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The Role of Intestinal Stem Cells in Epithelial Regeneration Following Radiation-Induced Gut Injury

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Abstract
Purpose of Review Intestinal epithelial cells show remarkable plasticity in regenerating the epithelium following radiation injury. In this review, we explore the regenerative capacity and mechanisms of various populations of intestinal stem cells (ISCs) in response to ionizing radiation.
Recent Findings Ionizing radiation targets mitotic cells that include "active" ISCs and progenitor cells. Lineage-tracing experiments showed that several different cell types identified by a single or combination of markers are capable of regenerating the epithelium, confirming that ISCs exhibit a high degree of plasticity. However, the identities of the contributing cells marked by various markers require further validation.
Summary Following radiation injury, quiescent and/or radioresistant cells become active stem cells to regenerate the epithelium. Looking forward, understanding the mechanisms by which ISCs govern tissue regeneration is crucial to determine therapeutic approaches to promote intestinal epithelial regeneration following injury.

This article is part of the Topical Collection on *Radiation Biology and Stem Cells*

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Abbreviations

ALPI	Alkaline phosphatase
ASCL2	Achaete-scute family BHLH transcription factor 2
ATOH1 (MATH1)	Atonal BHLH transcription factor 1
BMI1	B lymphoma Mo-MLV insertion region 1 homolog polycomb ring finger proto-oncogene
DCLK1	Doublecortin like kinase 1
DLL1	Delta-like canonical notch ligand 1
DTR	Diphtheria toxin receptor
GFP	Green fluorescent protein
HOPX	HOP homeobox
KLF4	Krüppel-like factor 4
KRT19	Keratin 19
LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5
LRIG1	Leucine-rich repeats and immunoglobulin like domains 1
MEX3A	Mex-3 RNA binding family member A
mTERT	Telomerase reverse transcriptase
OLFM4	Olfactomedin 4
PROM1	Prominin 1
PUMA	p53 Upregulated modulator of apoptosis
RFP	Red fluorescent protein
SMOC2	SPARC-related modular calcium binding 2
SOX9	SRY-box 9
STAT5	Signal transducer and activator of transcription 5

Introduction

Radiation therapy (RT) is a common treatment modality for malignant cancers and is used in approximately half of all cancer patients [1]. The major dose-limiting factor of RT is the damage to normal, non-cancerous tissues. Almost all patients undergoing RT to the abdomen, pelvis, or rectum develop acute enteritis [2]. In addition, 5–15% of the patients develop chronic enteritis due to fibrotic epithelium, ulceration, and damaged submucosa [3]. With the increasing cancer survival rate for all types of cancers [4], it is important to address the RT-induced side effects in patients. The major advances in radiation oncology have been in the increased precision of radiation dose delivery to reduce the damages to normal tissues and amplify effects on tumor cells [5]. Such advancements include image-guided radiation therapy and tumor-targeted radiosensitizers. However, currently available treatments for patients experiencing radiation enteritis are symptomatic cares for diarrhea, dehydration, malabsorption, and abdominal or rectal discomfort [1]. Amisfostine, an organic thiophosphate that can act as a free radical scavenger, is the only drug used in clinics to protect normal tissues from radiation-induced toxicity [1]. Considering a large number of cancer patients receiving radiotherapy and the critical functions it plays in cancer cures, the adverse effects of RT on the intestinal epithelial physiology warrant investigation.

Intestinal Stem Cells (ISCs)

The mammalian intestinal epithelium is a rapidly self-renewing tissues, with the entire epithelium replaced in approximately 3–5 days. Intestinal stem cells and progenitor cells that reside within the proliferative compartment of intestinal crypts are responsible for the rapid renewal of the tissue [6]. The cells within the crypts divide and differentiate as they migrate up the differentiated compartment of intestinal villi, which are primarily composed of absorptive enterocytes, goblet cells, enteroendocrine cells, and tuft cells [6].

Until now, two populations of ISCs have been identified. Active intestinal stem cells (aISCs), also called crypt base columnar (CBC) cells, rapidly divide to upwardly give rise to progenitor cells within the transit-amplifying (TA) zone [7]. The aISCs are major contributors to epithelial renewal, yet are sensitive to injuries incured from radiation or chemotherapy. Markers that identify aISCs include LGR5, ASCL2, OLFM4, SMOC2, PROM1, and SOX9^{lo} [8–13]. Located at "+4 to +6" positions from the bottom of the crypts are quiescent stem cells that are resistant to stress but become activated upon perturbation to aISCs, thus named reserve intestinal stem cells (rISCs). This population was initially proposed by Potten and colleagues as the labeling-retaining cells (LRCs) that are long-living dormant cells but with proliferative potential [14]. Several proposed markers for rISCs include BMI1, MTERT, HOPX, LRIG1, SOX9^{hi}, DCLK1, and KRT19 [15–22]. The development of lineage-tracing mouse models using promoter-driven expression of reporter genes, such as *GFP*, *RFP*, or *LacZ*, and inducible Cre recombinase system enormously contributed to identifying ISC markers. These mice showed that both active and reserve ISCs possess two major stem cell characteristics: the capacity to self-renew and to generate differentiated cells. In addition, the crypt TA zone harbors lineage-committed progenitors that express specific cell markers, such as ALPI for enterocyte progenitors [23], DLL1 or ATOH1 (MATH1*)* for secretory progenitors [24], and KRT19 for TA cells and rISCs [22]. Known ISC markers or those that regulate ISC function are summarized in Appendix Table 1.

Intestinal Epithelial Tissue Response to Radiation Injury

The clinically relevant γ-irradiation doses of 8 to 15 Gy are sufficient to induce intestinal epithelial injury in mice, although these doses are lethal due to bone marrow failure [73]. The mouse intestinal epithelium is capable of regenerating when exposed to total body irradiation (TBI) at doses below 14 Gy, as indicated by the increase in survival with bone marrow transplant or partial bone marrow shielding [73, 74]. However, doses above 15 Gy result in gastrointestinal acute radiation syndrome (GI-ARS) and mortality even with bone marrow transplant [74]. The regenerative responses by the intestinal epithelium can be divided into three phases: apoptotic phase, proliferative phase, and normalization phase (Fig. 1a). The apoptotic phase consists of first 2 days following irradiation. Histology and immunostaining analysis of irradiated mouse intestines show continuous crypt loss, shrinkage in crypt size, and shortening of the villi during this phase [73]. The regenerative phase follows the apoptotic phase between 2 to 4 days post irradiation, where surviving crypt cells regenerate entire crypts. While the overall number of crypts decrease in both small and large intestines, the regenerative crypts enlarge in size due to an approximately twofold increase in the number of proliferating cells. During the normalization phase, the size of the crypts and the length of the villi are restored to "homeostatic" (pre-irradiation) condition.

Intestinal Epithelial Cellular Response to Radiation Injury

Recent studies using lineage-tracing mouse models demonstrated remarkable plasticity within ISCs and progenitor cells in their capability to drive regeneration of the intestinal epithelium upon injury (Fig. 1b). The LGR5^{+} stem cell population is highly susceptible to ionizing radiation and is diminished upon injury [25]. One prominent source of regenerative

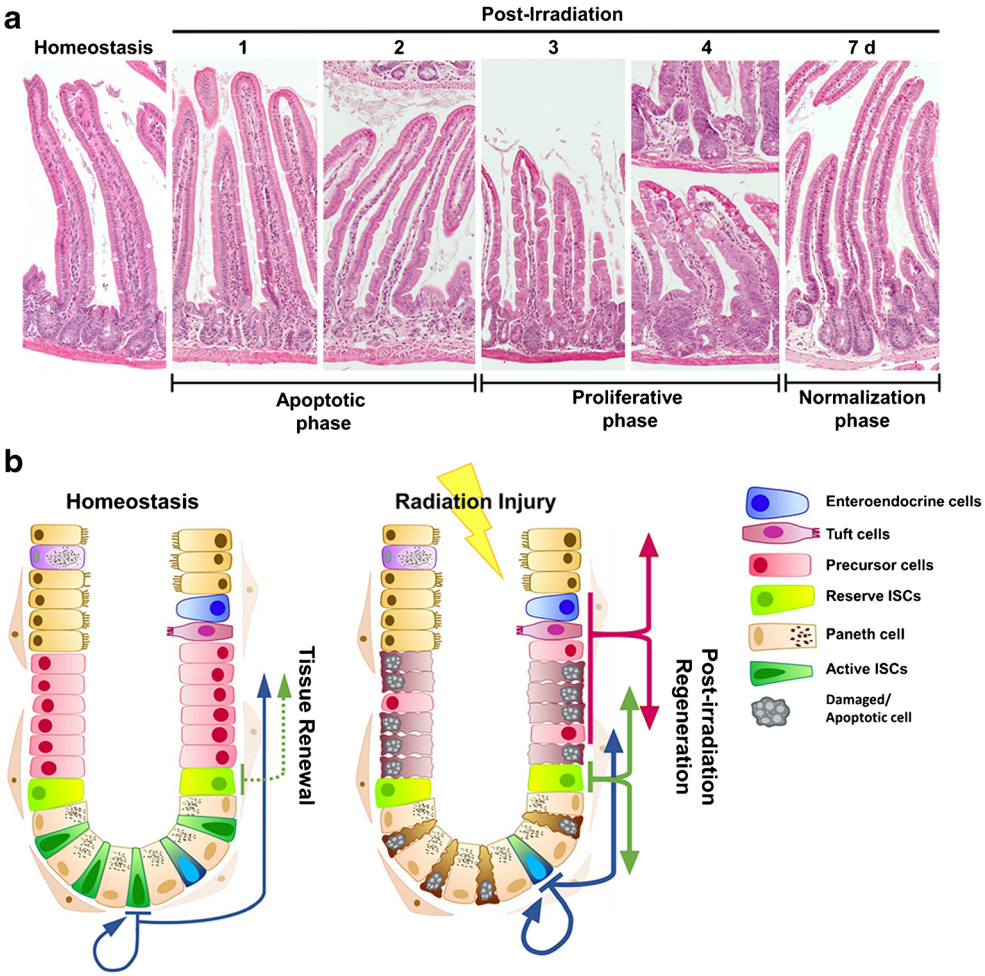

Fig. 1 **a** The regenerative responses by the intestinal epithelium can be divided into three phases, apoptotic phase (first 2 days), proliferative phase (2 to 4 days), and normalization phase (7 days) following radiation injury. **b** The intestinal epithelial cells demonstrate remarkable plasticity during regeneration following irradiation, where multiple populations of cells contribute to regenerative responses. (Fig. 1**b)** Adapted from Kuruvilla et al. with permission from Elsevier) [70]

responses are rISCs, regarded as a radioresistant population owing to its quiescence. When LGR5$^+$ aISCs are specifically depleted, rISCs enter the cell cycle, repopulate the crypt, and give rise to aISCs [44]. Of note, colonic and small intestine LGR5$^+$ cells respond differentially to irradiation damage. Under low- to medium-dose radiation, small intestinal LGR5$^+$ cells tolerate better compared to colonic LGR5$^+$ cells in terms of DNA damage repair and cell proliferation at 72 h following 0.1-, 1-, or 4-Gy irradiation [75]. On the other hand, colonic LGR5$^+$ aISCs are more resilient to high-dose irradiation injury compared to small intestine LGR5$^+$ aISCs and survive better following 19-Gy irradiation with complete DNA double-strand break repair [76].

Multiple studies have reported that rISCs are responsible for the regenerative response following radiation injury using *Bmi1*, *mTert*, *Lrig1*, or *Hopx* promoters as the lineage-tracing drivers [17, 19, 25, 39] (Appendix Table 1). These studies confirmed that cells expressing these markers were slow-cycling, distinguishably expressed in "position +4 to +6," and able to regenerate the epithelium by their lineages. Supporting the regenerative functions of non-LGR5-expressing stem cells, KRT19$^+$-expressing cells at "position +4" were also determined to have stem cell functions, distinguished from CBCs, and able to regenerate the epithelium following radiation injury in colon [22].

Maintenance of quiescence in rISCs is a crucial mechanism of radioresistance. A subpopulation of BMI1$^+$ rISCs also express KLF4, a zinc-finger transcription factor that is involved in regulation of the cell cycle by decelerating the rate of cell proliferation [70, 77]. Mice in which *Klf4* is deleted from BMI1$^+$ rISCs failed to regenerate the epithelium following 12-Gy irradiation, suggesting that the quiescence regulation by KLF4 also regulates cellular responses to radiation damage. In addition to KLF4, the transcription factor SOX9 [18] and RNA-binding proteins MSI1/2 [53] are involved in regulation of rISC quiescence via differential expression levels. The intestinal epithelial crypt cells with high levels of *Sox9* expression (EGFPHI) maintain quiescence through SOX9-mediated WNT signaling suppression [18]. Lineage-tracing experiments using *Sox9-CreERT2* showed that under 12-Gy irradiation, where aISCs are supposedly depleted due to their radiosensitivity, SOX9$^+$ cells were able to regenerate crypts, whereas conditional deletion of *Sox9* in the intestinal epithelium impaired this regeneration. On the contrary, MSI proteins

in rISCs are dispensable in homeostatic condition, but their increased levels are required to exit from quiescence during regeneration following 12-Gy irradiation [53]. Collectively, these studies confirm our understanding that quiescence in stem cell population provides a regenerative mechanism for the intestinal epithelium to restore homeostasis when aISCs are depleted.

In addition to rISCs, contributors to regeneration following radiation injury include the short-lived secretory progenitor cells in the TA zone. Abrogation of LGR5$^+$ aISCs in diphtheria toxin-treated *Lgr5*-DTR mice did not alter the epithelial structure, indicating that the intestinal epithelial renewal was maintained without LGR5$^+$ aISCs, but the number of enteroendocrine cells in the crypts doubled [44]. The increase in secretory cells suggested that secretory progenitor cells may serve as a source of tissue renewal in the absence of LGR5$^+$ cells. Using markers for lineage-committed secretory or enterocyte progenitor cells, lineage-tracing experiments showed that DLL1$^+$ secretory progenitor cells participate in regeneration of the epithelium when the homeostasis is disrupted with 6-Gy radiation injury [24]. The progenitor cells stop proliferating once they are committed to secretory lineages, and they stimulate NOTCH signaling pathways in neighboring cells by expressing NOTCH ligands to induce their fate to non-secretory lineages [78]. The stalled proliferation may also provide advantages to cell survival following irradiation injury, allowing them to de-differentiate to stem cells.

While multiple studies have reported that aISCs are more susceptible to radiation injury and undergo apoptosis within approximately 24 h following irradiation [22, 25], other studies suggest that LGR5$^+$ aISCs also contribute to tissue regeneration [30, 79]. According to Hua et al., not all LGR5$^+$ cells undergo apoptosis within 24 h following 10- and 12-Gy irradiation, and surviving LGR5$^+$ cells at 48 h are an important source of tissue regeneration [28]. The authors suggest that LGR5$^+$ cells are more efficient in DNA damage repair by homologous recombination compared to progenitor and villi cells, although further studies are necessary to confirm this observation. Supporting the notion that surviving LGR5$^+$ cells are involved in the regenerative response, depletion of LGR5$^+$ cells using the *Lgr5*-DTR mouse model immediately after 10-Gy irradiation decreased the number of regenerating crypts [30]. This study demonstrates that high-dose irradiation does not completely eliminate LGR5$^+$ cells, allowing surviving LGR5$^+$ cells to replenish the intestinal epithelium.

The contribution of LGR5$^+$ aISCs in the regenerative response is reasonable, considering aISCs have relatively slow proliferation rate of one cell cycle every 21.5 h, whereas progenitor cells divide once every 12 h [80]. It may be that the surviving LGR5$^+$ aISCs were at a cell cycle phase that is less sensitive to DNA damage. Another hypothesis is that there are subpopulations of cells within LGR5$^+$ cells identified in *Lgr5*-reporter mice that are differentially responsive to radiation injury. Indeed, single-cell transcript profiling of LGR5$^+$ aISCs isolated from *Lgr5*-EGFP mice showed that cells with high levels of GFP expressions are not exclusively aISCs but also include early progenitors derived from aISCs [27]. Interestingly, these early progenitor cells expressed high levels of cell cycle inhibitors, such as *Cdkn1a*, compared to "true" aISCs, indicating that these cells proliferate at a relatively slower rate. Recently, a subpopulation of slow-cycling LGR5$^+$ cells was identified, which expresses high levels of the RNA-binding protein Mex3a and is capable of organoid formation in ex vivo culture [56••]. Upon 12-Gy irradiation, LGR5$^+$MEX3A^{high} cells survived and regenerated crypts. Collectively, these data demonstrate the existence of slow-cycling LGR5$^+$ cell populations that are resistant to irradiation and may play a role during regeneration.

Reserve ISCs were first identified as label-retaining cells (LRCs) that incorporated labels in DNA and resided at position +4 [81]. A study using H2B-YFP mice for nuclear labeling showed that LRCs are secretory progenitor cells that ultimately differentiate to Paneth and enteroendocrine lineages, but with retained ability to de-differentiate into stem cells when homeostasis is perturbed [82]. Under 6-Gy irradiation, these LRCs were also capable of regenerating the crypts. Further supporting the notion that rISCs may be secretory progenitor cells, the transcriptome and open chromatin structure analyses of GFP$^+$ cells from transgenic reporter *Bmi1*-GFP mice showed strong similarities to secretory progenitor cells and enteroendocrine cells, suggesting *Bmi1*-GFP$^+$ cells are predetermined enteroendocrine cells [45••]. Additionally, *Bmi1*-GFP$^+$ cells were enriched for enteroendocrine marker messenger RNAs (mRNAs), such as *Gip*, *Pax6*, *Chga*, and *Chgb*, confirming that these cells are enteroendocrine lineages with the plasticity to convert to regenerative population after radiation injury [45••, 83••]. Interestingly, these cells also exhibited open chromatin structures in regions that are lineage-restricted, which potentially explains the high plasticity of these cells in response to aISC depletion. These observations provide first mechanistic explanations of how BMI1$^+$ rISCs are capable of regenerating crypts following irradiation injury.

Beside the secretory progenitors, mRNAs of putative rISC markers, *Bmi1*, *mTert*, *Hopx*, and *Lrig1*, have been found to be expressed in LGR5$^+$ cells using transcriptome analysis and in situ hybridization [11]. Curiously, the lineage-tracing mouse models using some of these rISC markers as driving promoters did not show tracings that occurred from CBCs or with similar mitotic kinetics. This may be that the lineage tracing of cells expressing these markers does not solely depend on the mRNA or protein levels.

A comparison study using *Lgr5*-EGFP, *Bmi1-CreERT2*, and *Hopx-CreERT2* transgenic mice with reporter genes and inducible Cre system showed that long-term LRCs are

homogenous Paneth or enteroendocrine lineage that have lost stem cell functions, thus distinct from aISC and rISC populations [39]. In addition, LRCs have differentially expressed cell cycle genes that were distinct from rISCs identified using reporter mouse models. This suggests that position +4 are variably occupied by LRCs or rISCs, which may be two different cell populations.

Secretory progenitor cells limit their numbers in the crypts through a process called "lateral inhibition," which induces neighboring cells to commit to highly proliferative absorptive progenitor cells [78]. On the contrary, absorptive progenitor cells are the dominant residents in the crypts, and $ALPI^+$ absorptive progenitor cells are capable of de-differentiating to give rise to the epithelium under circumstances that obliterate crypt aISCs [23]. Despite the highest proliferation rate, which may render them more sensitive to radiation injury, absorptive progenitor cells may contribute to regeneration following radiation injury, and further studies are warranted.

Mesenchymal Stem Cells in Intestinal Stem Cell Proliferation and Regeneration

Mesenchymal stem cells (MSCs) are undifferentiated fibroblast-like cells with the capacity to differentiate into various cell types, including muscle, bone, cartilage, and fat cells [84]. Recently, MSCs have been identified as a potential treatment for regenerative disease due to their ability to regulate inflammation, angiogenesis, and regeneration [85]. In the intestinal epithelium, MSCs also function as a critical mediator of injury repair by creating microenvironments that are suitable for ISCs and progenitor cells to regenerate crypts. For example, systemic administration of MSCs in irradiated mice resulted in improved tissue regeneration by increasing animal survival and reducing colonic ulceration [86, 87] and stimulating production of growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) that induce anti-inflammation and stimulate angiogenesis [87, 88]. The supportive function of MSCs in the intestinal epithelial regeneration is not specific to subpopulation of cells, but rather a global effect. It is important to understand the cell-to-cell and tissue-to-tissue interactions in studying tissue regeneration in order to account for complex environments of organs. The supporting function of MSCs is a potential therapeutic target for alleviating side effects of irradiation injury. MSCs can be pre-activated by pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and nitric oxide (NO), in amplifying the paracrine effects of MSCs on the intestinal epithelial regeneration after irradiation [89]. Priming local MSCs in preparation for radiation therapy may be advantageous for patients in recovering faster from the GI symptoms.

Following up with the involvement of pro-inflammatory cytokines in potentially creating a beneficial environment for the intestinal epithelial regeneration, signal transducer and activator of transcription 5 (STAT5) activation by cytokines are critical for crypt regeneration by ISCs after irradiation [72]. While *Stat5* deletion in $LGR5^+$ aISCs resulted in impaired crypt regeneration, *Stat5* activation in $LGR5^+$ cells resulted in increased proliferation and crypt regeneration following 15 Gy irradiation. In addition to MSCs, other cell types involved in the regenerative response following irradiation injury include endothelial cells [74, 90] and immune cells [91]. Collectively, these data indicate that external environments created by supporting cells are crucial to ISC proliferation and regeneration.

Factors Involved in Intestinal Epithelial Regeneration

As previously discussed, global effects of non-epithelial cells play critical functions in the regenerative response following radiation injury. Several factors have been implicated in acceleration of the regenerative process. Such factors include general inducer of proliferation, including R-spondin 1 [92, 93] and Slit guidance ligand 2 (Slit2) [93], secretory proteins that cooperatively promote the WNT/β-catenin signaling, growth factors insulin-like growth factor 1 (IGF-1) [94] and bFGF [95] that promote growth of the regenerative crypts and impair radiation-induced apoptosis through inhibition of PUMA expressions, and prostaglandins [96]. Other factors involved in the regenerative response after irradiation injury are Pectin [97] and glucagon-like peptide 2 (GLP-2) that directly stimulates $BMI1^+$ rISCs to promote stem cell proliferation during homeostasis and crypt regeneration following 10-Gy radiation injury. We can postulate that many other external modulatory factors are available to enhance the regeneration of the tissue. These factors may be dietary or secretory proteins contributed by epithelial or non-epithelial cells. It is of great interest to study natural molecules involved in maintenance of tissue homeostasis and regeneration.

Cellular Response to Radiation-Induced DNA Damage

The lethal effect of radiotherapy arises from cellular and molecular responses to DNA damage induced by free radicals causing double-strand breaks [98]. Ionizing radiation induces water radiolysis in cells, which generates reactive oxygen species (ROS) that become the major source of DNA damage. This type of damage is more detrimental to cells in the early DNA synthesis [99], which undergo apoptosis at a higher incidence than cells in other stages of the cell cycle. In the

intestinal epithelium, aISCs and progenitor cells in the crypts are more radiosensitive. The ultimate fate of these cells can be predicted based on the presence of apoptotic factors and DNA damage repair proteins. Indeed, stem cell populations with large accumulation of DNA damages may be more detrimental to overall health of the tissue, while survival of cells capable of DNA damage repairs and tissue regeneration is crucial.

P53/P21/PUMA

The transcription factor P53 is the key regulator of DNA damage response in the intestinal crypt. Following high-dose ionizing radiation, activated P53 upregulates the expressions of P21 and PUMA [100]. The protein P21 is a cyclin-dependent kinase (CDK) inhibitor that blocks the G1-to-S phase transition of the cell cycle and allows DNA repairs in the crypts [101]. On the other hand, activation of PUMA results in aISC and progenitor cell apoptosis following DNA damage, whereas the absence of PUMA leads to improved survival of intestinal stem cells and crypt regeneration [101]. Modulation of p53 activity following irradiation increases the cell survival. KLF4-mediated inhibition of p53 resulted in suppression of apoptosis and proliferation, thus allowing DNA damage repair [102, 103]. In addition to this observation, the pro-survival effects of GSK-3 inhibitor CHIR99021 on in vitro 3D culture of $LGR5^+$ aISCs following radiation was due to inhibition of p53-dependent induction of PUMA [104]. This suggests that inhibition of apoptotic pathways may be a therapeutic target for radiation-induced injuries.

NOTCH

The cell-to-cell NOTCH signaling in ISCs and progenitor cells has been shown to be important for stem cell functions and lineage commitments in the intestinal epithelium [33]. Deletion of *Notch1* increased the number of post-mitotic goblet cells, whereas deletion of *Notch2* had no effect on the lineage commitment [33]. However, inhibition of NOTCH with difluorophenacetyl-l-alanyl-S-phenylglycine *t*-butyl ester (DAPT) or deletions of *Notch1* or *Notch2* impaired crypt regeneration following irradiation injury due to absence of proliferation, suggesting that NOTCH receptors play important functions that are beyond cell fate determination, and strong NOTCH signaling is required for tissue regeneration [31, 33, 59]. Further investigations are necessary to elucidate the effect of irradiation on intestinal NOTCH signaling and its potential role in post-IR regeneration.

PI3K-AKT-mTORC1

Another important regulatory signaling pathway in intestinal epithelial tissue homeostasis is PI3K-AKT-mTROC1 signaling cascade. PTEN is a negative regulator of the PI3K-AKT-mTORC1 signaling pathway implicated in various cellular functions, such as proliferation and metabolism. PTEN is also implicated in maintenance of quiescence of $mTERT^+$ cells at "position +4" in the crypt, and deletion of *Pten* resulted in loss of quiescent ISCs [105], potentially due to aberrant proliferation and exhaustion of stem cells. Interestingly, PTEN functions to regulate dormancy of $mTERT^+$ rISCs. The GFP^+ cells' population obtained from the *mTert*-GFP mouse model showed lack of expression of inactive form of PTEN (P-PTEN) during homeostasis [17]. Yet, when these mice were irradiated with dose of 16 Gy, which is sufficient to eliminate the aISCs, the intestinal epithelium was able to regenerate 96 h later, possibly due to activation of rISCs, whereas *Pten*-deleted mice showed significantly less regenerative colonies [105]. Of note, the radiation dose used in this study induces GI-ARS, resulting in complete loss of intestinal epithelial regeneration. Thus, it is difficult to conclude that $mTERT^+PTEN^+$ rISCs play critical functions in regenerative response after irradiation, unless lower doses are used to study during the actual regenerative process.

The functions of the PI3K-AKT-mTORC1 pathway in the intestinal crypts may be cell type-specific. While the inhibition of this pathway is important for maintenance of quiescence in rISCs, mTOR plays critical functions in lineage generation and intestinal stem cell physiology of $OLFM4^+$ aISCs through mechanisms that are not mediated by WNT activity [34]. During intestinal crypt regeneration after 10-Gy irradiation injury, deletion of *mTOR* in the intestinal epithelium resulted in impaired regeneration, as well as decreased level of *Olfm4*. This result is interesting, because the expressions of *Ascl2* and *mTert*, which are WNT target genes and putative aISC markers, increase even with *mTOR* deletion [34]. This suggests that the putative stem cell markers may be indicators of active signaling pathways, and there is no bona fide single marker for ISCs, just as there are multiple essential signaling pathways involved in the maintenance of stem cell functions.

Conclusions

Radiotherapy is an essential part of cancer treatment, although clinical advancements in addressing its adverse effects on non-cancerous tissues have been insufficient. Understanding the mechanisms of intestinal epithelial regenerative processes is a critical step in developing therapeutic approaches for protection of normal tissue and may be beneficial to reestablish homeostasis post injury. The application of lineage-tracing mouse models has greatly contributed to determining cell populations responsible for the tissue homeostasis and regeneration following irradiation (Fig. 1b). However, the attempts to use a single marker to identify a cell population have resulted in an intricate list of cell markers and types. The recent focuses have been placed on delineating ISC or progenitor markers

using various methods, such as transgenic reporter mouse models, RNA sequencing, in situ hybridization, and chromatin structure analysis. Looking forward, it may be worthwhile to understand the global effects on the intestinal epithelium instead of targeting a cell population that contributes to regeneration, at least in achieving our goal to develop therapeutic approaches to ameliorate the adverse effects on normal tissue by radiotherapy. This will include determining the inter- and intra-cellular key mediators of the tissue regeneration that globally influence the tissue homeostasis and regeneration.

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Compliance with Ethical Standards

Conflict of Interest Chang-Kyung Kim, Vincent W. Yang, and Agnieszka B. Bialkowska declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Appendix

Table 1 Intestinal stem cell markers and functional proteins during homeostasis and post-irradiation regeneration

Gene name	Name	Expression pattern	Functions during homeostasis and post-IR regeneration	References
Active ISC markers (CBCs)				
Lgr5	Leucine-rich repeat-containing G protein-coupled receptor 5	aISCs	*Lgr5* is a WNT target gene that encodes a receptor for R-spondins that are involved in maintaining the expression of surface frizzled receptors to enhance WNT signaling Marks rapidly cycling stem cells that maintains the homeostasis of the intestinal epithelium with the capacity for multilineage differentiation and self-renewal $LGR5^+$ cells are more susceptible to radiation injury and undergo apoptosis within 24 h following irradiation A subpopulation of $LGR5^+$ cells can survive radiation injury and regenerate crypts	[8, 25–30]
Olfm4	Olfactomedin-4	aISCs	*Olfm4* encodes secretory glycoprotein olfactomedin 4, where the molecular function of OLFM4 is unknown. *Olfm4* gene expression is WNT-independent; however, it is regulated by the NOTCH signaling pathway in CBCs and progenitor cells Lineage tracing with *Olfm4-IRES-eGFPCreERT2* mice showed that $OLFM4^+$ cells are long-lived and multipotent mTOR signaling is important for $OLFM4^+$ aISC-driven crypt regeneration following irradiation	[10, 31–34]
Smoc2	SPARC-related modular calcium binding 2	aISCs	SMOC2 is a matricellular protein involved in matrix assembly Lineage-tracing with Smoc2-EGFP-ires-CreERT2 mice showed that $SMOC2^+$ cells are long-lived and multipotent cells The function of $SMOC2^+$ cells in IR response is not determined	[11]
Ascl2	Achaete scute-like 2	aISCs	*Ascl2* is a WNT target gene, where its expression is enriched in $LGR5^+$ aISCs It maintains the stemness of $LGR5^+$ aISC *Ascl2* gene is expressed in the entire crypt during crypt regeneration, but the function of $ASCL2^+$ cells in IR response is not determined	[9, 26, 35]
Rnf43 and *Znrf3*	Ring finger protein 43/zinc and ring finger 3	aISCs	RNF43/ZNRF3 are RING-type E3 ubiquitin ligases that negatively regulate the WNT signaling pathway by targeting frizzled receptors for degradation Their roles in IR response are not determined	[36]
Tnfrsf19 (Troy)	TNF (tumor necrosis factor) receptor superfamily, member 19	aISCs	*Tnfrsf19* is a WNT target gene that encodes a receptor tyrosine kinase required for cell migration and positioning in the intestinal crypts Lineage tracing with *Troy-CreERT2;Rosa-LacZ* mice shows that $TROY^+$ cells are long-lived and multipotent Troy negatively regulates LGR5-mediated signaling Its role in IR response is not determined	[37]
Reserve ISC markers				
Lrig1	Leucine-rich repeats and immunoglobulin-like domains 1	qISCs (+4 position)	LRIG1 is a negative inhibitor of ERBB signaling and functions to regulate aberrant proliferation by stem cells $LRIG1^+$ cells are slowly cycling and long-living	[19, 38]

Table 1 (continued)

Gene name	Name	Expression pattern	Functions during homeostasis and post-IR regeneration	References
			LRIG1^{+} cells are radioresistant and capable of crypt regeneration following 8-Gy irradiation	
Hopx	Homeodomain-only protein	rISCs (+4 to +7 position)	*Hopx* encodes a homeodomain-only protein, but the function of this protein in ISCs is unknown HOPX^{+} cells are slow-cycling stem cells found at +4 position and capable of giving rise to rapidly cycling ISCs (CBCs) mRNA and protein expressions detected in the CBCs; however, *Hopx*-driven reporter proteins are usually found at +4 to +7 positions HOPX $^{+}$ cells have differential expressions of CD24/CD44 compared to *Lgr5*$^{+}$ cells HOPX $^{+}$ ISCs contribute to crypt regeneration following 12-Gy irradiation	[11, 20, 39–41]
mTert	Mouse telomerase reverse transcriptase	rISCs (+4 position)	*mTert* encodes mouse telomerase reverse transcriptase that regulates telomerase activity mTERT^{+} cells are slow-cycling and multipotent cells found at +4 position mTERT^{+} cells are radioresistant and capable of surviving 1- or 10-Gy irradiation and contributing to crypt regeneration	[17, 42]
Markers with differential expression pattern				
Bmi1	Polycomb group RING finger protein 4	Reserve ISCs (+4 position) and predetermined enteroendocrine cells	BMI1 is a member of polycomb group of transcription repressors that are expressed in slow-cycling stem cells found at +4 position BMI1^{+} cells can replace aISCs when LGR5^{+} cells are depleted BMI1^{+} cells are radioresistant and capable of regenerating crypts following 12-Gy irradiation Notably, the *Bmi1*-lineage tracing model (*Bmi1-CreER*) has a penetrance of 10% and is limited to the proximal region of the small intestine mRNA expressions detected in LGR5^{+} and TA cells; however, *Bmi1*-driven reporter proteins are usually found at supra-Paneth positions (+1 to +6 positions) Transcriptome and open chromatin structure analyses showed that *Bmi1*$^{+}$ cells may be predetermined enteroendocrine cells	[11, 25, 26, 39, 43, 44, 45••]
EphB2	EPH receptor B2	Highest in active ISCs and gradually decreased as cells differentiate	*EphB2* is a WNT target gene and a receptor tyrosine kinase that is required for cell migration and positioning in the intestinal crypts EphB2^{hi} cells express mRNA of ISC-specific markers, such as *Lgr5* and *Ascl2*, capable of forming in vitro organoid Its role in IR response is not determined	[46–49]
Prom1 (CD133)	Prominin-1	Active ISCs and TA cells	Prominin-1 is a transmembrane protein PROM1^{+} cells that co-expresses *Lgr5* are capable of self-renewal and multilineage differentiation *Prom1-CreERT2-IRES-nLacZ-PGK-Neo mice showed that PROM1*$^{+}$ *cells are long-living and multipotent cells* Its role in IR response is not determined	[12, 50]
Msi1	Musashi-1	Active ISCs and reserve ISCs (+4 position)	*Msi1* encodes an RNA-binding protein that regulates proliferation through activation of WNT and NOTCH signaling pathways Using *Msi1*-eGFP mice, GFP^{Hi} cells co-express mRNA of rISC markers *mTert*, *Hopx*, and *Lrig1*, whereas GFP^{lo} cells express high levels of *Lgr5*, *Ascl2*, *Olfm4*, and *Smoc2* MSI1 and MSI2 expressions in rISCs are required for effective regeneration following 12-Gy irradiation.	[51–53]
Sox9	SRY (sex-determining region Y)-box 9	*Sox9*^{EGFPLO} (using *Sox9*^{EGFP} transgenic mouse) in active ISCs; *Sox9*^{EGFPHI} in +4 to +7 reserve ISCs; also expressed in enteroendocrine cells	SOX9 is a transcription factor that has dose-dependent functions in stem cells and precursor cells *SOX9*^{EGFPLO} cells are expressed in rapidly cycling stem cells that reside at the crypt base at a relatively low level *Sox9*^{EGFPHI} cells are slow-cycling and are enriched for *Bmi1* and *Hopx* *Sox9*^{EGFPHI} cells are radioresistant, and SOX9 is required for crypt regeneration following 12-Gy irradiation	[13, 18, 54]
Mex3a	Mex-3 RNA-binding family member A	Subpopulation of slow-cycling LGR5^{+} cells at +3 to +4 positions	MEX3A is an RNA-binding protein that regulates a transcription factor CDX2 LGR5^{+}MEX3A^{high} cells are a slow-cycling and multipotent subpopulation of LGR5^{+} cells	[55, 56••]

Table 1 (continued)

Gene name	Name	Expression pattern	Functions during homeostasis and post-IR regeneration	References
			$LGR5^{+}MEX3A^{high}$ cells are radioresistant and survive at 48 h following 12-Gy irradiation	
Dclk1	Doublecortin and CaM kinase-Like-1	rISCs (+4 position) and differentiated Tuft cells	DCLK1 is expressed in a subpopulation of $MSI1^{+}$ cells at +4 position and long-lived intestinal Tuft cells In situ hybridization showed that mRNA of *Dclk1* is also expressed in a subpopulation of $Lgr5^{+}$ cells at the crypt base $DCLK1^{+}$ Tuft cells are capable of self-renewing and functioning as stem cells following dextran sodium sulfate-induced injury Inhibition of the NOTCH pathway following 12-Gy irradiation decreases $DCLK1^{+}$ population and reduces crypt regeneration The protein kinase ataxia-telangiectasia mutated (ATM)-mediated DNA repair following radiation injury requires interaction with DCLK1	[21, 26, 57–62]
Nkx2.2	NK2 homeobox 2	Subset of enteroendocrine cells, $BMI1^{+}$ cells, and $LGR5^{+}$ cells	*Nkx2.2* encodes the transcription factor NK2 homeobox 2 that plays a critical function in enteroendocrine cell fate determination $NKX2.2^{+}$ cells retain stem cell-like characteristics, such as the capacity to multilineage differentiation $NKX2.2^{+}$ cells are radioresistant to 12-Gy irradiation	[63]
Krt19	*Keratin*-19	Reserve ISCs and TA cells	Keratin-19 is an intermediate filament that maintains the cytoskeleton Lineage tracing from *Krt19*-CreERT mice showed that $KRT19^{+}$ cells are a distinct population from $LGR5^{+}$ cells $KRT19^{+}$ cells are able to regenerate the epithelium following radiation injury in colon	[22]
Dll1	Delta-like 1	Secretory precursor cells and differentiated secretory cells	DLL1 inhibits neighboring cells become secretory cells by stimulating NOTCH signaling pathways $DLL1^{+}$ cells are capable of regenerating crypts following radiation injury	[64–66]
Math1 (Atoh1)	Atonal BHLH transcription factor	Secretory precursor cells	MATH1 is a basic helix-loop-helix transcription factor that regulates secretory cell fate determination Deletion of *Math1* results in loss of Paneth cell, without apparent consequences on CBCs during homeostasis or regeneration following radiation injury	[67, 68]
Klf4	Krüppel-like factor 4	Subpopulation of $BMI1^{+}$ rISCs at +4 position and differentiated cells	KLF4 is a transcription factor that regulates cell lineage differentiation and maintains epithelial homeostasis $BMI1^{+}$ and $KLF4^{+}$ cells are located at +4 position KLF4 is critical for crypt regeneration following 12-Gy irradiation	[69, 70]
Stat5	Signal transducer and activator of transcription 5	Intestinal epithelial cells, including $LGR5^{+}$ cells	STAT5 is transcription factor that plays a functional role in maintenance of intestinal epithelial cell integrity and response to gut injury STAT5 expression in $LGR5^{+}$ cells is required for aISC proliferation during homeostasis STAT5 is required for crypt regeneration following 12- and 15-Gy irradiation by $Lgr5^{+}$ aISCs	[71, 72]

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