FG-4592 Protects the Intestine from Irradiation-Induced Injury by Targeting TLR4 Signaling Pathway

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Abstract

Background: Severe ionizing radiation (IR) induced intestinal injury has high mortality, which is a worldwide problem to be solved urgently. In recent years, studies have found that PHD-HIF signaling pathway may play key roles in IR induced intestinal injury, and we found that FG-4592, the PHD inhibitor, has significant radio-protective effects on IR induced intestinal injury.

Methods: With/without FG4592 treatment, the survival time, pathology, cell viability, cell apoptosis and organoids of mice after irradiation were compared, and the mechanism was verified after transcriptome sequencing. The data were analyzed using SPSS ver. 19.

Results: Our results show that FG-4592 has significant radio-protective effects on the intestine. FG-4592 improves the survival of irradiated mice, inhibits the radiation damage of intestinal tissue, promotes the regeneration of intestinal crypt after IR, and reduces the apoptosis of intestinal crypt cells. Through organoid experiments, it is found that FG-4592 promotes the proliferation and differentiation of intestinal stem cells (ISCs). Moreover, the results of RNA sequence and WB show that FG-4592 significantly upregulated the TLR4 signaling pathway, and FG-4592 has no radioprotection on TLR4 KO mice, suggesting that FG-4592 may play protective role against IR by targeting TLR4.

Conclusion: Our work proves that FG-4592 may promote the proliferation and regeneration of ISCs through the targeted regulation of TLR4 and WNT/β-catenin signaling pathway, and ultimately play radio-protective roles in IR induced injury. These results enrich the molecular mechanism of FG-4592 to protect IR induced injury and provide new methods for the radioprotection of intestine.

Introduction

Acute radiation exposure is a serious public and military health problem[1]. Ionizing radiation (IR) can cause a variety of types of radiation damage. Intestinal tissue is extremely sensitive to IR, which is a common pathological injury after high dose of IR[2]. When the radiation dose is ≥ 10.0 Gy, intestinal tissue necrosis can be caused, leading to the death of patients with acute intestinal radiation sickness[3]. In addition, IR induced intestinal injury is also a common complication of with abdominal tumor, which limits the dose and efficacy of radiotherapy for patients with tumor[4]. Although great progress has been made in the radioprotection, there is still no ideal treatment for IR induced intestinal injury caused by high dose radiation, which is one of the key problems to be solved urgently in the field of radioprotection.

Over the years, there have been many studies on the mechanism and prevention of intestinal ionizing radiation injury[5]. It is found that the occurrence of acute IR induced intestinal injury is a complex process involving cell death and inflammatory activation[6]. IR can directly damage intestinal cells, leading to the death of intestinal epithelial cells and intestinal crypt stem cells and the destruction of the epithelial barrier, resulting in digestion and absorption disorders, electrolyte imbalances, and even bacteria translocation, which is the main cause of death of intestinal radiation injury[7]. In addition, intestine

exposed to large doses of IR will cause swelling and apoptosis of microvascular endothelial cells, release inflammatory factors, promote the formation of microthrombosis, and then lead to intestinal tissue ischemia and hypoxia, aggravate intestinal injury[8]. Much research have been carried out in prevention and treatment of IR induced intestinal injury, including stem cell transplantation, cytokines treatment, chemical drugs, comprehensive therapy and so on[9–11]. The basic research progress has been made from different aspects, but the overall radio-protective effects are not ideal[12]. For example, some research results show that some compounds have no obvious therapeutic effect. Although some compounds can alleviate the occurrence and development of intestinal ionizing radiation injury, these compounds cannot be directly applied in clinical treatment due to their high toxicity or some unpredictable side effects[13].

PHD-HIF signaling pathway, one of the research hotspots in recent years, is involved in inflammation, cancer, immunity, and other physiological processes[14, 15]. HIF (Hypoxia-inducible factor) is a DNA-binding protein consisting of an α subunit (HIF-1α or HIF-2α) and a β subunit (HIF-1β). Since it was discovered by Semenza et al. in 1990s, the role of HIF in hypoxia tolerance, tumor proliferation, erythropoiesis and other aspects has been gradually recognized[16]. PHD (HIF Prolyl hydroxylase), an important negative regulator of HIF, can reduce the stability of HIF-1α through hydroxylation modification. PHD-HIF pathway is not only related to hypoxia, but also involved in various pathophysiological processes such as inflammation, tumor, and immunity[17, 18].

In recent years, it has been found that PHD-HIF pathway is a new target for the treatment of treated IR-induced intestinal injury[19]. For example, dimethyloxalylglycine (DMOG), as a PHD inhibitor, has been reported that it mediated radioprotection mainly through inhibition of PHD2 and upregulation of HIF-2α[20]. Our group has previously reported that FG-4592 has significant radio-protection effects on hematopoietic system[21]. FG-4592, also known as Roxadustat, is a novel oral PHD inhibitor. It was first used in the treatment of anemia to enhance the synthesis and release of EPO by inhibiting PHD and increasing HIF level[22]. In 2014, FG-4592 was applied to China for clinical trial as a class 1.1 new drug. Now, phase □ clinical study has been carried out in China, showing great safety[23–25]. In this study, we discover that FG-4592 protects the intestine from IR induced injury by targeting TLR4 signaling pathway and FG-4592 might be a potentially highly effective and selective intestinal radioprotector.

Materials And Methods

Chemicals and reagents

FG-4592 was purchased from Cayman Chemical Company (www.caymanchem.com), and normal saline (NS) was obtained from ChangHai Hospital (Shanghai, China). The apoptosis detection kit was purchased from Transgen (Beijing, China). The PCR kit (RR036A and RR420A) was purchased from TAKARA. RPMI 1640 □DMEM and fetal bovine serum (FBS) were supplied by Gibco. Organoid culture was obtained from STEM CELL. The Antibody of BCL2□BAX□C-CASPASE3□GAPDH□NF-κB□P-IKK-β were

supplied by CST. The Antibody of TLR4 were supplied by Proteintech. Cell-Light EdU Apollo488 In Vitro Kit was obtained from RIBOBIO (Guangzhou, China). In Situ Cell Death Detection Kit was obtained from Roche. The primes were obtained from Shenggong Biotech (Shanghai, China). The list of Primeis shown in Table 1.

Table 1
qRT-PCR primers for the 8 genes evaluated.

Gene symbol	Forward	Reverse
TLR4	AAATGCACTGAGCTTTAGTGGT	TGGCACTCATAATGATGGCAC
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IGFBP2	CAGACGCTACGCTGCTATCC	CCCTCAGAGTGGTCGTCATCA
SOX2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
REG2	CTGATGTTCCTGTCATACAGCC	CCAGGTCAAACGGTCTTCAATTA
REG3B	ACTCCCTGAAGAATATACCCTCC	CGCTATTGAGCACAGATACGAG
REG3D	GACTCCATGATCTGTCACTTGG	CATAGGGAAATGTTGGGTCACAA
HK1	CAAGAAATTACCCGTGGGATTCA	CAATGTTAGCGTCATAGTCCCC

Cell culture and treatment

MODEK cell was obtained from American Type Culture Collection and cultured in RPMI 1640 with 10% FBS at 37°C in a 5% CO2 humidified chamber. HIEC was obtained from American Type Culture Collection and cultured in DMEM with 10% FBS at 37°C in a 5% CO2 humidified chamber. Cells were treated with FG-4592 (10 μM) 12 and 2 h before irradiation.

Cell viability and apoptosis assay.

Cell viability was analyzed by CCK-8. Pretreated cells were seeded into 96-well plates at 5000 cells/well. The cells were counted by absorbance measurements at 450 nm 24 h post-radiation. The cell apoptosis was analyzed using the apoptosis detection kit. After radiation, the cells were stained using Annexin V-fluorescein isothiocyanate (AV-FITC) and propidium iodide-Phycoerythrin (PI-PE). The cells were then analyzed by flow cytometry (Beckman Cytoflex) in accordance with the manufacturer's instructions.

Animals and treatment

Male wildtype C57BL/6 mice were obtained from China Academy of Science (Shanghai, China). TLR4 KO mice aged 6–8 weeks old were purchased from Model Animal Research Center, Nanjing University. All mice were housed in a laboratory animal room under standard conditions. The experiments were approved by the Laboratory Animal Center of the Naval Medical University, China in conformance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The mice were treated with FG-4592 (25.0 mg/Kg, dissolved in NS) via peritoneal injection 24h and 2 h before IR.

Irradiation

^{60}Co source in the radiation center (Naval Medical University, China) was used to irradiate mice and cells. Mice were irradiated at 8.5 Gy to build Total Body Irradiation (TBI) model and irradiated at 25.0 Gy at abdomen to build Abdominal Irradiation (ABI) model.

Histological examination

Mice small intestinal tissues were removed and then fixed in 4% paraformaldehyde after IR. Hematoxylin and Eosin (HE), TUNEL (Terminal deoxynucleotidyl transferase dUTP nick-end labelling) staining, and Ki-67 assay were did according to the manufacturer's instructions. The TUNEL + cells were counted in 10 crypts per section. The Ki67 positive area per section was measured using ImageJ software (National Institutes of Health, USA).

FISH (Fluorescence in situ hybridization)

FISH was used to detect the expression of Lgr5, the intestinal stem cell marker, in intestinal tissues. Mice small intestinal tissues were removed and then fixed in 4% paraformaldehyde after IR. FISH were conducted according to the manufacturer's instructions. And the fluorescence microscopy was used to observe FISH results.

Intestinal organoid culture

The isolated whole crypts efficiently expand into three-dimensional spherical structures recapitulating the intestinal crypt-villus organization. In this work, the whole crypts were abstracted from mice according to the manufacturer's instructions. Then the crypts were seeded into 24-well plate, and the intestinal organoid culture medium was changed every 3 days. The mature organoids were observed under microscope, and the surface area and budding situation of the organoids were measured using ImageJ software (National Institutes of Health, USA). The EDU proliferation ability detection kit was used to detect the proliferation ability of intestinal organoid. And TUNEL detection kit was used to detect the apoptotic ability of irradiated organoid

Intestine Immunofluorescence

Immunofluorescence analysis was used to detect OLFM4. The intestinal tissues or intestinal organoid were fixed in 4% paraformaldehyde for 20 min and permeabilized in 0.5% Triton X-100 for 10 min. After blocked in BSA, The intestinal tissues or intestinal organoid were stained with antibodys, and then stained with the secondary antibody (1:1000). The images were obtained using fluorescent microscope.

RNA sequencing and functional enrichment analysis

Total RNA was isolated from intestine of mice using Trizol (Invitrogen, USA) 24h after radiation. NanoVue (GE, USA) was used to assess RNA purity. Each RNA sample had an A260:A280 ratio greater than 1.8 and

an A260:A230 ratio greater than 2.0. Sequencing was performed at Oebiotech (Shanghai, China) with the Illumina HiSeq 2500. Prior to sequencing, the raw data were filtered to produce high-quality clean data. All the subsequent analyses were performed using the clean data.

Statistical analysis

Data were expressed as means ± the standard errors of means. Two-tailed Student's t-test was used to analyze the difference between 2 groups. One-way ANOVA was employed to analyze the difference among 3 groups. Kaplan–Meier analysis was applied to estimate the difference of overall survival between 2 groups. The data were analyzed using SPSS ver. 19 (IBM Corp, Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

Results

FG-4592 exhibited a significant radio-protective effect in vivo **and** in vitro

To prove the radio-protective effects of FG-4592 on IR-induced intestinal injury, we took C57BL/6 mice as the research object. C57BL/6 mice were given 25.0 mg/kg FG-4592 intraperitoneally 24 hours and 2 hours before irradiation, followed by total body irradiation of 8.5 Gy or local abdominal irradiation of 25.0 Gy. FG-4592 can significantly improve the survival rate of mice after IR (Fig. 1A/B). Meanwhile, MODEK and HIEC cells were treated with FG-4592 at 10 μM 12 hours and 2 hours before irradiation, and cell viability and apoptosis rate were detected 24 hours after irradiation. As shown in the Fig. 1C/D, compared with the IR group, the cell viability after irradiation was significantly increased and the apoptosis rate was decreased in the FG-4592 treatment group. Meanwhile, the levels of apoptosis-related proteins were detected by using WB. FG-4592 reduced the levels of Bax and Cleaved-Caspase-3, the apoptosis promoting proteins, which were up-regulated by IR and increased BCL2, which inhibited apoptosis and were down-regulated after IR (Fig. 1E).

FG-4592 protected the intestinal issue against radiation-induced injury

Subsequently, intestinal tissues of mice were collected 3.5 days after IR for HE staining to observe the degree of intestinal injury(Fig. 2A). HE staining results showed that FG-4592 could improve the intestinal integrity of mice after irradiation, and the villi length and crypt cell number were better than that of the IR group. Ki67 staining showed that FG-4592 group exhibited greater intestinal structure, taller villi, and more surviving crypts(Fig. 2B). TUNEL staining showed that FG-4592 could significantly inhibit the apoptosis of intestinal crypts after radiation injury(Fig. 2C). $Lgr5^+$ FISH also showed that irradiation could significantly reduce the number of $Lgr5^+$ ISCs, while FG-4592 significantly increased the number of $Lgr5^+$ ISCs(Fig. 2D). These results proved that FG-4592 had great radio-protective effects on IR induced intestinal injury.

FG-4592 protected the intestinal organoid against radiation-induced injury

Intestinal organoid is a great technology to study the proliferation, differentiation, and regeneration of ISCs. In this work, Intestinal organoids were also used to explore the radio-protective effects of FG-4592 on intestinal radiation injury. Intestinal crypts of C57BL/6 mice were extracted for organoid culture, and then it was stimulated with FG-4592 (50 μM) before 6.0 Gy IR. As shown in Fig. 3A, FG-4592 could improve the ability of organoid formation. Compared with the IR group, the number and volume of single organoid buds in FG-4592 group were increased. At the same time, HE, Ki-67 and TUNEL staining were also performed on the organoids, and it was found that FG-4592 could significantly promote the proliferation and inhibit apoptosis of the intestinal organoids after 6.0 Gy (Fig. 3B/C/D). These were consistent with the results of intestinal tissue in mice. OLFM4 is another ISCs marker and immunofluorescence analysis was used to detect the expression of OLFM4 in intestinal organoid. The result was showed as Fig. 3E. FG-4592 increased the expression of OLFM4 which were down-regulated by IR. These results suggested that FG-4592 could significantly improve the proliferation and differentiation of irradiated ISCs.

Identification of DEGs after FG-4592 treatment

To explore the regulatory mechanism of FG-4592 on intestinal radiation injury, RNA-sequence (4:4) of intestinal issues was conducted. 476 up-regulated and 157 down-regulated differential expression genes (DEG) were found (Fig. 4A). The differential gene heat map was drawn for analysis (Fig. 4B). At the same time, KEGG and GO analyses were conducted for differences, and significant enrichment pathways of DEGs (Fig. 4C/D). KEGG result showed that Immune system and Signal transduction were significant enrichment (Fig. 4C). GO result showed that cell wall disruption in other organism (GO:0044278), wound healing, spreading of epidermal cells (GO:0035313), negative regulation of cell death (GO:0060548), and antimicrobial humoral immune response mediated by antimicrobial peptide (GO:0061844) were significant enrichment (Fig. 4D and Table 1). And these pathways have been reported to be related to the process of IR induced injury.

Table 2
The list of Top 30 Go Term.

ID	Term	Category	P val	Enrichment_score
GO:0044278	**cell wall disruption in other organism**	**biological_process**	**0.000**	**33.89672131**
GO:0035313	**wound healing, spreading of epidermal cells**	**biological_process**	**0.000**	**20.33803279**
GO:0009247	glycolipid biosynthetic process	biological_process	0.000	15.64464061
GO:0060548	**negative regulation of cell death**	**biological_process**	**0.000**	**5.332068521**
GO:0010466	negative regulation of peptidase activity	biological_process	0.000	4.635449068
GO:0006811	ion transport	biological_process	0.000	2.433610761
GO:0017158	regulation of calcium ion-dependent exocytosis	biological_process	0.000	8.217386985
GO:0048791	calcium ion-regulated exocytosis of neurotransmitter	biological_process	0.000	7.532604736
GO:0016079	synaptic vesicle exocytosis	biological_process	0.000	7.136151855
GO:0061844	**antimicrobial humoral immune response mediated by antimicrobial peptide**	**biological_process**	**0.000**	**5.467213115**
GO:0005576	extracellular region	cellular_component	0.000	2.479256595
GO:0005615	extracellular space	cellular_component	0.000	2.321368755
GO:0016020	membrane	cellular_component	0.000	1.440847291
GO:0016324	apical plasma membrane	cellular_component	0.000	3.122066437
GO:0016021	integral component of membrane	cellular_component	0.000	1.367007432
GO:0009897	external side of plasma membrane	cellular_component	0.000	2.965963115
GO:0005886	plasma membrane	cellular_component	0.000	1.421755063
GO:0009986	cell surface	cellular_component	0.001	2.135226539
GO:0005811	lipid droplet	cellular_component	0.001	4.580638015
GO:0005581	collagen trimer	cellular_component	0.001	4.519562842
GO:0019911	structural constituent of myelin sheath	molecular_function	0.000	20.33803279

ID	Term	Category	P val	Enrichment_score
GO:0005544	calcium-dependent phospholipid binding	molecular_function	0.000	7.975699132
GO:0004190	aspartic-type endopeptidase activity	molecular_function	0.000	10.84695082
GO:0070492	oligosaccharide binding	molecular_function	0.000	14.52716628
GO:0030414	peptidase inhibitor activity	molecular_function	0.000	4.519562842
GO:0042834	peptidoglycan binding	molecular_function	0.000	12.71127049
GO:0004198	calcium-dependent cysteine-type endopeptidase activity	molecular_function	0.000	10.70422778
GO:0030246	carbohydrate binding	molecular_function	0.001	3.097162353
GO:0030276	clathrin binding	molecular_function	0.001	5.021736491
GO:0008375	acetylglucosaminyltransferase activity	molecular_function	0.001	6.163040238

TLR4 signaling pathway play a critical role in the radioprotection of FG-4592

In the list of DEGs (Table 2), we selected TLR4 as a key downstream molecule. In our previous works, TLR4 plays a key role in the process of IR induced injury. Through quantitative PCR, we found that FG-4592 could significantly up-regulate the level TLR4 mRNA. IL-6, IGFBP2, SOX2, HK1, REG3B, REG2, REG3D and other DEGs were significantly up-regulated(Table 3). Moreover, the changes of TLR4 signaling pathway in of FG-4592 treated MODEK cells were also increased by using WB Blot. The results of WB showed that the expression of TLR4 and Phospho-IKK-β was increased after FG-4592 stimulated. Next, TLR4 KO mice were used to verify the function of TLR4 in FG-4592 induced radioprotection. TLR4 KO mice were divided into the IR + PBS group and the IR + FG-4592 group (n = 8). By recording the survival period after 8.0 Gy IR, we found that the mortality rate of TLR4 KO mice was not statistically significant between the IR + PBS group and the IR + FG-4592 group (Fig. 5C), indicating that TLR4 KO reversed the radioprotection of FG-4592. These results proved that the radioprotection of FG-4592 was dependent on TLR4.

Table 3
Partial differential gene list.

gene_id	foldChange	P val	Regulation
Hk1	**1.46035**	**0.001071**	**Up**
IL-6	**2.013457**	**0.08727**	**Up**
Tlr4	**2.277509**	**1.70E-05**	**Up**
Igfbp2	**2.345283**	**0.023405**	**Up**
Reg3b	**2.837572**	**0.008005**	**Up**
Reg3d	**3.050849**	**0.006954**	**Up**
Sox2	**6.727548**	**0.000878**	**Up**
Reg2	**17.74323**	**0.004824**	**Up**
Pde2a	1.633734	0.002347	Up
Pglyrp1	1.698371	0.001095	Up
Arhgap24	1.914116	9.08E-05	Up
Nov	2.23212	0.004262	Up
Defa28	2.509691	0.018393	Up
Agr2	2.671268	1.54E-06	Up
Clu	3.250905	8.03E-06	Up
Wnt11	3.569708	0.000169	Up
Plet1	3.675288	1.35E-05	Up
Ltf	3.68403	0.000989	Up
Scnn1g	5.190979	0.000447	Up
Ntf5	5.831077	0.003121	Up
Scnn1b	7.607157	6.84E-06	Up

Discussion

Acute severe radiation-induced damage has been a worldwide problem[1]. Although researchers have made some progress in this field, there is still no effective means for prevention and treatment of acute intestinal radiation sickness caused by high-dose irradiation[26]. Therefore, it is of great significance to figure out the key mechanism of IR-induced injury and to screen new radio-protective agents.

In recent years, it has been found that PHD-HIF pathway is a new target for the treatment of treated IR-induced intestinal injury[19]. Taniguchi CM et.al report a radioprotective compound DMOG that regulates the PHD2-HIF pathway. At 16.0 Gy lethal TBI or 20.0 Gy ABI in mice, the survival rate of the DMOG treated mice was significantly improved, with 2/3 of the mice still alive 60 days after IR exposure, and all untreated control animals died 10 days after IR. Taniguchi CM et al. also found that DMOG-mediated radioprotection is achieved mainly through inhibition of PHD2 and upregulation of HIF-2[20]. Moreover, Forristal CE et al. reported similar results that DMOG played radioprotective effects on hematopoietic system through PHD-HIF pathway[27]. Cummins EP et al. demonstrated that the up-regulation of HIF with PHD inhibitors could promote NF-κB translocation by activating IKK-β[28]. Greten FR et al. demonstrated that the activation of NF-κB in intestinal epithelial cells could inhibit apoptosis of epithelial cells[29]. Olcina MM and Giaccia AJ published a review in J Clin Invest suggesting that the upregulation of HIF pathway, especially the inhibition of PHD, may be a new and effective way for the prevention and treatment of IR-induced intestinal injury[19].

In this study, we systematically studied the radioprotective effects of PHD inhibitor FG-4592 on IR-induced intestinal injury and found that FG-4592 significantly improved the survival of mice exposed to TBI and ABI. Moreover, FG-4592 also promoted cell proliferation and inhibited cell apoptosis. Furthermore, HE staining, Ki-67 staining and TUNEL staining of intestinal tissue showed that FG-4592 could significantly reduce the damage degree of intestinal tissue, promote the proliferation of intestinal crypt cells and reduce the apoptosis of intestinal crypt cells caused by IR. Lgr5 + FISH also showed that FG-4592 significantly increased the number of Lgr5 + ISCs.

Intestinal organoids have unlimited proliferation ability and can simulate many characteristics of the real intestine, which is an important model for studying intestinal development, function, and diseases. In 2009, Sato T et al. successfully cultured single ISCs into a three-dimensional structure containing crypt-like region and villous epithelial region that can grow and differentiate into all intestinal epithelial terminal cells, which can accurately simulate the physiological state of intestinal epithelium and provide a new research approach for ISCs. In this study, we constructed an intestinal organoid culture system, and found that FG-4592 could improve the ability of crypt organoid formation. Compared with the IR group, FG-4592 group had more crypt organoid formation, and the number and volume of single organoid buds were increased. HE staining, Ki-67 staining and TUNEL staining on intestinal organoids showed that FG-4592 could promote the proliferation of intestinal organoids and inhibit the apoptosis of intestinal organoids after IR. In addition, OLFM4 immunofluorescence assay confirmed that FG-4592 significantly upregulated the number of ISCs in intestinal organoids after IR.

As a high-throughput sequencing method RNA sequence technology is used to study all mRNAs produced by a specific organization or cell in a state of characteristic function. In this study, RNA sequence was used to further investigate the potential mechanism of FG-4592, and 633 DEGs were identified, including 476 up-regulated DEGs and 157 down-regulated DEGs, using $|\log2 \text{Fold Change}| > 0.58$ and $p < 0.05$ as the criteria. Subsequently, we conducted heat map analysis, KEGG analysis and GO analysis for DEGs

between the two groups and found that cell wall disruption in other organism (GO:0044278), wound healing, spreading of epidermal cells (GO:0035313), negative regulation of cell death (GO:0060548), and antimicrobial humoral immune response mediated by antimicrobial peptide (GO:0061844) were significant enrichment. And these pathways have been reported to be related to the process of radiation damage regulation. In the expression verification of DEGs, we found that FG-4592 could significantly up-regulate TLR4 mRNA level, and Cycld1, SOX2, SOX21, HK1 and other differential genes were significantly up-regulated through QT-PCR. TLR4 plays key role in radioprotection. Next, the changes of TLR4 signaling pathway after FG-4592 treated were also increased by using WB Blot, and the WB results showed that FG-4592 could significantly up-regulate TLR4-MYD88 signaling pathway. Furthermore, TLR4 KO mice were used to verify the function of TLR4 in FG-4592 induced radio- protection and we found that TLR4 KO reversed the radioprotection of FG-4592. These results proved that the radioprotection of FG-4592 was dependent on TLR4.

Conclusion

In conclusion, in this study, we explored the radioprotection and internal mechanism of FG-4592 in IR-induced intestinal injury through animal survival, intestinal organoid, RNA sequence and TLR4 KO mice. These results suggest that FG-4592 may protects the intestine from IR-induced injury by targeting TLR4 signaling pathway.

Declarations

Ethics approval and consent to participate

All animal experiments conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals' (NIH Publication No. 85-23, National Academy Press, Washington, DC, revised 1996), with the approval of the Laboratory Animal Center of the Naval Medical University, Shanghai.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Founding

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Authors' contributions

Jianming Cai, Jicong Du, and Cong Liu designed the study. Zhenlan Feng, Qinshu Xu, Xiang He, Yuedong Wang performed the experiments. Ying Cheng、Lan Fang and Jianpeng Zhao analyzed the data. Zhenlan Feng and Jicong Du wrote the paper, Jianming Cai, Jicong Du, and Cong Liu supported fund assistance. All authors read and approved the final manuscript.

Conflict of interest statement

The authors confirm that there are no conflicts of interest.

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Figures

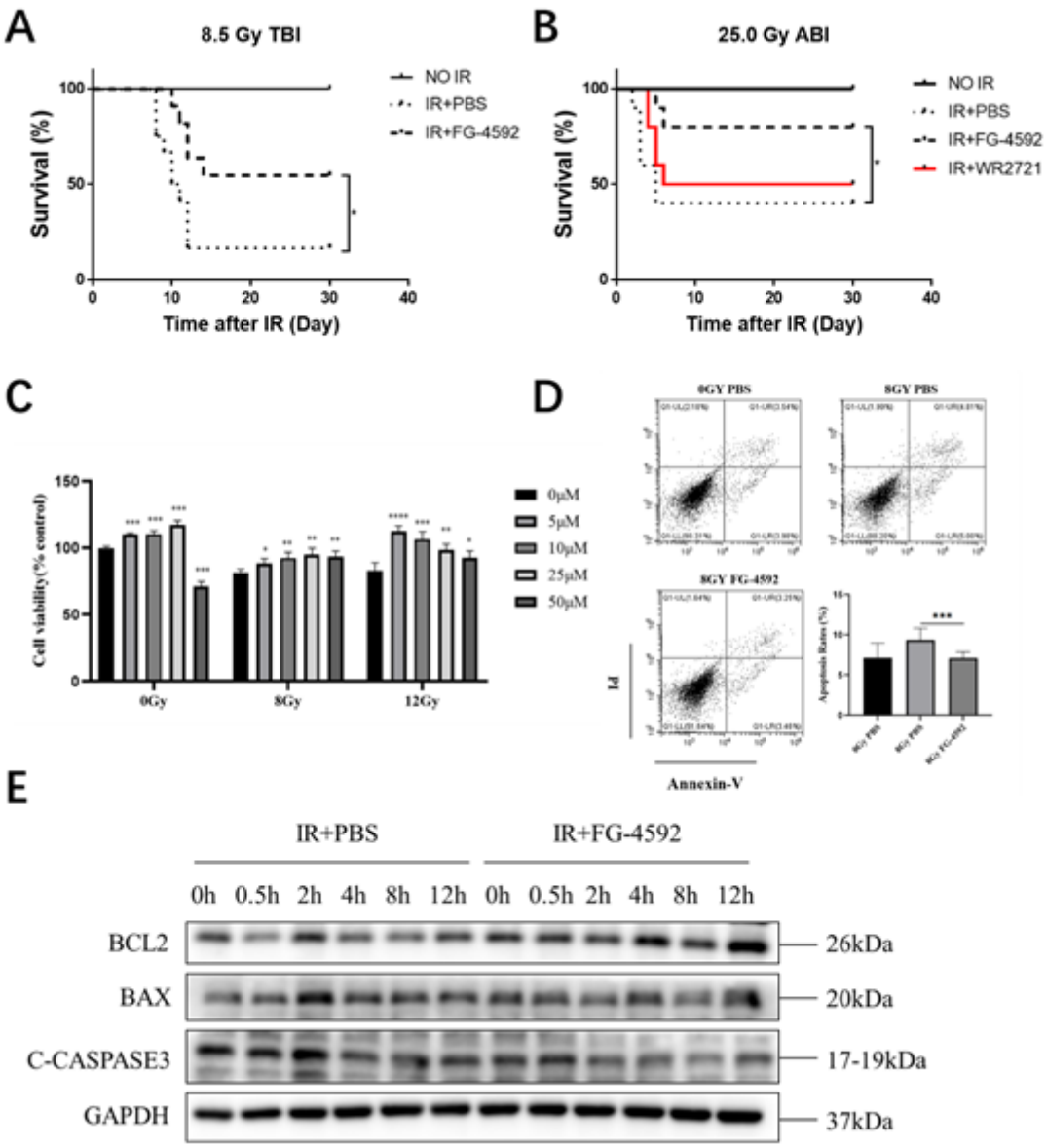

Figure 1

FG-4592 exhibited a significant radio-protective effect *in vivo* and *in vitro*.

A. The survival of mice after total body irradiation of 8.5 Gy. B. The survival of mice after local abdominal irradiation of 25.0 Gy. C. MODEK cell viability was detected by using CCK-8. D. HIEC cell apoptosis was detected by using flow cytometry. E. Expression of HIEC cell apoptosis pathway related proteins. The data were presented as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001 for control versus FG-4592 treatment.

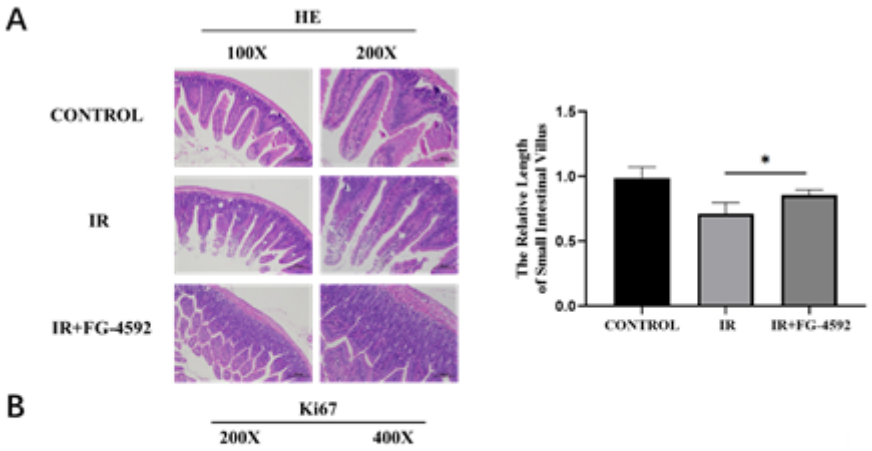

Figure 2

FG-4592 protected the intestinal issue against radiation-induced injury. C57BL/6 mice were pre-treated with PBS or FG-4592 before 8.5 Gy TBI. A. Representative images of HE-stained intestinal sections with the indicated treatment at 3.5 d after TBI. B. The representative images of Ki67-stained intestinal sections with the indicated treatment at 3.5 d after TBI. C. The representative images of TUNEL-stained intestinal sections with the indicated treatment at 3.5 d after TBI. D. The representative images of Lgr5+ FISH intestinal sections with the indicated treatment at 3.5 d after TBI.

Figure 3

FG-4592 protected the intestinal organoid against radiation-induced injury. A. Organoid regeneration after IR with PBS and FG-4592. B. Representative images of HE-stained intestinal organoids with the indicated treatment after IR. C. Representative images of TUNEL-stained intestinal organoids with the indicated treatment after IR. D. Representative images of Ki-67-stained intestinal organoids with the indicated treatment after IR. E. The expression of OLFM4 receptor was detected by fluorescence microscopy.

Figure 4

Identification of DEGs after FG-4592 treatment.

A. volcano map. B. heatmap. C. KEGG Pathway Classification. D. Top 30 Go Term.

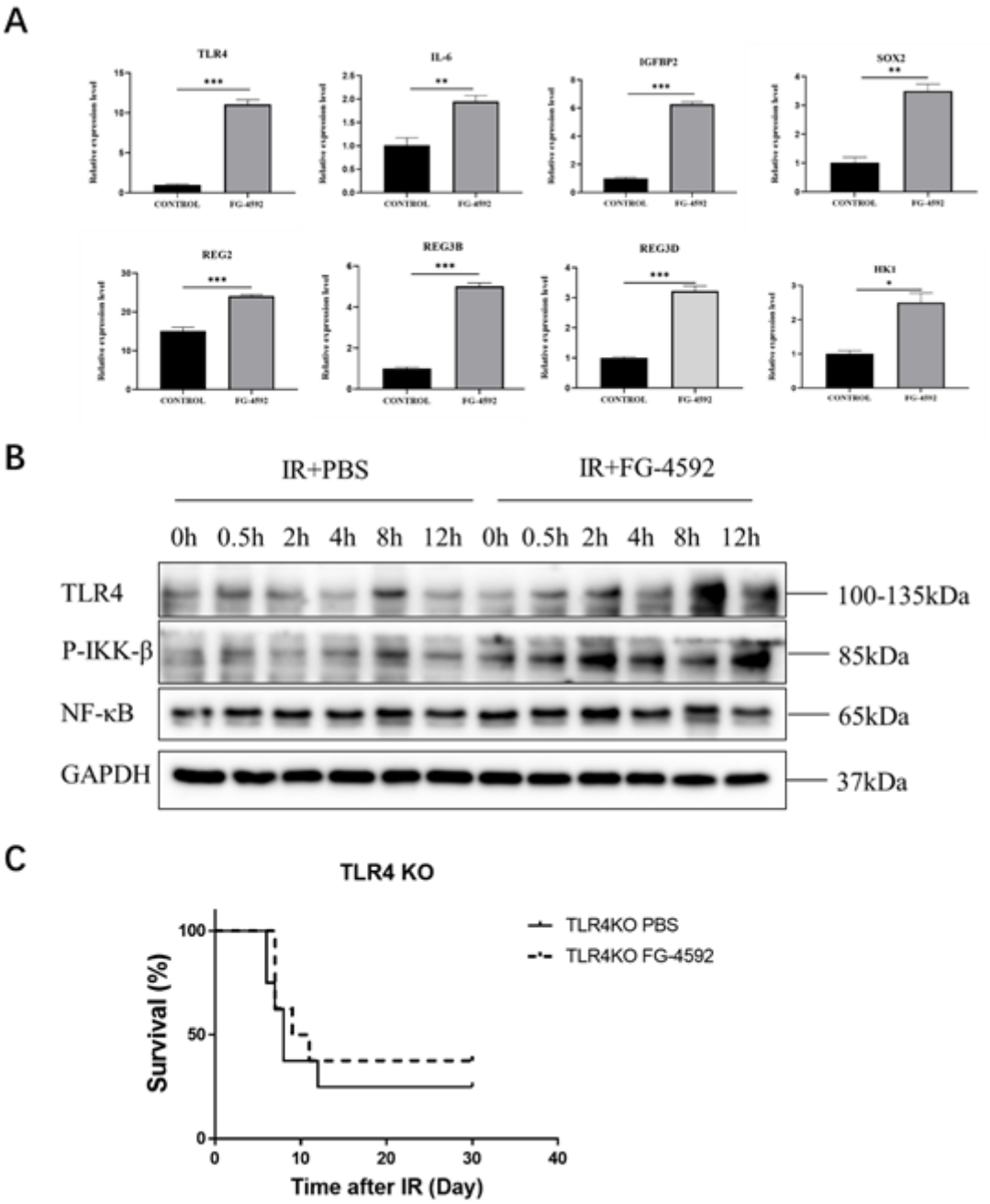

Figure 5

FG-4592 activates TLR4 related pathways. A. RNA level to verify the expression of DEGs such as IL-6, IGFBP2, SOX2, HK1, REG3B, REG2, REG3D. B. The expression of TLR4 pathway related proteins. C. The radioprotection of FG-4592 on TLR4 KO mice.