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SOX9 Maintains Reserve Stem Cells and Preserves Radioresistance in Mouse Small Intestine

Kyle C. Roche[1], **Adam D. Gracz**[2], **Xiao Fu Liu**[1], **Victoria Newton**[1,2], **Haruhiko Akiyama**[3], and **Scott T. Magness**[1,2]

[1]Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

[2]Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

[3]Department of Orthopedics, Gifu University, Gifu, Gifu Prefecture, Japan

Abstract

BACKGROUND & AIMS—Reserve intestinal stem cells (rISCs) are quiescent/slowly cycling under homeostatic conditions, allowing for their identification with label-retention assays. rISCs mediate epithelial regeneration after tissue damage by converting to actively proliferating stem cells (aISCs) that self renew and demonstrate multipotency, which are defining properties of stem cells. Little is known about the genetic mechanisms that regulate the production and maintenance of rISCs. High expression levels of the transcription factor Sox9 (Sox9^{high}) are associated with rISCs. This study investigates the role of SOX9 in regulating the rISC state.

METHODS—We used fluorescence-activated cell sorting to isolate cells defined as aISCs (*Lgr5*^{high}) and rISCs (*Sox9*^{high}) from *Lgr5*^{EGFP} and *Sox9*^{EGFP} reporter mice. Expression of additional markers associated with active and reserve ISCs were assessed in *Lgr5*^{high} and *Sox9*^{high} populations by single-cell gene expression analyses. We used label-retention assays to identify whether Sox9^{high} cells were label-retatining cells (LRCs). Lineage-tracing experiments were performed in *Sox9*-CreERT2 mice to measure the stem cell capacities and radioresistance of *Sox9*-expressing cells. Conditional SOX9 knockout mice and inducible-conditional SOX9 knockout mice were used to determine whether SOX9 was required to maintain LRCs and rISC function.

RESULTS—Lgr5^{high} and a subset of crypt-based Sox9^{high} cells co-express markers of aISC and rISC (*Lgr5. Bmi1. Lrig1*, and *Hopx*). LRCs express high levels of *Sox9* and are lost in SOX9-knockout mice. SOX9 is required for epithelial regeneration after high-dose irradiation. Crypts from SOX9-knockout mice have increased sensitivity to radiation, compared with control mice, which could not be attributed to impaired cell-cycle arrest or DNA repair.

Address requests for reprints to: Scott T. Magness, PhD, University of North Carolina at Chapel Hill, 111 Mason Farm Road, CB# 7032, MBRB Room 4337, Chapel Hill, North Carolina 27599. magness@med.unc.edu; fax: (919) 843-6899.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.07.004.

Conflicts of interest

The authors disclose no conflicts.

CONCLUSIONS—SOX9 limits proliferation in LRCs and imparts radiation resistance to rISCs in mice.

Keywords

Irradiation Injury; Intestinal Epithelium; Radio-resistant Stem Cells; Gene Expression

Radiation therapies and many chemotherapeutics treat cancer by targeting rapidly dividing cells, but also have off-target effects that damage normal cells in highly proliferative tissues such as the intestinal epithelium.[1] Consequently, the majority of patients undergoing radiation treatment to the abdomen develop acute enteritis owing to apoptosis of rapidly proliferating crypt-based cells.[1,2] Similarly, accidental or combat exposure to high doses of radiation can result in gastrointestinal syndrome, which is characterized by massive mitotic arrest, apoptosis, and the clinical sequelae associated with the loss of epithelial barrier function.[2,3] Radiation damage studies in animal models have uncovered radioresistant stem cells that are defined by their slowly proliferating or quiescent states and primarily localize to the +4–5 cell position from the base of the crypt.[4] These radioresistant stem cells are very rare and have been shown in a number of studies to act as a reserve intestinal stem cell (rISC) population when the more abundant active intestinal stem cell or rapidly dividing intestinal stem cell (aISC) population is depleted.[4] The genetic and cellular mechanisms required for rISC production and maintenance are unknown. Elucidating these mechanisms could have a profound impact on developing new interventions to protect against damage and promote epithelial repair in a number of intestinal-related health conditions.

In the past decade, efforts have been made to develop models that enable identification and isolation of rISCs to study their contribution to epithelial regeneration. Many of these studies have focused on identifying unique genetic biomarkers useful for distinguishing between aISCs and rISCs.[4] *Lgr5* was one of the earliest ISC biomarkers to be validated by genetic lineage tracing and its expression generally is considered to be highly restricted to aISCs that are intercalated between Paneth cells at the crypt base.[5] Since then, a number of biomarkers have been reported to mark rISCs including *Bmi1*. *Hopx. Tert*, and *Lrig1*.[6–10] However, a recent study reported overlapping expression of these biomarkers with the aISC biomarker *Lgr5*,[11] suggesting that these markers may not be as useful as previously thought for discriminating between active and reserve ISC states. A less controversial functional marker of rISCs is their slowly dividing or quiescent nature, which can be assayed based on nuclear retention of detectable nucleotide analogs.[12,13] A modification of this assay using histone 2B-YFP transgenic mice recently showed that quiescent, label-retaining cells (LRCs) function as secretory progenitors that predominantly give rise to Paneth cells under homeostatic conditions, but also show plasticity and function as rISCs after radiation-induced injury.[13] A key property that distinguishes aISCs from rISCs is that aISCs are radiosensitive and undergo apoptotic death whereas rISCs are radioresistant and are capable of surviving exposure to high doses of radiation.[4]

Although rISCs can give rise to aISCs, the reverse also has been shown and highlights the dynamic relationship between these 2 ISC states.[8,14] It is unclear whether this state transition follows a progression through stereotyped gene expression patterns characterized

by enrichment or de-enrichment of the classic aISC and rISC biomarkers. What is clear is that the state transition is characterized by changes in proliferative capacity.[4] The genetic mechanisms that regulate this process are likely the key to understanding how tissues such as the intestine maintain a diverse pool of ISCs that have different phenotypic behaviors during homeostatic epithelial renewal and injury-induced regeneration. Our work and other studies have shown that the SOX9 transcription factor regulates proliferation[15–17] and is associated in a dose-dependent manner with different stem/progenitor populations in the small intestine and colon.[15,18–20]

In vivo, cells expressing high levels of the transcription factor *Sox9* (*Sox9*^{high}) show extremely rare staining with the general proliferative marker Ki-67 and show gene expression patterns that are consistent with rISCs and some secretory lineages.[15,18,20] Although fluorescence-activated cell sorting (FACS)-isolated *Sox9*^{low} cells actively divide and maintain functional stemness in vitro, *Sox9*^{high} cells divide very infrequently and are incapable of growing in culture conditions that recapitulate endogenous ISC niche signaling.[18,20] Interestingly, during the regenerative phase after irradiation damage, *Sox9*^{high} cells express genes associated with cell cycle re-entry, DNA repair, and anti-apoptosis, and, importantly, acquire functional stemness in culture.[20] Together, these reports provide strong evidence that *Sox9* plays a role in rISC biology. In the present study, we used a combination of single-cell gene expression analysis, *Sox9* lineage tracing, and intestinal epithelial SOX9 ablation to determine whether SOX9 is directly responsible for generating and maintaining the rISC state.

Materials and Methods

Mice Models

Characterization of the enhanced green fluorescent protein Sox9 reporter (*Sox9*^{EGFP}) mouse model has been described in previous studies.[15,18,20] Intestinal epithelial-specific conditional SOX9 knockout mice (*Sox9*fl/fl;*Villin*Cre) were generated as previously described.[17] To acutely ablate SOX9, *Sox9*fl/fl;*Villin*CreERT2 mice were fed chow containing tamoxifen citrate (200 mg/kg) for 8 days. Lineage tracing assays were conducted by administering 2 mg of tamoxifen intraperitoneally to *Sox9*-CreERT2[45];ROSA26-loxP-STOP-loxP-tdTomato mice.

Ethynyl Deoxyuridine Administration

For proliferation assays, control and conditional *Sox9* knockout mice (SOX9^{cKO}) were injected intraperitoneally with 100 μg/25 g body weight 5-ethynyl-2'-deoxyuridine (EdU). To identify label-retaining cells, osmotic minipumps containing 115 mg EdU were implanted subcutaneously. After labeling, pumps were removed and EdU was allowed to washout for 8–12 days. Intestines subsequently were harvested and processed for histology and FACS.

Microscopy/Histology

A Zeiss LSM 700 (Thornwood, NY) confocal microscope was used to acquire 1-μm optical sections for image analysis. For all histologic quantification, more than 50 crypts/mouse were counted and statistical significance was determined using an unpaired *t* test.

Tissue Dissociation/FACS

Jejunal crypt fractions were dissociated into single cells as previously described.[18] Viable single epithelial cells were isolated based on the gating scheme shown in Supplementary Figure 1*A*.

Single-Cell Analysis and Quantitative Polymerase Chain Reaction

Single cells were isolated by FACS and applied to the Fluidigm C1 autoprep integrated microfluidics chip (Fluidigm, South San Francisco, CA) to generate specific target amplified complementary DNA libraries using TaqMan probes (Carlsbad, CA) (Supplementary Table 1). The libraries then were applied to the Biomark HD autoprep platform to assess relative gene expression levels by quantitative reverse-transcription polymerase chain reaction. Delta-cycle threshold values were calculated based on a limit of detection of 35 cycles. Statistical significance was determined using 1-way analysis of variance.

Results

Sox9^{high} Cells Show a Secretory Progenitor/rISC Signature

Cells expressing the highest levels of *Sox9* have been shown at the population level to co-express rISC biomarkers and secretory transcripts, suggesting population heterogeneity.[15,18,20] We sought to determine whether the *Sox9*^{high} population is a mixed population or a homogeneous population with characteristics of both ISCs and differentiated lineages. We conducted single-cell gene expression analysis on *Sox9*^{high} cells to compare and contrast expression patterns with those observed in cells expressing high levels of the ISC biomarker *Lgr5*, which primarily marks aISCs (*Lgr5*^{high}).[5] Crypt-enriched preparations from *Lgr5*^{EGFP} and *Sox9*^{EGFP} reporter mice were used to FACS-isolate *Lgr5*^{high} and *Sox9*^{high} cells for single-cell gene expression analysis of ISC and lineage-specific gene expression patterns (Figure 1*A*, Supplementary Figure 1*A* and *D*, Supplementary Table 1).

Our data show at the single-cell level that *Lgr5*^{high} cells are relatively homogenous by principal component analysis (Figure 1*B*). All *Lgr5*^{high} cells express aISC biomarkers *Lgr5* and *Sox9*, and nearly all *Lgr5*^{high} cells express aISC biomarkers *Lgr4* and *Ascl2* (Figure 1*C*). Consistent with ISCs, *Lgr5*^{high} cells generally lack expression of biomarkers associated with differentiated absorptive (*Sis*), enteroendocrine (*Tac1*), and goblet cells (*Muc2*) (Figure 1*D*). Interestingly, all *Lgr5*^{high} cells express Paneth cell biomarker *Lyz2* and a minority express *Defcr-2*, suggesting that these biomarkers may not be restricted to the Paneth cell lineage at the messenger RNA level (Figure 1*C* and *D*). Consistent with gene expression analysis conducted at a population level,[11] nearly all single *Lgr5*^{high} cells express high levels of biomarkers classically associated with rISCs including *Bmi1*. *Lrig1*. *Hopx*, and *Dll1* (Figure 1*C*).

Principal component analysis bisects *Sox9*^{high} cells into distinct populations when *Lgr5* expression status is used as a distinguishing criterion (Figure 1*B*). A fraction of crypt-enriched *Sox9*^{high} cells (40%) express *Lgr5*, whereas the remaining *Sox9*^{high} cells do not (60%) (Supplementary Figure 2). Further analysis indicated that *Sox9*^{high}:*Lgr5*^{pos} cells co-express aISC biomarkers *Lgr4* and *Ascl2*, and rISC biomarkers *Bmi1*. *Lrig1*, and *Hopx* (Figure 1*C*). *Ascl2* expression is nearly absent in *Sox9*^{high}:*Lgr5*^{neg} cells and only a small fraction of these cells express the aISC biomarker *Lgr4* and other rISC biomarkers that were analyzed (Figure 1*C*). Although *Sox9*^{high} cells are heterogeneous with respect to *Lgr5* expression, most show expression of secretory lineage genes including *Chga* (98%), *Chgb* (87%), *Dcamkl1* (62%), and *Dll1* (51%) (Supplementary Figure 2). The gene expression pattern in the *Sox9*^{high}:*Lgr5*^{pos} population is comparable with previously described secretory progenitor LRCs that express a set of aISC and rISC biomarkers and the secretory transcripts *Chga* and *Chgb* (Figure 1*E*).[13] These data suggest that *Sox9*^{high}:*Lgr5*^{pos} cells may represent previously described LRCs that act as rISCs after damage.[13]

Label-Retaining rISCs Express High Levels of Sox9

To directly test whether *Sox9*^{high} cells are LRCs, we performed a label-retention assay in *Sox9*^{EGFP} reporter mice. For this assay, all cells in the intestinal epithelium were labeled with a detectable nucleotide analog (EdU) over the course of 28 days by osmotic minipumps (Supplementary Figure 3). In this assay, only cells having undergone a proliferative event over the 28-day EdU administration period are labeled and thus are very long-lived LRCs, and postmitotic Paneth cells remain unlabeled. The intestinal epithelium then was allowed to undergo a washout period, during which time EdU labeling was passively lost either through sloughing of differentiated lineages into the lumen or proliferation-dependent dilution. EdU-labeled cells that remained after a 10-day washout period were considered LRCs in agreement with previous definitions.[12,13] The level of *Sox9* expression in LRCs was characterized by assessing EdU expression by flow cytometry in *Sox9*^{EGFP} populations (Supplementary Figure 1*B*). Contaminating Paneth cells, which are long-lived and remain labeled by EdU, conveniently were excluded from this analysis because they do not express the *Sox9*^{EGFP} transgene.[15] Consistent with previous reports, we confirmed that non-Paneth LRCs predominantly localize to the +4 to the +7 crypt position (Supplementary Figure 4).[12,13] Flow cytometric analysis showed that *Sox9*^{high} cells show the highest incidence of label retention (Figure 2*A*). The LRC distribution among *Sox9*-expressing populations then was calculated by accounting for the relative abundance of *Sox9*^{sublow}, *Sox9*^{low}, and *Sox9*^{high} populations, showing that 70% of LRCs are *Sox9*^{high} (Supplementary Figure 1*C* and Figure 2*B* and *C*). These data show that most LRCs express high levels of *Sox9*.

SOX9 Is Necessary for the Generation and Maintenance of LRCs

A number of studies have established that high levels of SOX9 inhibit proliferation in the intestinal epithelium.[15–17,20] These findings suggest that SOX9 might be controlling the proliferative capacity of ISCs in a dose-dependent manner and may be responsible for controlling the slowly proliferative/quiescent state of rISCs. To determine if SOX9 is necessary for the generation of LRCs, we performed a label-retention study in conditional SOX9^{cKO}. In these mice, *Sox9* was ablated genetically at embryonic day 10.5 specifically in the intestinal epithelium (Supplementary Figure 5).[16,17] Again, the intestinal epithelium was

labeled using osmotic minipumps implanted subcutaneously in SOX9^{cKO} mice and littermate controls (*Sox9*$^{fl/fl}$). Pumps then were removed and EdU was allowed to washout for 8 and 12 days, 2 time points that were chosen to flank the 10-day washout period previously used to define LRCs.[12,13] LRCs in this assay were defined as EdU^{pos} (LRCs), epithelial cell adhesion molecule positive (epithelial cells), and lysozyme^{neg} (non-Paneth cells). After both washout periods, SOX9-deficient intestines contained markedly lower numbers (between 80% and 95% reduction) of LRCs, indicating that SOX9 plays an essential role in the generation of LRCs in vivo (Figure 3*A* and *B*). To determine if continued SOX9 expression is necessary for maintaining the LRC state, a label-retention assay was conducted in inducible SOX9 knockout animals (SOX9^{iKO}). In these mice, *Sox9* is acutely ablated in the adult intestinal epithelium after tamoxifen administration (Supplementary Figure 5). In this assay, *Sox9* was ablated during the EdU washout period after LRCs had been labeled with EdU. We found that acute loss of SOX9 in pre-existing LRCs resulted in the loss of EdU retention, showing that sustained SOX9 expression is necessary for LRC maintenance (Figure 3*C*).

Sox9-Expressing Cells Lineage Trace After Injury

Although our data show that LRCs express high levels of *Sox9*, we used genetic lineage tracing to determine whether *Sox9* is expressed in all rISCs, which might include non-LRCs (Figure 4*A*). *Sox9*-expressing cells have been shown to lineage-trace under homeostatic conditions,[21] a finding that we have confirmed independently (Figure 4*B*). To promote lineage tracing events from rISCs, we depleted the aISC pool using radiation exceeding 12 Gy, a broadly applied method for depleting aISCs[7,22,23] and eliciting rISC-dependent regeneration.[7–10] In this assay, *Sox9*-expressing cells were marked genetically before the administration of 14 Gy of abdominal radiation. To negate the possibility that *Sox9*-positive cells acutely give rise to *Sox9*-negative rISCs within the 24-hour window preceding irradiation, we also initiated lineage tracing at the time of irradiation. In both conditions tested, *Sox9*-expressing cells consistently gave rise to crypt/villi units completely labeled by the lineage tracing reporter gene (Figure 4*B*), and, notably, complete lineage tracing was observed throughout the entire proximal–distal axis of the small intestinal epithelium (>98%) (Supplementary Figure 6). Although the *Sox9*-CreERT2 allele driving the lineage tracing cannot distinguish between different *Sox9*-expressing populations in the crypt, our results indicate that the rISC pool expresses *Sox9*.

SOX9 Knockout Intestinal Epithelium Shows Loss of Regenerative Capacity After Radiation Damage

Although our data show that rISCs express *Sox9*, we wanted to determine whether SOX9 was required to maintain the ability of rISCs to mediate intestinal regeneration after damage. To determine if SOX9 maintains rISC function, we challenged SOX9^{cKO} intestinal epithelium with 14 Gy of abdominal irradiation and assessed the ability of rISCs to initiate epithelial regeneration using in vivo microcolony assays, which are considered a functional read-out of ISC activity.[24,25] Microcolonies are defined as proliferative epithelial units that appear 3.5 days after irradiation, a time point at which new epithelium emerging from surviving rISCs in the epithelial-depleted mucosal space is first observed. In SOX9^{cKO} epithelium, microcolonies were nearly absent 3.5 days after irradiation (Figure 5*A* and *C*).

Epithelial coverage, measured by epithelial cell adhesion molecule immunostaining, was diminished and only present on the villi and flat luminal aspects of SOX9^{cKO} tissue (Supplementary Figure 7*A* and *B*). The lack of epithelial regeneration in SOX9^{cKO} intestine was accompanied by severe weight loss requiring humane euthanasia at 7 days after irradiation owing to morbidity (Supplementary Figure 7*C*, Supplementary Table 2).

SOX9^{cKO} mice lack Paneth cells,[16,17] which previously have been shown to support ISC function in vitro.[26,27] To negate the possibility that the impaired epithelial regeneration observed in SOX9^{cKO} animals was owing to a lack of Paneth cells, we acutely ablated SOX9 in the adult intestines of SOX9^{iKO} mice, a process that does not affect Paneth cell numbers (Supplementary Figures 5 and 8). Consistent with observations made in SOX9^{cKO} animals, the number of microcolonies present in SOX9^{iKO} was decreased markedly, indicating that the impaired regeneration observed in SOX9^{cKO} mice is not owing to a lack of Paneth cells (Figure 5*B* and *D*). Together, these results further support that *Sox9* is required for rISC generation and maintenance.

SOX9^{cKO} Intestinal Crypts Show Increased Apoptosis After Radiation Challenge Despite Normal Cell-Cycle Arrest and DNA Repair

After radiation damage, ISCs avoid apoptosis by undergoing cell-cycle arrest to repair DNA.[23] Given that SOX9 is known to inhibit proliferation, we sought to determine whether SOX9 maintains rISC function by preserving the ability of crypt-based cells to undergo cell-cycle arrest. SOX9^{cKO} and control mice were exposed to 14 Gy of whole-body radiation, a dose known to result in sufficient levels of DNA damage to initiate apoptosis in ISCs.[7,22,23] Immunostaining for the apoptotic marker cleaved caspase 3 shows increased apoptosis in SOX9^{cKO} crypts at 1, 6, and 24 hours after irradiation (Figure 6*A*). To determine if this increased radiosensitivity was caused by impaired radiation-induced, cell-cycle arrest, we quantified EdU incorporation (S-phase) and phosphohistone H3 staining (M-phase) in SOX9^{cKO} crypts after radiation damage. In both control and SOX9^{cKO} crypts, DNA replication was halted at 6 hours after irradiation and a lack of cells present in the M-phase was observed at 1 and 6 hours after irradiation (Figure 6*B* and *C*). These results indicate that SOX9 is dispensable for damage-induced cell-cycle arrest.

After radiation damage, DNA repair mechanisms fix double-stranded DNA breaks, allowing for avoidance of apoptosis. To determine if apoptosis in SOX9^{cKO} crypts is the result of impaired DNA repair mechanisms, we performed immunostaining for γ H2A histone family member X (γH2AX) foci, which appear at DNA double-stranded breaks after radiation-induced damage and disappear over time as DNA is repaired.[28] Radiation exposure resulted in rapid formation of γH2AX foci, which equivalently diminished in SOX9^{cKO} and control crypts over 24 hours. These results indicate that SOX9 is dispensable for γH2AX-mediated DNA repair (Supplementary Figure 9). Despite competence to arrest the cell cycle and repair DNA, SOX9^{cKO} cells still show a higher incidence of apoptosis.

Discussion

Stemness in somatic tissues is defined classically as the functional ability of a cell to give rise to all of the postmitotic lineages of its resident tissue and to produce progeny with self-

renewing stem cell capacity.[4] Although the nature of this definition is reliant on functional properties, stem cell biology has been largely dependent on the use of genetic biomarkers as proxies for cell function.[4] Currently, stemness is shown experimentally through in vivo lineage tracing or ex vivo culture of cells carrying ISC biomarker reporter alleles.[4] The increase in ISC biomarkers over the past 5–10 years has presented the research community with multiple theories on whether or not ISCs exist in distinct states marked by specific sets of genes, or whether stemness in the intestine is a relatively plastic property shared across many cell populations expressing different combinations of biomarkers.[29–31]

Emerging studies that highlight the dynamic ability of ISCs to convert between actively dividing and slowly dividing proliferation states have forced a re-examination of biomarker attribution.[8,13,14] Transcriptomic, proteomic, and single molecule Florescent in situ hybridization analysis have shown that many of the biomarkers originally attributed to a rISC population are expressed in aISCs ($Lgr5^{high}$ cells).[11] Data from our study confirm these findings and show at the single-cell level that $Lgr5^{high}$ cells uniformly co-express the rISC biomarkers *Bmi1*. *Hopx*, and *Lrig1*. Despite the current volatility of rISC biomarker attribution, what has become apparent is that mechanisms exist to produce a pool of ISCs with functionally equivalent stem cell properties but with different proliferative capacities and distinct gene expression patterns.[30,31] Attributing biomarkers to aISC and rISC populations has occurred largely in the absence of understanding the mechanisms that regulate the proliferative capacities that in part define these states. As a conceptual framework, the present study examines *Sox9*, a broadly expressed ISC/progenitor biomarker previously identified by our laboratory from the perspective of its functional role in rISCs.[15,18]

rISCs are distinguishable from aISCs based on 2 phenotypic criteria: proliferation rate and sensitivity to damaging agents.[30] ISCs residing in the reserve state are slowly proliferating/quiescent, a property that can be assessed using label retention assays.[30] Our study shows that LRCs express high levels of SOX9 and that SOX9 is indispensible for LRC production and maintenance. In addition to regulating cellular quiescence, we found that SOX9 also maintains the other defining criterion of rISCs, resilience to DNA damaging agents.[30] Regardless of Paneth cell presence, we found that crypt-based cells become radiosensitive after SOX9 ablation, showing increased apoptotic cell death despite no apparent changes in cell-cycle arrest or γH2AX-mediated DNA repair. Consistent with a radiosensitive phenotype, SOX9 knockout intestinal epithelium failed to mount a regenerative response after radiation damage, resulting in a loss of epithelial coverage accompanied by severe weight loss. Although our results clearly indicate that SOX9 is required to maintain the properties that define rISCs, the mechanisms by which SOX9 preserves quiescence and radioresistance remains a point of interest. Previous studies have shown that SOX9 is able to repress Wnt signaling and inhibit proliferative capacity,[15–17] both of which have been shown to be involved in maintaining radioresistance of crypt-based cells.

Recently, it was shown that high levels of canonical Wnt signaling predispose crypt-based cells to radiation-induced apoptosis by enhancing pro-apoptotic DNA damage responses in a proliferation-independent manner.[32] Both in vivo and in vitro assays have shown that enhanced canonical Wnt signaling increases radiosensitivity, whereas attenuation of Wnt

signaling has the opposite effect.[33] The mechanisms that SOX9 uses to inhibit Wnt signaling have been explored using in vitro overexpression assays. Expression of high levels of SOX9 in vitro has been shown to result in down-regulation of Wnt target genes, *c-Myc* and *Ccnd1*, and increased expression of Wnt-pathway inhibitors, *Grg/TLE* family members, and *ICAT*.[15,16] Conversely, the loss of SOX9 both in vitro and in vivo has been shown to increase the expression of canonical Wnt target genes.[16] Taken together, these studies support the interpretation that SOX9 imparts radioresistance to the ISC pool through repression of canonical Wnt signaling.

In addition to inhibition of Wnt signaling, SOX9 might regulate radioresistance by controlling the proliferative capacity of ISCs. A common feature among radioresistant cell populations is a slowly proliferating/quiescent state.[30] Studies conducted 40 years ago indicated that dividing jejunal cells in the early DNA synthesis phase (S-phase) are highly sensitive to ionizing radiation and undergo apoptosis at a higher incidence than those in other stages of the cell cycle.[33] This raises the possibility that heightened radiosensitivity of cells in $SOX9^{cKO}$ crypts may be the result of impaired cell-cycle control. Increased rates of cell division and crypt hyperplasia observed in $SOX9^{cKO}$ intestines supports this interpretation,[16,17] and suggests that high levels of *Sox9* might promote radioresistance by limiting proliferation. In this regard, SOX9 has been shown to modulate the proliferative capacity of intestinal epithelial cells by repressing pro-proliferative pathways. Although the ability of SOX9 to repress Wnt signaling has been known for some time,[15–17] recently it was shown that SOX9 can repress pro-proliferative insulin signaling through direct regulation of the insulin-like growth factor binding protein 4 gene.[34] Whether SOX9 represses proliferation solely through modulation of these genetic networks remains unknown.

Taken together, our data support a model in which the dynamic interconversion between active and reserve ISC states is regulated by SOX9 dosage. Under homeostatic conditions, active ISCs convert to a rISC state through up-regulation of SOX9. Increased SOX9 doses actively preserve the cellular quiescence of secretory progenitor cells, which over time become Paneth cells. After radiation damage and loss of aISCs, rISCs down-regulate SOX9 expression to levels that are permissive for cell proliferation and acquisition of the aISC state. This model also provides a cellular mechanism in which SOX9 is necessary for the production of the Paneth cell lineage (Figure 7).

Our study provides the conceptual foundations to show detailed genetic mechanisms controlling the production and maintenance of the rISCs and their reactivation by intrinsic and extrinsic signaling. This becomes important from a clinical perspective because the rISCs play a critical role in epithelial regeneration and repair after events that deplete aISCs including ionizing radiation and chemotherapy. Our results suggest that developing strategies to transiently increase SOX9 levels may prove effective for protection against off-target effects of radiation therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

aISC	active intestinal stem cell
EdU	ethynyl deoxyuridine
EGFP	enhanced green fluorescent protein
FACS	fluorescence-activated cell sorting
γH2AX	γ H2A histone family member X
ISC	intestinal stem cell
LRC	label-retaining cell
rISC	reserve intestinal stem cell
Sox9	sex determining region y–box 9
SOX9^{cKO}	conditional *Sox9* knockout
SOX9^{iKO}	inducible SOX9 knockout.

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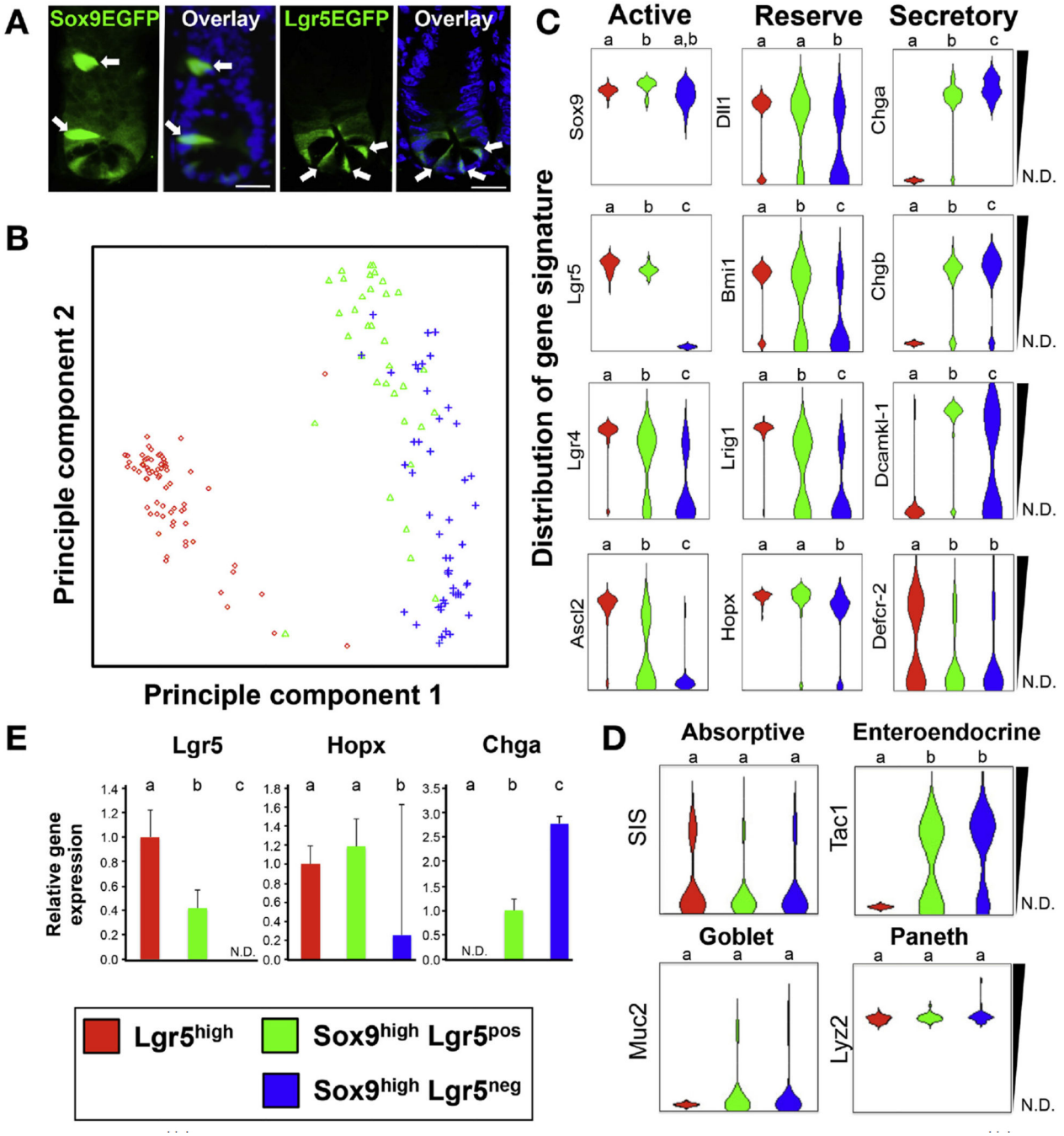

Figure 1.

The *Sox9*^{high} population comprises *Lgr5*^{pos} and *Lgr5*^{neg} cells. (*A*) Representative confocal images of *Sox9*^{high} and *Lgr5*^{high} cells (*white arrows*) in situ. *Scale bar*: 20 μm. (*B*) Principal component analysis of single-cell gene expression data shows that Sox9^{high} cells bisect into 2 populations when *Lgr5* expression is used as a distinguishing criterion. *Lgr5*^{high} cells (n = 71), *Sox9*^{high} *Lgr5*^{pos} cells (n = 34), and *Sox9*^{high} *Lgr5*^{neg} cells (n = 54). (*C*) Violin plots of single-cell gene expression analysis show increased expression of aISC and rISC biomarkers in *Sox9*^{high} *Lgr5*^{pos} cells when compared with *Sox9*^{high} *Lgr5*^{neg} cells. (*D*) Violin plots of

single-cell gene expression analysis show limited expression of absorptive and goblet cell biomarkers in Lgr5^{high} and *Sox9*^{high} cells and ubiquitous expression of the Paneth cell biomarker *Lyz2*. Unlike *Lgr5*^{high} cells, a subset of *Sox9*^{high} cells express the enteroendocrine marker *Tac1*. (*E*) *Sox9*^{high} *Lgr5*^{pos} cells show a gene expression pattern consistent with the reported LRC gene signature. *Bars* represent means ± standard error of the respective groups. Statistical significance is indicated with letters above ($P < .05$). Groups that share the same letter are not significantly different. SIS, sucrase isomaltase (α-glucosidase).

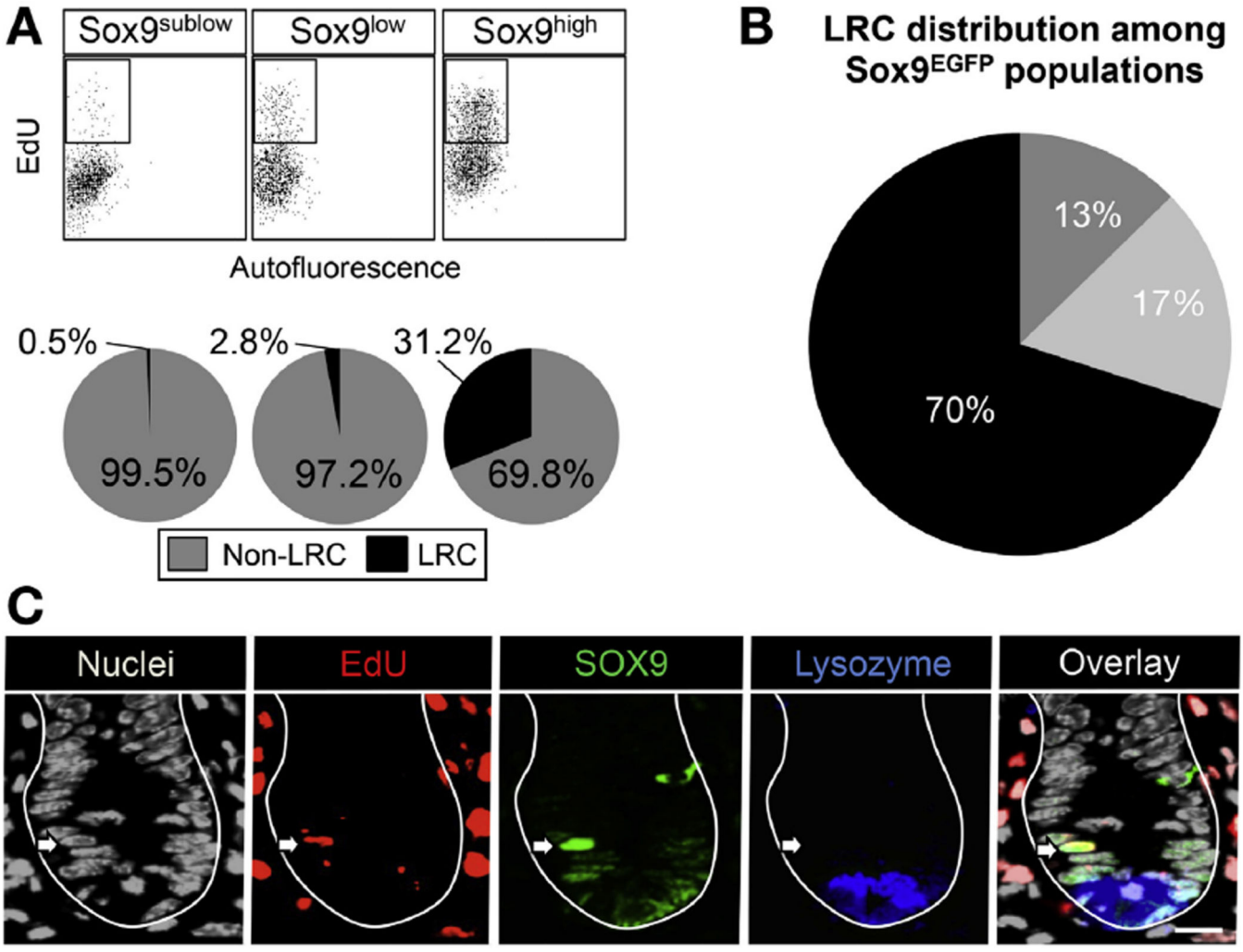

Figure 2.

LRCs express high levels of *Sox9*. (*A*) Representative flow cytometry plots analyzing the percentage of EdU-positive LRCs in FACS-isolated *Sox9*^{EGFP} populations. Quantification is shown in the *pie charts below*. (*B*) LRC distribution among *Sox9*^{EGFP} shows that 70% of LRCs express high levels of *Sox9*. (*C*) Representative confocal image of an LRC expressing high levels of SOX9. *Scale bar*: 20 μm. n = 6 mice.

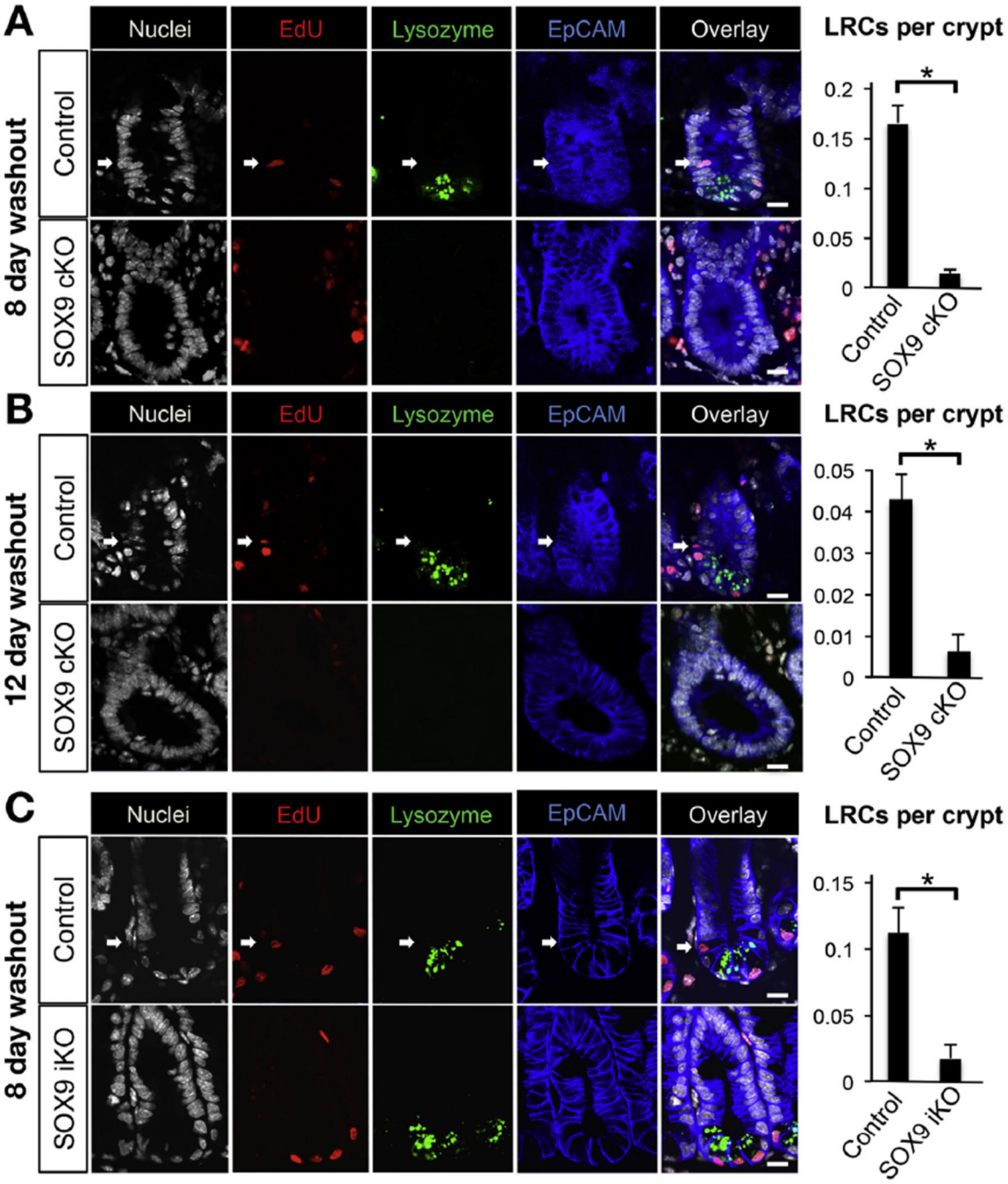

Figure 3.

LRCs are reduced in SOX9-ablated intestines. (*A* and *B*) Confocal images of LRCs (*white arrows*) present in control and absent in $SOX9^{cKO}$ animals after 8- and 12-day washout periods. (*C*) Confocal images of LRCs (*white arrows*) present in control mice and absent in $SOX9^{iKO}$ animals after an 8-day washout period. Note that LRCs in this assay were defined as EdU^{pos} (LRCs), $EpCAM^{pos}$ (epithelial cells), and $lysozyme^{neg}$ (non-Paneth cells). Quantification is shown to the *right*. *Bars* represent means ± SD of each group. *Scale bar*: 20 μm. n = 4 mice per group. **P* < .05.

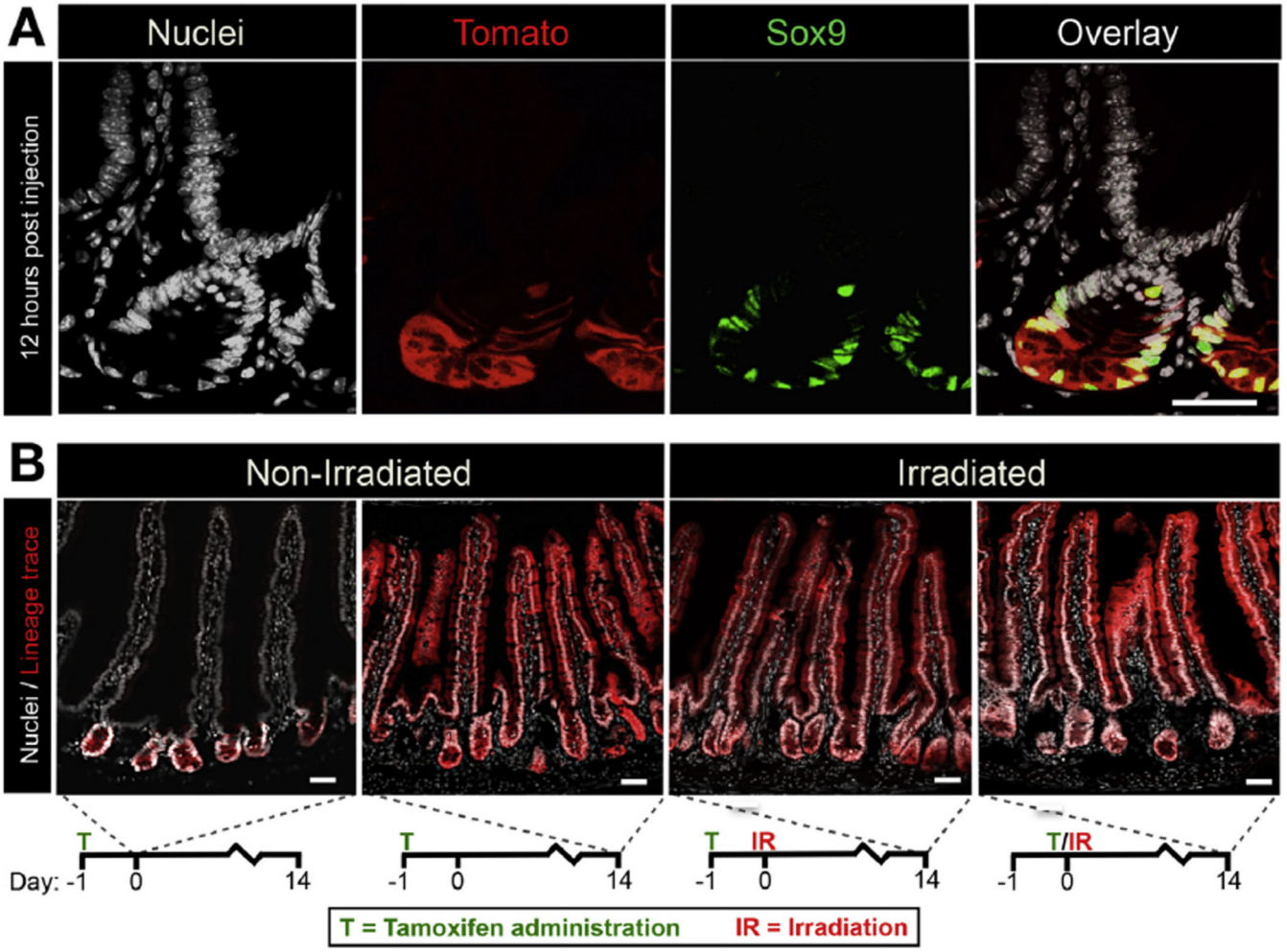

Figure 4.

Sox9-expressing cells lineage trace after irradiation damage. (*A*) Representative image of a crypt 12 hours after injection with tamoxifen showing that lineage tracing is initiated in SOX9-expressing cells. (*B*) *Sox9*-expressing cells lineage trace under both homeostatic conditions and after administration 14 Gy of radiation. Experimental timelines are shown below, indicating the time of tamoxifen injection (T) and irradiation damage (IR). *Dotted lines* reflect the time point at which images were acquired. *Scale bar*: 20 μm. n = 3 mice per time point.

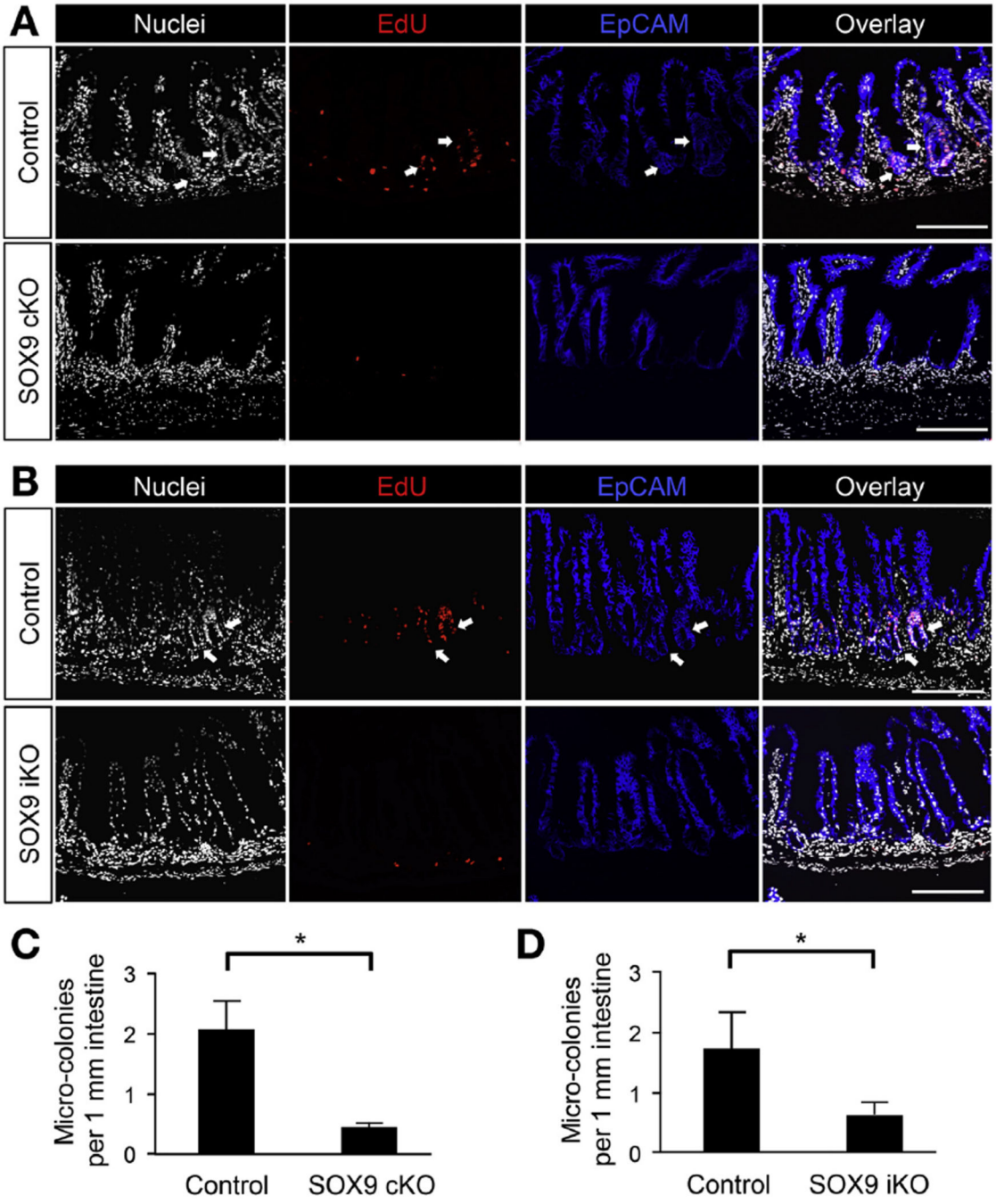

Figure 5.

Regeneration of SOX9-ablated epithelium is impaired severely after irradiation-induced damage. (*A* after *B*) Representative confocal images of microcolonies (*white arrows*) present in control and absent in SOX9^{cKO} and SOX9^{iKO} intestines after irradiation damage. *Scale bar*: 100 μm. (*C* and *D*) Quantification of microcolonies in control, SOX9^{cKO}, and SOX9^{iKO} animals show that Sox9 knockout intestines lack competent ISCs after irradiation damage. *Bars* represent means ± SD. n = 6 mice per group. *$P < .05$

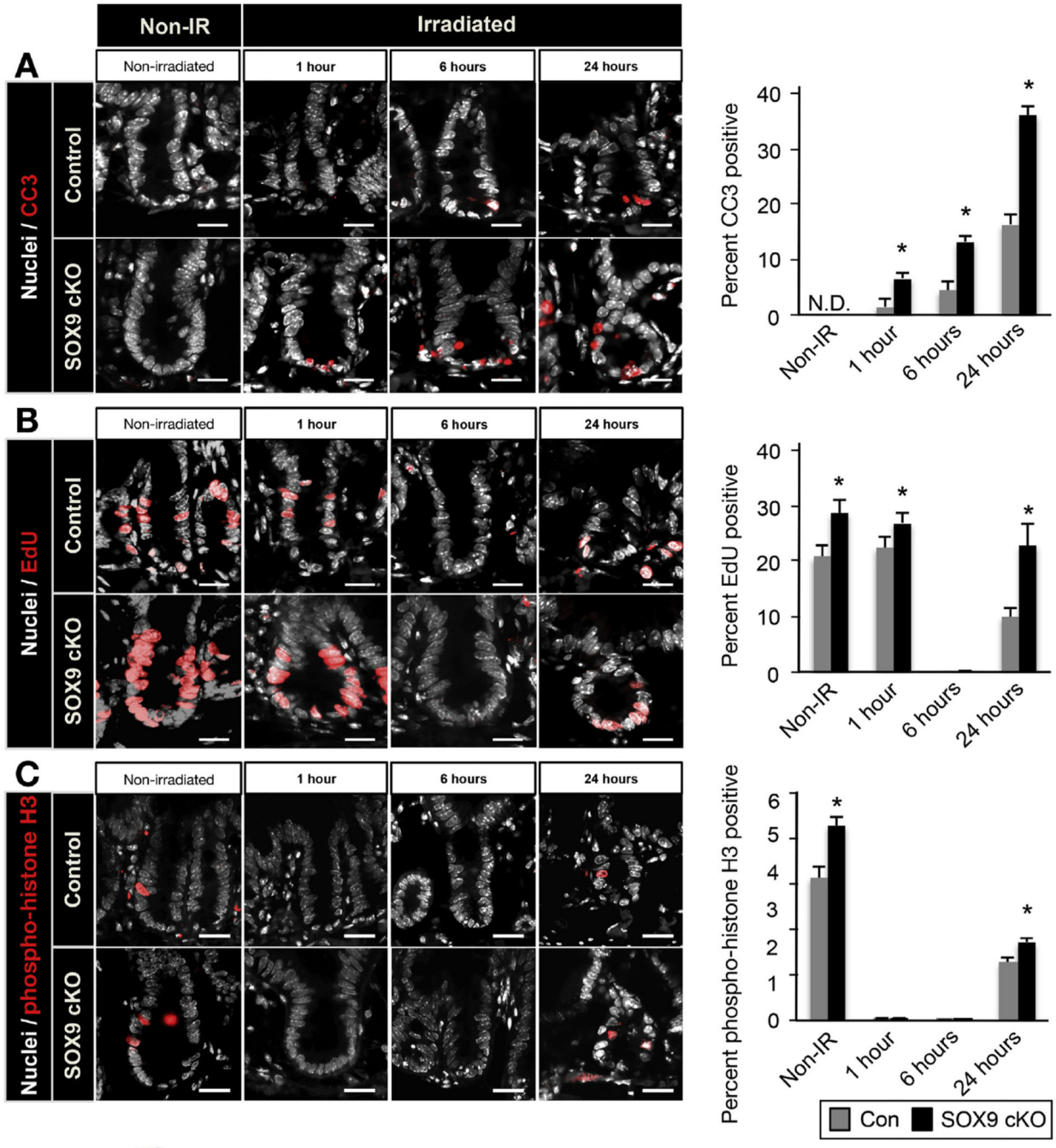

Figure 6.

SOX9^{cKO} crypts show increased apoptosis despite normal cell-cycle arrest after irradiation damage. (*A*) Representative confocal images of control and SOX9^{cKO} crypts immunostained for the apoptotic marker cleaved caspase-3 at 1, 6, and 24 hours after irradiation. Quantification, shown to the right, shows that crypt-based cells in SOX9^{cKO} mice experience a higher incidence of damage-induced apoptosis. (*B*) Confocal images depicting EdU incorporation in crypt-based cells after irradiation damage show a halt of S-phase entry 6 hours after irradiation in control and SOX9^{cKO} crypts. (*C*) Confocal images of

phosphohistone H3 show a lack of M-phase occurring at 1 and 6 hours after irradiation in control and SOX9^{cKO} crypts. These results indicate that SOX9 is dispensable for damage-induced cell-cycle arrest. *Bars* represent means ± standard error. n = 3 mice for each time point. *Scale bar*: 20 μm. *$P < .05$.

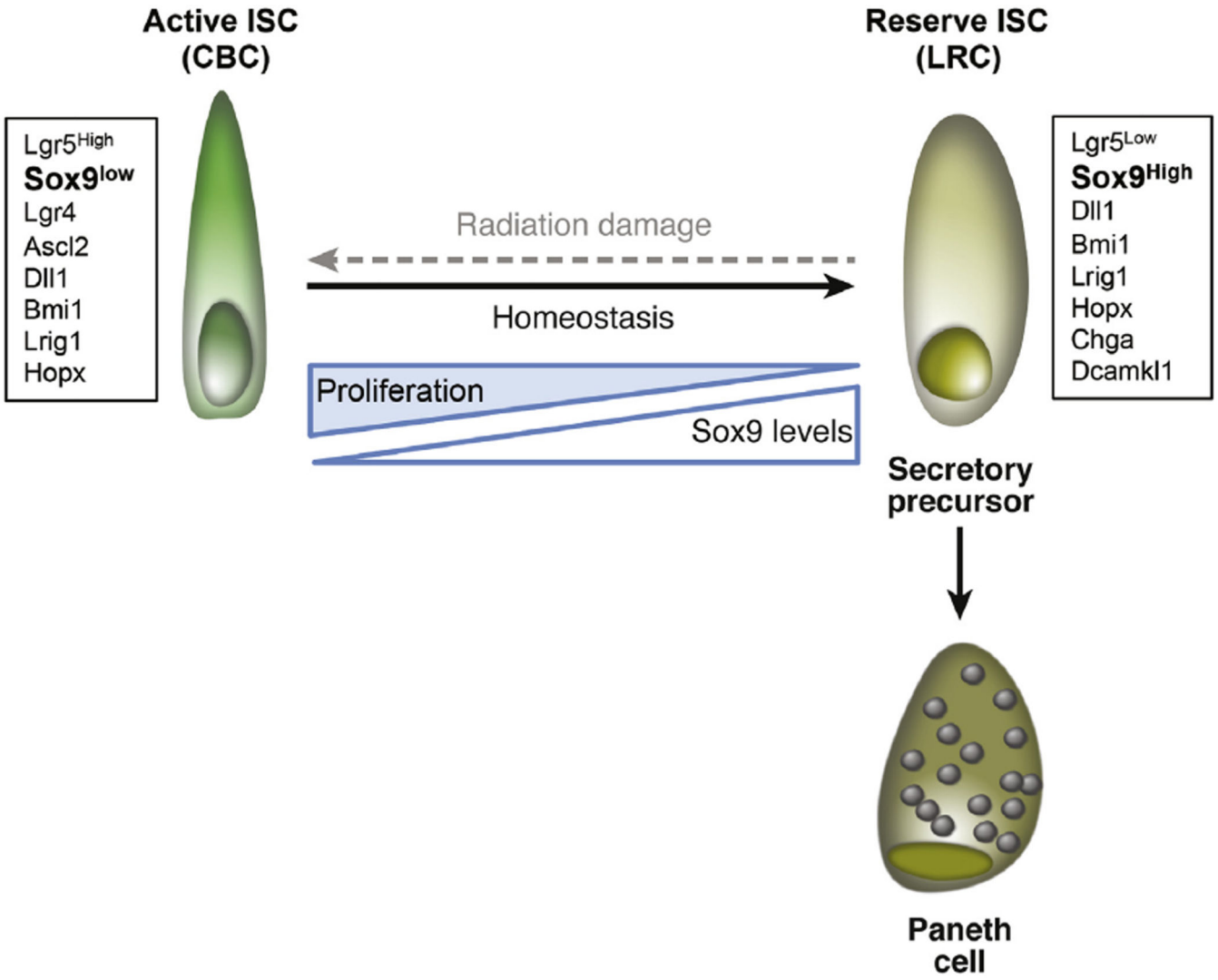

Figure 7.

A model for a dose-dependent role of SOX9 in rISC maintenance and Paneth cell differentiation. aISCs give rise to daughter progenitors consisting of absorptive and secretory precursors. Secretory progenitor cells become nonproliferative LRCs as expression of SOX9 increases. In the absence of SOX9, secretory LRC progenitors, which give rise to Paneth cells, are not generated, resulting in the loss of the Paneth cell lineage. After damage, LRCs down-regulate SOX9, re-acquire aISC properties, and facilitate epithelial repair.