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

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

Keywords: Irradiation Injury; Intestinal Epithelium; Radioresistant 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

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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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