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Crypt Base Columnar Stem Cells in Small Intestines of Mice Are Radioresistant

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Abstract

BACKGROUND & AIMS—Adult stem cells have been proposed to be quiescent and radiation resistant, repairing DNA double-strand breaks by nonhomologous end joining. However, the population of putative small intestinal stem cells (ISCs) at position +4 from the crypt base contradicts this model, in that they are highly radiosensitive. Cycling crypt base columnar cells (CBCs) at crypt positions +1–3 recently were defined as an alternative population of ISCs. Little is known about the sensitivity of this stem cell population to radiation.

METHODS—Radiation-induced lethality of CBCs was quantified kinetically in *Lgr5-lacZ* transgenic mice. γ -H2AX, BRCA1, RAD51, and DNA-PKcs foci were used as DNA repair surrogates to investigate the inherent ability of CBCs to recognize and repair double-strand breaks. 5-ethynyl-2'-deoxyuridine and 5-bromo-2'-deoxyuridine incorporation assays were used to study patterns of CBC growth arrest and re-initiation of cell cycling. Apoptosis was evaluated by caspase-3 staining.

RESULTS—CBCs are relatively radioresistant, repairing DNA by homologous recombination significantly more efficiently than transit amplifying progenitors or villus cells. CBCs undergo apoptosis less than 24 hours after irradiation (32% $\pm$ 2% of total lethality) or mitotic death at 24–48 hours. Survival of CBCs at 2 days predicts crypt regeneration at 3.5 days and lethality from gastrointestinal syndrome. Crypt repopulation originates from CBCs that survive irradiation.

CONCLUSIONS—Adult ISCs in mice can cycle rapidly yet still be radioresistant. Importantly, homologous recombination can protect adult stem cell populations from genotoxic stress. These findings broaden and refine concepts of the phenotype of adult stem cells.

Keywords

Radioresistance; GI Syndrome; BrdU; Cancer Treatment

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Conflicts of interest The authors disclose no conflicts.

A body of recent work proposed the hypothesis that adult normal tissue stem cells are resistant to DNA damage-mediated cell death compared with more differentiated cells along the same lineage.^{1,2} In 2 systems in which the phenotype of stem cells has been well defined, the bone marrow (BM) and hair follicle, irradiated hematopoietic stem cells (HSCs) and bulge stem cells (BSCs) of the hair follicle, respectively, are resistant to p53-mediated apoptosis compared with their committed progeny. This radioresistant stem cell phenotype is attributed to attenuated p53 expression in the case of the HSC and enhanced Bcl-2 expression in the BSC.^{1,2} An attribute that contributes to radioresistance in this stem cell phenotype is increased nonhomologous end joining (NHEJ)-mediated repair of radiation-induced DNA double-strand breaks (DSBs).

A functional consequence of this status is that normal tissues are preserved at the expense of accrued DNA damage and genomic instability, leading at times to enhanced tumorigenesis. A corollary to this concept is that embryonic stem cells are proliferative and repair by homologous recombination (HR), which allows for generation of stem cell populations with high-fidelity DNA required for sustaining life of the organism.³ The cost of this HR predilection is increased apoptosis to remove damaged cells from the gene pool.³ It has been suggested that an outlier to this concept is the p53-dependent quiescent intestinal stem cell (ISC) at the +4 position relative to the crypt base,⁴ which purportedly undergoes enhanced apoptotic death compared with its differentiated lineages. At low radiation doses (1 Gy), virtually all of this putative ISC population undergoes rapid (within 3–6 h) apoptotic death. It has been argued that such radiation sensitivity protects the bowel from accumulating potentially mutagenic lesions that could result in eventual tumorigenesis.⁴ However, whether the p53-dependent +4 quiescent cells truly represent a relevant ISC population has been questioned because apparent deletion of these cells by low-dose irradiation (~1 Gy) does not lead to intestinal injury,⁵ whereas higher doses (8–15 Gy) are required for tissue damage, organ failure, and animal lethality.⁶

The gastrointestinal (GI) mucosa is a rapid turnover system, driven by mitotic activity of its clonogens consisting of self-renewing stem cells and their transit amplifying (TA) daughter cells, together referred to as the stem cell clonogen (SCC) compartment. At baseline, TA cells traffic up through the crypt to the villus while differentiating into the functional cells of the small intestines, eventually being extruded from the villus tip into the gut lumen. Exposure to high-dose ionizing radiation (IR) can cause lethal GI injury, a process known as *radiation GI syndrome*. The earliest relevant crypt SCC response to radiation seems to be delayed progression through a late S-phase checkpoint and mitotic arrest, coupled with continued epithelial cell migration along the crypt-villus axis and extrusion from the villus tip.⁷ Outcome of this combined response is progressive crypt shrinkage during the first 24–48 hours after irradiation.⁸ Release from mitotic arrest is associated with hyperproliferative activity of SCCs that have not migrated from their original crypt location.^{7,9} Resumption of mitotic activity, however, leads to rapid depletion of crypt SCCs dying of mitotic cell death^{8,10} (although this has never been formally proven). Mitotic cell death is generic to irradiated mammalian cells, resulting from residual or misrepaired DNA DSBs, which confer genomic instability and postreplication generation of mutations and chromosomal aberrations, eventually leading to death of injured progeny.¹¹ However, direct evaluation of ISC mitotic death is incompletely understood because until recently there were no markers specific for GI stem cells. In this study, the contribution of ISC death to organ loss was assessed quantitatively and kinetically in vivo using the leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5)-lacZ transgenic mouse model.

Self-renewing ISCs divide about a thousand times throughout life, and are essential for tissue homeostasis and repair. This enormous functional demand and stem cell longevity suggest ISCs are equipped with effective DNA damage response mechanisms to ensure

genomic integrity over a lifetime. Normally, IR-induced DSBs enlist either error-prone NHEJ for DSB repair or high-fidelity HR, feasible only in S/G2-phase cells.¹² Although the mechanism driving choice between alternative DSB repair systems remains unknown, spatiotemporal engagement of each repair mode can be monitored via immunohistochemical staining of mode-specific repair mediators incorporated into discrete IR-induced repair foci (IRIF), rapidly produced at sites of DSBs.¹³ Early in the DNA damage response, ataxia-telangiectasia mutated (ATM) phosphorylates serine 139 on the C-terminus of H2AX histone at multiple chromatin sites flanking DSBs, generating γ -H2AX. This master IRIF regulator is involved in assembly of mediator and adaptor proteins (ie, MDC1, RNF8/RNF168, RAP80, BRCA1, and 53BP1), producing a platform for efficient signal amplification of cell-cycle checkpoint control and HR repair. Although NHEJ does not depend on γ -H2AX activity,¹⁴ NHEJ effectors nonetheless co-localize with γ -H2AX at IRIF.¹⁵ Here we used co-registration of γ -H2AX with BRCA1 and Rad51 as markers of HR, and with DNA-PKcs as a marker of NHEJ, to define radiation-induced DNA damage responses of ISCs.

Recent studies have defined crypt base columnar cells (CBCs), cycling cells most often located between Paneth cells at positions +1–3 from the crypt base,¹⁶ as an ISC population.¹⁷ CBCs are characterized by high-level expression of the Wnt target gene *Lgr5* (also known as *Gpr49*).¹⁷ Lineage-tracing experiments showed that a *Lgr5*-positive cell generated all mouse intestinal terminally differentiated epithelial lineages over a 1-year period,¹⁷ and that a single-sorted *Lgr5*⁺ stem cell is capable of generating ever-expanding crypt/villus organoids in vitro, in which all differentiated intestinal mucosa cell lineages are present.¹⁸ These observations provide definitive evidence that CBCs constitute at least a major component of the ISC compartment. Little is known regarding sensitivity of this stem cell population to radiation damage. Here, we show that the CBC ISC, similar to the quiescent HSC and BSC, shows DNA repair-mediated radiation resistance, allowing for generalization of the concept that normal adult tissue stem cell populations display increased DNA repair to survive genotoxic insults.

Materials and Methods

Mice

Lgr5-lacZ mice were genotyped and used as described.¹⁷ Mouse protocols were approved by Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Foci Quantification

Fluorescence images were captured by a Zeiss LSM5 Live line-scanning confocal microscope (Zeiss, Jena, Germany) to map 3-dimensional distribution of nuclear foci into several 2-dimensional z-stack images. Step size between slices was 0.4 μ m (z-direction). Images of 30 slices were captured/z-stack to map the entire nucleus.¹⁹ At least 30 images were collected randomly for each time point. For γ -H2AX, DNA-PKcs, and BRCA1 foci, MetaMorph 7.6 software (Molecular Devices) was used for data analysis. Foci were scored within a nucleus whose boundary was defined from a 4',6-diamidino-2-phenylindole image. The focus threshold was set manually and 8.28 pixels determined the average focus size based on discrete γ -H2AX foci generated in a 2 Gy-treated sample (at 15 min). At early time points after 12 Gy and 15 Gy, owing to extensive γ -H2AX focus formation, individual foci could not be distinguished accurately. By applying the standard 8.28 pixels/foci to the entire fluorescent nuclear region, we estimated approximate numbers of foci/nucleus for these early times. RAD51 foci number were counted by eye. All quantitative foci studies were performed using mice receiving irradiation without BM.

β-Galactosidase (LacZ) Staining

Lgr5-lacZ mice were euthanized after radiation, and four 2.5-cm sequential segments of proximal jejunum from the ligament of Treitz were obtained. Staining for the presence of β-galactosidase was as described by Barker et al.¹⁷

5-Ethynyl-2'-Deoxyuridine and 5-Bromo-2'-Deoxyuridine Incorporation Assay

Mice were injected intraperitoneally with 350 μL of 3 mmol/L 5-ethynyl-2'-deoxyuridine (EdU) solution in phosphate-buffered saline, or 300 μL 5 mg/mL 5-Bromo-2'-Deoxyuridine (BrdU) solution 4 hours before death.²⁰

Statistical Analysis

Statistical analysis was performed using the Student *t* test. Full methods are available in the Supplementary Materials and Methods.

Results

IR Induces BM and GI Lethality in *Lgr5-lacZ* Transgenic Mice

The current studies used *Lgr5-lacZ* transgenic mice,¹⁷ commonly used to mark CBCs, because the *lacZ* gene is integrated into the last exon of the *Lgr5* allele (Figure 1A). We first defined the phenotypic response of *Lgr5-lacZ* mice to whole body radiation (WBR) with respect to induction of the lethal GI syndrome, considered a consequence of total or near-total depletion of the ISC compartment.⁸ As in the parental C57BL6 strain,⁶ 12 Gy WBR results in the death of *Lgr5-lacZ* mice (Figure 1B) from BM aplasia at 9–10 days, not the GI syndrome, as confirmed by autopsy (Figure 1C). BM damage was diagnosed as the cause of death when marrow showed extensive matrix necrosis, widespread hemorrhage, and complete depletion of hematopoietic elements.⁶ Accordingly, BM transplantation (5×10^6 cells at 16 h after irradiation) prevented animal mortality (Figure 1B). In contrast, 15 Gy induced more rapid death of this strain at 5–7 days from the GI syndrome as evidenced clinically by diarrhea and weight loss, and at autopsy by complete loss of crypt-villus units. In addition, BM transplantation had no impact on the temporal pattern of death or autopsy findings at time of death after 15 Gy. Moreover, in a separate experiment, we compared survival using heterozygous *Lgr5-lacZ* and wild-type (littermate) control mice. At 15 Gy WBR, all *Lgr5-lacZ* mice died at 5.2 ± 0.5 days from autopsy-proven GI death (data not shown). Wild-type littermates similarly died at 5.7 ± 0.7 days ($P = .14$, *Lgr5-lacZ* vs wild-type littermates). These studies show that loss of a single *Lgr5* allele has no significant impact on the pattern of death from the GI syndrome in C57BL6 mice.

CBCs Are Relatively Radioresistant

The microcolony assay (also termed the *clonogenic assay*), developed by Withers and Elkind,²¹ directly quantifies radiation dose-dependent lethality of the crypt SCC compartment⁸ and is predictive of eventual animal death from the GI syndrome. This assay, which measures the number of regenerating crypts per intestinal circumference at 3.5 days after radiation, serves as a surrogate for stem cell survival, and is a pure measure of GI damage because it is not influenced by concomitant damage to other organs such as BM.²² Coupling Kaplan-Meier animal survival with the microcolony assay has revealed that 10–15 regenerating crypts per circumference, or approximately 8%–12% crypt survival, is minimally required for full recovery of the GI mucosa and GI tract survival.^{6,23} Figure 2A shows that unirradiated *Lgr5⁺-LacZ* mice contain 122 ± 5 crypts/small intestinal circumference, reduced to 23 ± 5 crypts by 12 Gy treatment ($P < .001$; associated with full recovery of GI mucosa and no GI syndrome lethality; Figure 1) and further to 1 crypt/circumference by 15 Gy irradiation ($P < .001$ vs 12 Gy; and GI syndrome lethality; Figure

1), consistent with a large body of literature on this topic.^{24,25} CBCs show a similar response profile with 295 ± 6 CBCs detected in unirradiated *Lgr5⁺-LacZ* mice reduced to 19 ± 1 CBCs at 3.5 days after 12 Gy ($P < .001$; Figure 2B), a nonlethal dose to the GI tract, whereas <1 CBC/small intestinal circumference was detected using the lethal dose of 15 Gy ($P < .001$ vs 12 Gy). Paneth cells represent a niche cell for *Lgr5⁺* stem cells.²⁶ In this context, the number of Paneth cells slowly decrease in parallel with loss of CBCs (Supplementary Figure 1).

CBC Deletion Is a Predictor of Radiation GI Damage

Furthermore, at 2 days post-irradiation, a time preceding consistent loss of crypts,⁸ reduction in CBC number nonetheless can be detected (Figure 2C). Note that the number of surviving crypts at day 2 after radiation was unchanged, although crypt shrinkage had occurred.²⁷ At 8–10 Gy, doses minimally impacting crypt number at 2 days determined by the Withers and Elkind²¹ microcolony assay, significant CBC deletion already is observed ($P < .001$ each vs unirradiated control). Further, when comparing impact of these radiation doses at 2 days with 3.5 days, it is apparent that damage to CBCs at low radiation doses (8–10 Gy) is reversed upon transition to 3.5 days, whereas at the higher doses depletion is progressive (compare Figure 2B with C). At day 2, CBC numbers/circumference at 8 and 10 Gy are 72 ± 4 and 53 ± 3 , which increase at day 3.5 to 227 ± 5 and 151 ± 4 , respectively. A more detailed analysis of the temporal pattern of CBC loss (Figure 3) reveals that 2.0 days is the nadir after 12 Gy with recovery after 3.5 days (Figure 3, *right upper and lower panels*), whereas at 15 Gy depletion is evident by 1 day post-irradiation ($P < .001$ vs unirradiated control) and progresses to undetectable levels thereafter (Figure 3, *left upper panel*), shown histologically (Figure 3, *left lower panel*). Note that many crypts display a cluster of LacZ-positive CBCs (up to 12) at the crypt base at day 10 after 12 Gy, consistent with extensive crypt fission often observed at this time post-irradiation²⁸ (Supplementary Figure 2). A Spearman correlation analysis revealed number of CBCs and of surviving crypts were highly correlated at day 3.5 at multiple radiation doses ($R = 0.95$, $P < .0001$). These studies indicate that CBC death correlates closely to the clinical radiation GI syndrome. Because these studies depend on use of a *LacZ* reporter gene, we have repeated these studies identifying CBCs as wedge-shaped cells between lysozyme-stained Paneth cells and have obtained virtually identical results (Supplementary Figure 3).

CBCs Repair DNA Damage More Efficiently Than Differentiated GI Cells

Evolving concepts indicate that early phase (8 h) γ -H2AX IRIF detect DSB number, while kinetics of IRIF resolution serve as a surrogate for DSB repair.¹⁴ Alternately, late-phase (24 h) γ -H2AX foci are now considered indicative of chromatin remodeling associated with unrepaired DSBs or post-repair chromosomal aberrations and genomic instability,²⁹ precursors of cell lethality via the reproductive (also termed *mitosis-associated* or *clonogenic*) cell death pathway.¹¹ Figure 4 shows that although cells in all compartments of the crypt-villus unit rapidly accrue similar numbers of γ -H2AX foci after 12 Gy (for additional confirmation at 2 Gy see Supplementary Figure 4), only CBCs completely resolve γ -H2AX foci (at 24 h $P > .05$ vs unirradiated control, $P < .001$ vs TA cells; shown are typical $20\times$ low-magnification images in Figure 4A and $63\times$ high-magnification images in Figure 4C). In fact, after an early repair phase lasting 4–6 hours, the TA compartment, which contains progenitor cells, comprising positions +4–10 from the crypt base, and the differentiated cell populations of the villus, only minimally resolve γ -H2AX foci (Figure 4B and C). The most parsimonious interpretation of these data is that CBCs repair DSBs efficiently, preserving small intestinal integrity. In contrast to 12 Gy, at 15 Gy CBC γ -H2AX foci do not resolve completely by 24 hours after irradiation (Supplementary Figure 5), preceding CBC depletion at 2–3.5 days (Figure 3, *left panels*).

Consistent with this concept, although CBCs and TA cells rapidly incorporate BRCA1 and RAD51 into repair foci, villus cells do not (Figure 5A). Typical low- and high-magnification images of BRCA1 staining are shown in Figure 5B and C, respectively, whereas Figure 5D displays high-magnification RAD51 focus images. Note that the low-level staining detected in villus cells is not in foci but rather diffuse (Supplementary Figure 6). BRCA1 and RAD51 IRIF formation occurs rapidly in irradiated CBCs, reaching a maximum by 1 hour after IR. It takes 2 hours to reach maximum in TA cells. Furthermore, CBCs fully resolve BRCA1 (Figure 5A, *left*, and C) and RAD51 (Figure 5A, *right*, and D) foci (at 24 h $P > .05$ each vs unirradiated control, $P < .001$ each vs TA cells), whereas TA cells fail to do so, consistent with failure to resolve γ -H2AX foci at sites of DSBs. Lack of high-quality commercial anti-Lgr5 antibodies makes it difficult to perform double staining with DNA repair markers and Lgr5. To provide direct evidence of efficient DNA repair in Lgr5⁺ CBCs, we used Lgr5-enhanced green fluorescent protein (EGFP)-ires-Cre-ERT2 mice. γ -H2AX and BRCA1 were co-stained with GFP in small intestines of Lgr5-EGFP-ires-CreERT2 mice at key time points (6 and 12 h) after 12 Gy (Supplementary Figure 7). At 6 hours after 12 Gy, GFP^{high}-Lgr5⁺ CBCs display 15 ± 3 γ -H2AX foci vs 38 ± 8 in TA cells ($P < .001$), and 8 ± 3 and 22 ± 6 BRCA1 foci, respectively ($P < .001$). Similar data were obtained at 12 hours. Double-staining of γ -H2AX or BRCA1 with GFP thus confirm that Lgr5⁺ CBCs repair DNA significantly more efficiently than TA progenitors.

Given that apoptosis may affect DNA repair analyses, we performed experiments using p53 up-regulated modulator of apoptosis (*PUMA*)-/- mice. We observed *PUMA* deficiency resulted in an approximately 70% reduction of apoptosis in both CBCs and TA compartments 6 hours after 12 Gy, consistent with published data that indicated *PUMA* deficiency significantly blocks crypt epithelial apoptosis after high-dose radiation in this strain.³⁰ DNA repair foci (γ H2AX, Rad51, and BRCA1) data in *PUMA*-/- mice confirm that CBCs repair damage more efficiently than TA cells (Figure 5E). In irradiated small intestines, evidence of DNA-PKcs engagement in NHEJ also was detected in CBC IRIF using an antibody against the T2609 phosphorylation site of DNA-PKcs (Figure 5F), required for downstream activation of the NHEJ pathway.¹⁵ In contrast to inefficient resolution of HR foci by TA cells compared with CBCs, TA cells resolve DNA-PKcs foci only slightly less efficiently than CBCs (Figure 5F). Villus cell populations, however, fail to show DNA-PKcs foci. These data indicate that although CBCs and TA cells both use NHEJ effectively, CBCs are more effective in high-fidelity HR repair. Consistent with our data, very recently Merlos-Suarez et al³¹ reported that DNA-damage repair genes such as BRCA1 and RAD51 are enriched in CBCs.

Repopulation Begins From CBCs

The earlier-described studies showing CBC death occurs over 48 hours provided an opportunity to examine mechanisms of cell death in a validated stem cell population in vivo. To our knowledge there is no quantitative assessment of contributions of distinctive cell death events to ultimate organ loss in a solid tissue. Because mammalian cells have an elaborate cell-cycle checkpoint machinery, rapidly activated in the DNA damage response, and because available cell death mechanisms such as apoptosis and mitotic death relate to cycling status post-irradiation, we examined the pattern of CBC growth arrest and re-initiation of cell cycling using EdU staining over a 72-hour time course. Although EdU staining was observed throughout the CBC and TA compartment before irradiation, albeit at higher rates in TA cells, within 12 hours after 12 Gy no EdU labeling of crypts was detected, consistent with known cessation of proliferation post-irradiation. EdU-positive crypts begin to appear at 18 hours and peak at 24 hours after irradiation (with $88\% \pm 3\%$ of crypts positive), then decrease precipitously at 48 hours ($P < .001$ vs 24 h) (Figure 6A and B). We

suggest these data indicate EdU-positive cycling crypt cells began to die at or soon after the first post-irradiation mitotic peak.

Furthermore, we note that repopulation originates, at least in some crypts, from surviving CBCs after irradiation. By using lysozyme staining to identify Paneth cells, we observed that a population of wedge-shaped cells between Paneth cells, which likely represent CBCs, have re-entered S phase at 18 hours after irradiation (Figure 6A). Double staining with BrdU and lacZ on serial sections confirms the CBC population starts proliferating first post-irradiation (Figure 6C and D). At 18 hours after irradiation, $90\% \pm 5\%$ of BrdU-labeled cells are Lgr5⁺ stem cells (ie, CBCs), whereas only $9\% \pm 1\%$ of cells labeling with BrdU are Lgr5⁺ stem cells in unirradiated mice (Figure 6D; $P < .001$). In contrast, EdU-positive TA cells do not appear in the crypt until 24 hours after irradiation. These data are consistent with the observation that CBC stem cells need approximately 18 hours to finish DNA repair (Figure 4), whereas TA progenitor cells require more time. Consistent with these data, we found a 24-hour delay in migration of cells from the TA compartment into the villus in irradiated mice compared with control mice (Supplementary Figure 8).

To confirm repopulation originates from surviving CBCs upon radiation, we performed lineage tracing assays using Lgr5-EGFP-ires-CreERT2/Rosa26-lacZ mice. Lgr5⁺ CBC-derived lacZ lineage tracing is observed along crypt/ villus units after 12 Gy, showing that Lgr5⁺ CBCs represent a population that regenerates epithelium after irradiation (Figure 6E).

Apoptotic and Mitotic Death Occur Sequentially After Irradiation

In this study, a robust and immediate apoptotic response, detected by immunostaining for active caspase-3, was observed in the lower crypt after irradiation (Figure 7). Apoptotic cell death of CBCs peaked at 6 hours, decreasing rapidly thereafter, such that apoptosis was detected in only $5\% \pm 1\%$ of CBCs at 24 hours after irradiation and was negligible by 48 hours. These data indicate that growth-arrested noncycling CBCs make an early decision on cell fate coincident with or just after the majority of DNA repair is complete. The peak of TA cell apoptosis occurred somewhat later at 12 hours after irradiation and apoptosis remained increased at 24 hours. Quantitation of the extent of CBC loss during the first and second 24 hours after 12 Gy (Figure 3) revealed that $32\% \pm 2\%$ of CBCs died during the first 24-hour time period and $58\% \pm 3\%$ died during the second 24-hour period. Thus, the majority of CBC cell death occurred after apoptosis was complete and CBCs had re-entered the cell cycle (Figure 6), consistent with classic radiobiologic concepts that genomic instability after irradiation results in mitotic death of cells once repair is complete and cells re-initiate cell cycling, owing to residual misrepair and unrepair of DNA DSBs that lead to chromosomal aberrations during cell divisions.¹¹

Discussion

Our studies show that despite being a proliferative ISC compartment, the CBC stem cell is radiation resistant compared with the previously described p53-responsive ISC at position +4 from the crypt base, repairing DNA damage by HR more efficiently than other cells in the small intestines. Whether actively dividing CBCs are more radiation resistant than the newly described Bmi1⁺ and Hopx⁺ quiescent stem cells also at position +4, a population that can be derived from Lgr5⁺ CBCs (and vice versa),³² presently is uncertain because the literature is divided on this topic.^{33,34} As our dose-response curves show variations in CBC radiosensitivity, with a graded reduction in survival as a function of dose within the range of 8–15 Gy, these observations are consistent with CBCs cycling constantly with relatively short doubling times.²⁰ In fact, it is reasonable to assume that individual CBCs show cell-cycle-dependent variations in radiosensitivity, because some such variations are reported to be generic for irradiated cycling mammalian cells in vitro and in vivo (reviewed by

Denekamp³⁵). Consistent with this notion, Withers et al³⁶ reported fluctuations in survival of jejunal crypts exposed to radiation as they pass through the cell cycle after synchronization with hydroxyurea, with maximum sensitivity observed at or close to mitosis and maximum resistance at late S phase. In this regard, CBCs are highly efficient in DNA repair by the HR pathway, active at late S phase,³⁷ as compared with TA cells and differentiated populations of the villus. NHEJ-mediated repair is nonetheless important for integrity of the post-radiation DNA damage response because inactivation of DNA-dependent protein kinase in C57BL/6^{Prkdc/SCID} severe combined immunodeficient mice results in substantial radiosensitization of the murine GI tract.³⁸

Although CBC lethality increases as a function of dose, within the range of 8–14 Gy sufficient numbers of surviving CBCs support complete anatomic and functional GI tissue and organ recovery, whereas at 15 Gy insufficient CBCs survive to restore small intestinal viability. Our data and other data show highly similar dose-dependent reductions in crypt survival at 3.5 days,^{6,8,21,24} with a steep reduction from the minimum of 10–15 surviving crypts required to prevent animal lethality from GI syndrome at 14 Gy to <2 crypts/small intestinal circumference at the 100% lethal dose of 15 Gy.^{6,23} The apparent capability of CBC survival at 2.0 days to predict outcome of the clonogenic assay at 3.5 days suggests that counting residual CBCs may be as good or better a predictor of radiation GI damage than the established gold standard 3.5-day clonogenic assay, a notion that will require confirmation by further investigation, applying multiple modalities. In this regard, by using CBC deletion as a readout, it may be possible to develop a screen for agents that serve as protectors (delivered before irradiation) and/or mitigators (delivered post-irradiation) of the lethal radiation GI syndrome, which currently represents an untreatable toxicity to the human GI tract, such as might occur after a nuclear accident.

Although the data defining CBCs as the causative stem cell for colon cancer in human beings remain incomplete, the data derived from the cross of *Lgr5⁺-EGFP-ires-CREERT2* mice with *apc*-floxed mice, which die of small intestinal adenomas, are consistent with that notion.³⁹ Further, a recent clinical study showed that *Lgr5* overexpression was associated with poor prognosis in colorectal cancer patients.⁴⁰ In addition, a gene signature specific for ISCs that includes *Lgr5* predicts disease relapse in colorectal cancer.³¹ In this regard, our ability to evaluate the death response of the CBC should be invaluable as we search for better therapeutic strategies to alter survival of GI tumor stem cells.

Finally, this study quantified mechanisms of cell death available to a solid-tissue stem cell. Our data indicate that at a high yet sublethal dose of IR, 12 Gy, approximately one third of overall CBC death is via apoptosis induction and two-thirds is via mitotic death. These death responses appear to be determined by the proliferative status of the CBC, with apoptosis occurring during interphase and mitotic death occurring upon re-initiation of cell cycling. Thus, CBC stem cell death appears to occur by 2 separate mechanisms temporally distinguished by cycling status. This information, if found common for stem cell responses to IR and other genotoxic insults, may have substantive implications for the development of therapeutics to enhance or attenuate stem cell death in vivo.

In summary, our data broaden and redefine the phenotypic features of the normal adult stem cell. Stem cell populations in adult specialized tissues, whether quiescent or cycling, are radioresistant owing to proficient use of DNA damage repair pathways. Although quiescent adult stem cells use NHEJ exclusively because they do not have the option to engage HR, in at least one instance, that of the dividing CBC ISC, the HR mechanism also is used to repair radiation-induced DSBs. In fact, CBCs use HR more proficiently than their differentiated progeny. Hence, we suggest that a basic tenet of the adult stem cell phenotype is highly proficient and versatile engagement of DNA repair mechanisms in response to genotoxic

insults, maximizing the likelihood of stem cell survival, with preferential use of HR, when feasible, in addition to NHEJ, to protect the integrity of the genome. This hypothesis is consistent with the assumed extreme resistance of cancer stem cells to drugs and radiotherapy, with potential implications for human cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

BM	bone marrow
BRDU	5-Bromo-2'-Deoxyuridine
BSC	bulge stem cell
CBC	crypt base columnar
DSBs	double-strand breaks
EGFP	enhanced green fluorescent protein
GI	gastrointestinal
HR	homologous recombination
HSC	hematopoietic stem cell
IR	ionizing radiation
IRIF	ionizing radiation-induced repair foci
ISC	intestinal stem cell
Lgr5	leucine-rich-repeat-containing G-protein-coupled receptor 5
NHEJ	nonhomologous end joining
PUMA	p53 up-regulated modulator of apoptosis
SCC	stem cell clonogen
TA	transit amplifying
WBR	whole body radiation

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Figure 1.

IR induces dose-dependent BM and GI lethality in *Lgr5-lacZ* transgenic mice. (A) Representative full-transverse section of proximal jejunum from *Lgr5-lacZ* transgenic mice stained for lacZ. LacZ⁺ CBCs are visible at the crypt base (blue cells with arrow). (B) Actuarial survival of 8-to 12-week-old *Lgr5-lacZ* transgenic mice treated with 12 Gy and 15 Gy WBR with/without administration of 5×10^6 syngeneic BM cells 16 hours after irradiation. Actuarial survival was calculated by the Kaplan–Meier method. Number of animals/group is shown in parentheses. (C) Tissue damage in *Lgr5-lacZ* mice dying after 12 Gy or 15 Gy. H&E-stained sections of femur and proximal jejunum were obtained from animals displaying an agonal-breathing pattern. Murine small intestinal mucosa is well preserved at day 10 after 12 Gy, whereas BM elements appear depleted from the femur cavity. In contrast, mucosa is denuded at day 5 after 15 Gy, with almost no villi/crypts apparent, although BM shows only partial damage. Scale bar, 60 μ m.

Figure 2.

Close correlation of *Lgr5*⁺ stem cell loss to crypt loss. (A) GI damage assessed by the crypt microcolony assay of Withers and Elkind.²¹ (B and C) Dose-dependent radiation-induced CBC depletion in *Lgr5-lacZ* mice. CBC frequency in sectioned *LacZ*⁺-stained small intestines was scored at (B) 3.5 or (C) 2 days after irradiation. CBC number/circumference was assessed by counting crypt base *lacZ*-positive cells. CBC number and crypt survival were quantified from 4 mice/time point, with 3–5 circumferences scored per mouse.

Triangles represent the number of crypts or CBCs in individual circumferences. Data (mean $\pm$ standard deviation) are from 2 experiments.

Figure 3.

Quantitative comparison of CBC-depletion kinetics in small intestines of *Lgr5-lacZ* transgenic mice treated with 12 Gy and 15 Gy. Shown are CBC kinetic profiles after 12 Gy (*right panel*) and 15 Gy (*left panel*). Small intestines were obtained from irradiated *Lgr5-lacZ* mice and surviving CBCs identified after lacZ-staining as in Figure 1A. CBC survival data (mean $\pm$ standard deviation) was quantified from 4 mice per time point, with 3–5 circumferences/mouse. *Triangles* represent CBC number in individual circumferences. *Lower panel*: sectioned lacZ-stained small intestines of *Lgr5-lacZ* mice with/without IR. *Scale bar*, 20 μ m.

Figure 4.

CBCs repair DNA damage much faster than other differentiated cells. (A) Confocal images (objective, 20 $\times$) of γ -H2AX immunofluorescence staining on small intestinal sections in control and irradiated mice at 6 hours after 12 Gy. *Scale bar*, 70 μ m. (B) Kinetic analysis of γ -H2AX focus resolution in CBCs, TA cells, and villus cells after 12 Gy WBR. Foci/nucleus was determined by counting 3 classes of cells: CBC cells (located between Paneth cells), TA cells (at positions 4–10, with position 4 being directly above Paneth cells), and villus cells (midvillus). Paneth cells were stained for lysozyme (red). Data (mean $\pm$ standard error of the mean) are collated from 3 experiments. (C) Representative high-magnification confocal images (objective, 63 $\times$) showing γ -H2AX foci on small intestinal sections after 12 Gy. *Scale bar*, 10 μ m. *White arrows* identify CBCs. * P < .05; ** P < .001; each vs CBCs cells.

Figure 5.

CBCs manifest rapid formation/resolution of HR and NHEJ foci. (A) Quantification of BRCA1 (left panel) and RAD51 (right panel) foci in CBCs, TA cells, and villus cells as in Figure 4 after 12 Gy WBR. Data (mean $\pm$ standard error of the mean) were collated from 3 experiments. Note $36\% \pm 3\%$ of CBCs and $67\% \pm 4\%$ of TA cells display RAD51 foci, whereas 100% of both populations display BRCA1 foci. (B) Confocal images (objective, 20 $\times$) of BRCA1 immunofluorescence staining on small intestinal sections of control and irradiated mice 1 hour after 12 Gy. Scale bar, 30 μ m. (C) Representative high-magnification confocal images (objective, 63 $\times$) of (C) BRCA1 and (D) RAD51 foci in crypts after 12 Gy. (E) Quantification of γ -H2AX, BRCA1, and RAD51 foci in CBCs and TA cells at 6 hours after 12 Gy in PUMA knockout mice (right panel). Confocal image (objective, 63 $\times$) showing crypt γ -H2AX foci (left panel). Data (mean $\pm$ standard deviation) were scored in 100 cells, pooled from 3 independent mice. $**P < .001$ CBCs vs TA cells. (F) DNA-PKcs foci quantification (left panel) and confocal images (right panel, crypts only) at 30 minutes to 24 hours after 12 Gy. Data (mean $\pm$ standard error of the mean) were collated from 3 experiments. Note that at 6, 12, and 24 hours, $P < .005$ CBCs vs TA cells. Scale bars, 10 μ m. White arrows identify CBCs.

Figure 6.

Kinetic analysis of CBC cell-cycle arrest and re-initiation after irradiation. (A) Kinetics of cell proliferation after 12 Gy in intestinal sections of 8-week-old *Lgr5-lacZ* mice pulsed with EdU for 4 hours before death. CBCs (white arrows) are interspersed between Paneth cells stained red for lysozyme. (B) Frequency of EdU-positive crypts/circumference in small intestines of *Lgr5-lacZ* mice after 12 Gy. Data (mean $\pm$ standard error of the mean) were compiled from 3 experiments. (C) LacZ (blue) and BrdU (brown) double staining of consecutive intestinal sections from *Lgr5-lacZ* mice with/without 12 Gy at 18 hours. Black arrows highlight double-positive CBCs. Scale bar, 10 μ m. (D) Percentage of BrdU-labeled *Lgr5*⁺ small intestinal cells before and at 18 hours after 12 Gy. Two hundred crypts were counted per mouse with 5 mice/group. Bars represent mean $\pm$ standard error of the mean of 3 experiments. (E) Lineage tracing in small intestines of irradiated *Lgr5-EGFP-ires-CreERT2/Rosa26-lacZ* mice at days 5, 7, and 10 after 12 Gy. Adult mice were injected intraperitoneally with a single tamoxifen dose immediately after radiation. Scale bar, 30 μ m.

Figure 7.

Kinetic evaluation of apoptotic cell death in CBCs and TA cells. *Lgr5-lacZ* mice were irradiated with 12 Gy WBR. Apoptotic death of CBCs (*black arrows*) and TA cells (*white arrows*) was evaluated by staining for active caspase-3 fragments as described in the Materials and Methods section. CBC cells are located between Paneth cells, although approximately 10% of *Lgr5*+ CBCs may occur at the +4 position,¹⁷ with position 4 being directly above the most distal Paneth cell. TA cells are located at positions 4–10. Paneth cells are identified by the presence of granules, readily observed under brightfield microscopy. Although Paneth cell degranulation may occur after irradiation, this did not occur to an extent that altered Paneth cell detection in our studies. A minimum of 300 cells were scored for apoptosis per time point. Data (mean $\pm$ standard deviation) were collated from 4 mice. Scale bar, 10 μ m.