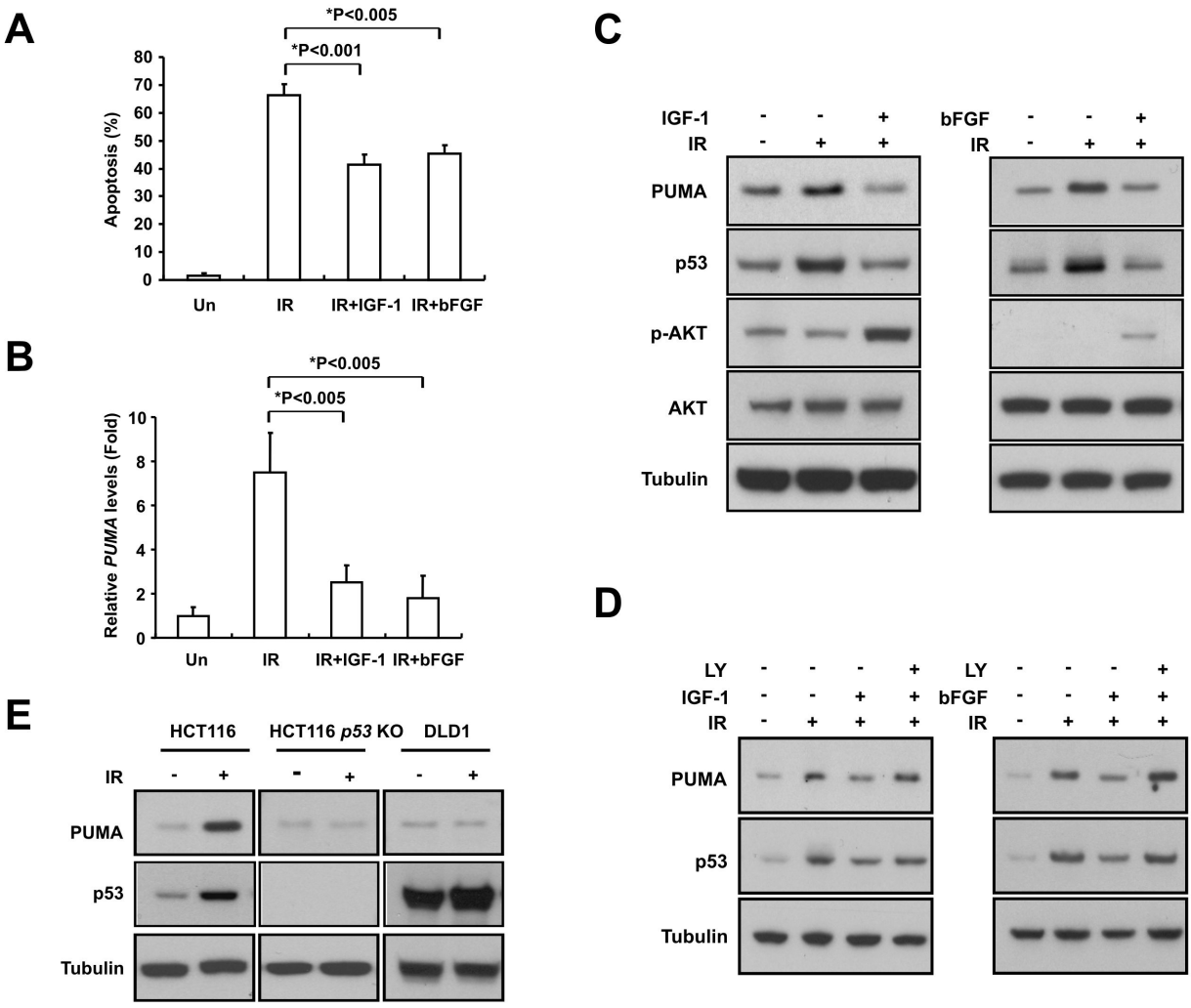

Figure 3. Growth factors protected against radiation-induced apoptosis through the PI3K/AKT pathway *in vitro*

(A) HCT116 *p21* KO cells with or without growth factor treatment were irradiated by 15 Gy. Apoptosis was determined by nuclear staining with DNA binding dye Hoechst 33258 48 hr after irradiation. Those with fragmented and condensed nuclei were counted as apoptotic cells. **(B)** *PUMA* mRNA expression in HCT116 *p21* KO cells following 15 Gy radiation with or without the treatment of growth factors was evaluated by quantitative real-time RT-PCR. Values are means ± SD. Each experiment was repeated for 3 times. **(C)** The expression of PUMA, p53, Akt, and p-Akt in HCT116 *p21* KO cells with the indicated treatments was determined by western blotting. **(D)** The effect of the PI3K inhibitor LY294002 on the expression of PUMA and p53 in HCT116 *p21* KO cells with the indicated treatments. The levels of the proteins were determined by western blotting. LY: LY294002, PI3K inhibitor. **(E)** The expression of PUMA and p53 in the indicated cell lines 24 hr after radiation was determined by western blotting. α-tubulin was used as the control for loading in **(C-E)**. The gene or protein expression in **(B)-(E)** was determined 24 hr after irradiation.

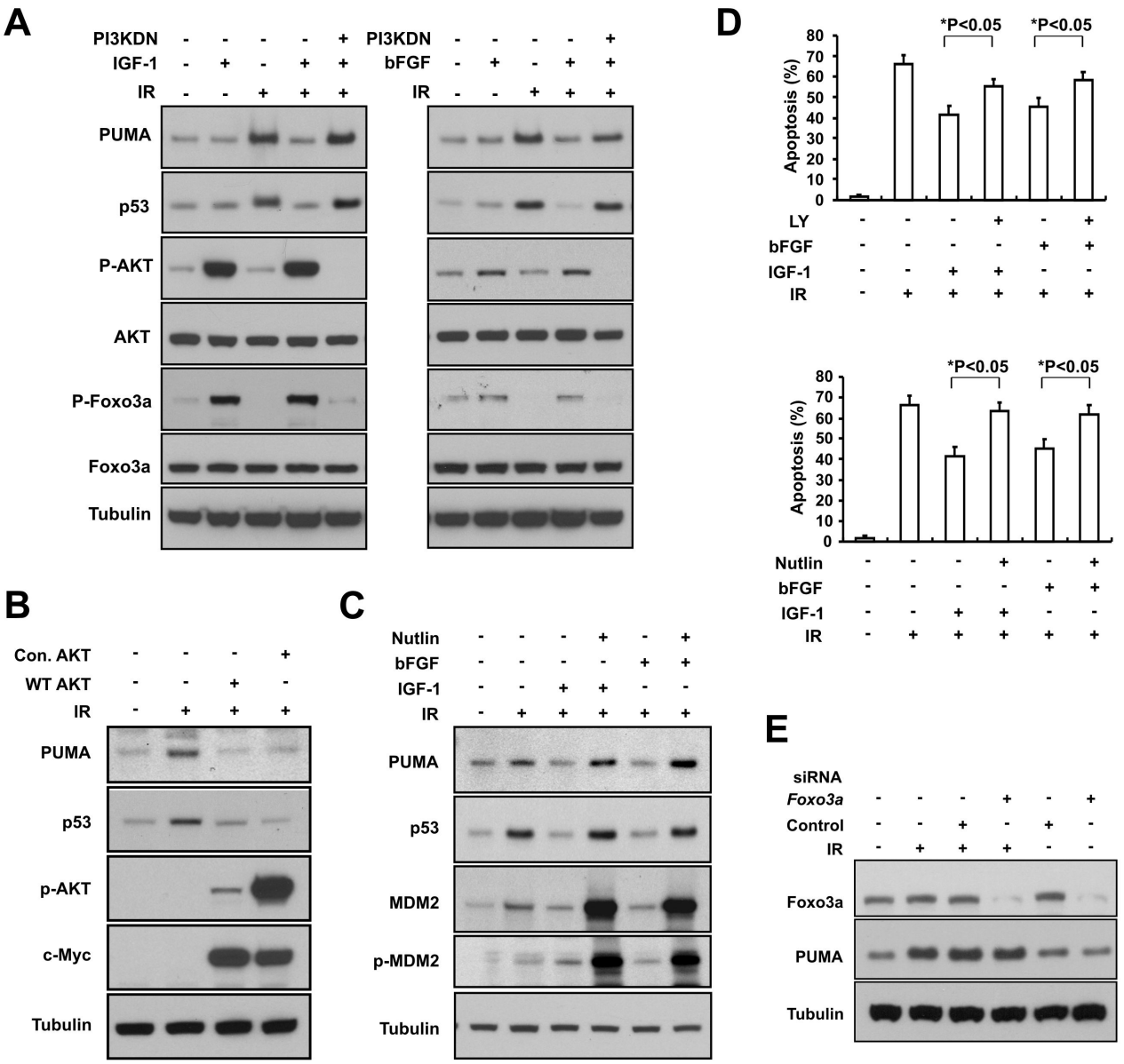

Figure 4. Growth factors inhibited PUMA induction by radiation through a p53-dependent mechanism *in vitro*

(A) The effect of dominant negative PI3K (DN PI3K) on the expression of PUMA, p53, AKT, p-AKT, Foxo3a, and p-Foxo3a in HCT116 *p21* KO cells with the indicated treatments. The protein levels were determined by western blotting. **(B)** The effects of AKT on the indicated proteins in HCT116/*p21* KO cells were determined by western blotting. The cells were transfected with WT or constitutively active AKT for 24 h followed by irradiation, and collected for analysis 24 hr later. **(C)** The effects of a MDM2 antagonist nutlin-3a on the expression of PUMA, p53, MDM2, and p-MDM2 in HCT116 *p21* KO cells with the indicated treatments. The protein levels were determined by western blotting. α-tubulin was used as the control for loading. **(D)** The effects of LY294002 and nutlin-3a on radiation-induced apoptosis in HCT116 *p21* KO cells. **(E)** HCT116 *p21* KO cells were

transfected with control or *Foxo3a* siRNA 24 hr before irradiation. Foxo3a and PUMA expression was analyzed by western blotting. The gene or protein expression in **(A-C)** and **(E)** was determined 24 hr after radiation, while the apoptosis in **(D)** was determined 48 hr after radiation.

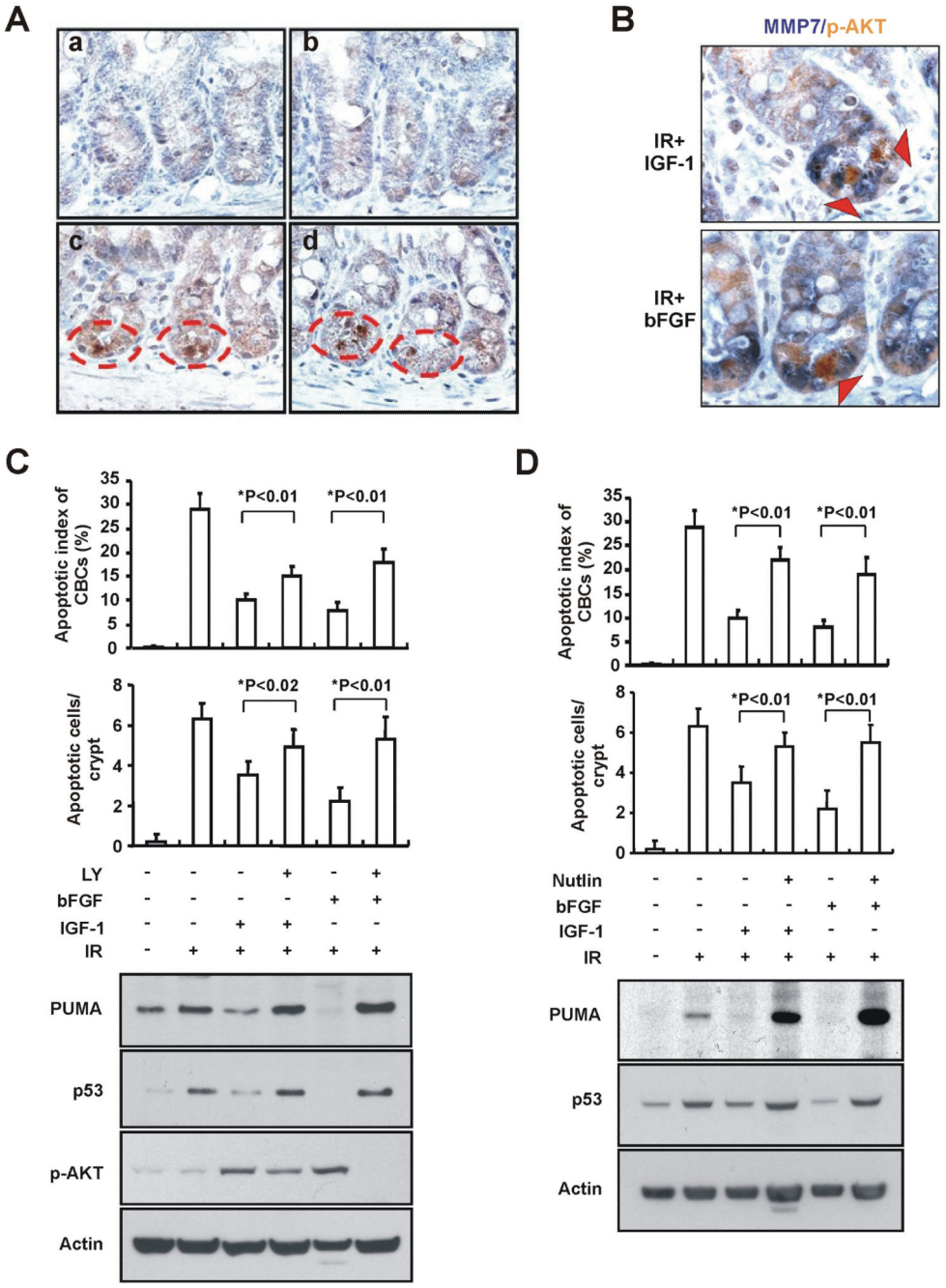

Figure 5. Growth factors protected the intestinal stem cells from radiation-induced apoptosis through the PI3K-AKT-p53-PUMA axis

(A) p-AKT expression in the intestinal crypts of mice with various treatments was evaluated by immunohistochemistry. a: UN; b: IR; c: IR+IGF-1; d: IR+bFGF. The CBC areas were circled in the groups with growth factor treatment. **(B)** An example of MMP-7/p-AKT double staining used to identify CBCs in the crypts of mice 4 hr after irradiation. Magnification × 600. Arrowheads indicate p-AKT expressed in CBCs. **(C)** The effect of the PI3K inhibitor LY294002 (LY) on the apoptosis and expression of indicated proteins in

mice subjected to the indicated treatments. **Top,** apoptotic index in the CBCs measured by TUNEL staining and quantitated. Values are means ± SD; n=3 in each group. **Middle,** apoptotic index in the crypts measured by TUNEL staining and quantitated. Values are means ± SD; n=3 in each group. **Bottom,** the expression of PUMA, p53, and p-Akt in the intestinal mucosa was determined by western blotting. β-actin was used as the control for loading. **(D)** The effects of the MDM2 antagonist nutlin-3a on apoptosis of the CBCs and crypts, and the expression of the indicated proteins were analyzed as in **(C)**.

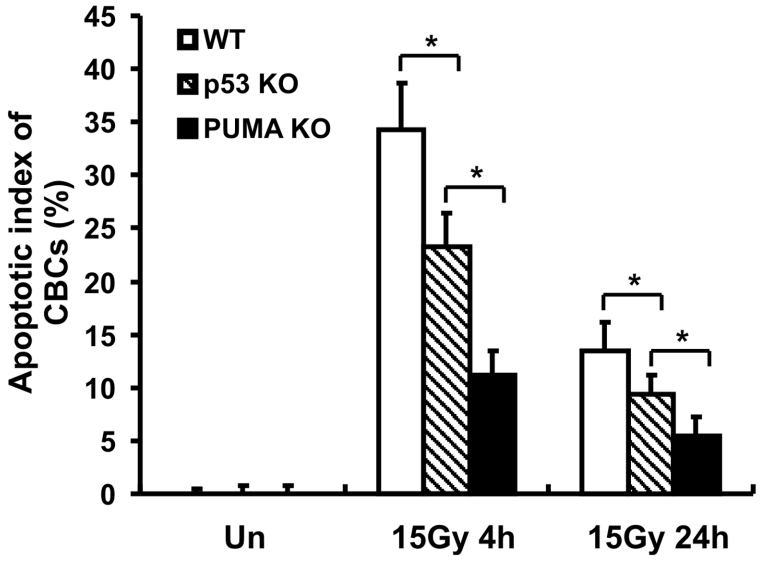

Figure 6. *PUMA* deficiency protected the CBCs against radiation-induced apoptosis better than *p53* deficiency

Apoptosis in the intestinal crypts of mice with indicated genotype 4 hr or 24 hr after 15 Gy WBR was assessed by TUNEL staining as in Fig. 5C. The fraction of crypts with at least one TUNEL-positive CBC was calculated by counting 100 crypts with well preserved Paneth cell areas. Values are means ± SD; n = 3 in each group. *$p < 0.05$.