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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Stroma provides an intestinal stem cell niche in the absence of epithelial Wnts

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ABSTRACT

Wnt/β-catenin signaling supports intestinal homeostasis by regulating proliferation in the crypt. Multiple Wnts are expressed in Paneth cells as well as other intestinal epithelial and stromal cells. *Ex vivo*, Wnts secreted by Paneth cells can support intestinal stem cells when Wnt signaling is enhanced with supplemental R-Spondin 1 (RSPO1). However, *in vivo*, the source of Wnts in the stem cell niche is less clear. Genetic ablation of *Porcn*, an endoplasmic reticulum resident O-acyltransferase that is essential for the secretion and activity of all vertebrate Wnts, confirmed the role of intestinal epithelial Wnts in *ex vivo* culture. Unexpectedly, mice lacking epithelial Wnt activity (*Porcn^{Del}*/*Villin-Cre* mice) had normal intestinal proliferation and differentiation, as well as successful regeneration after radiation injury, indicating that epithelial Wnts are dispensable for these processes. Consistent with a key role for stroma in the crypt niche, intestinal stromal cells endogenously expressing Wnts and *Rspo3* support the growth of *Porcn^{Del}* organoids *ex vivo* without RSPO1 supplementation. Conversely, increasing pharmacologic PORCN inhibition, affecting both stroma and epithelium, reduced *Lgr5* intestinal stem cells, inhibited recovery from radiation injury, and at the highest dose fully blocked intestinal proliferation. We conclude that epithelial Wnts are dispensable and that stromal production of Wnts can fully support normal murine intestinal homeostasis.

KEY WORDS: Wnt, PORCN, Radiation injury, R-spondin, Intestinal niche, Mouse

INTRODUCTION

The epithelial lining of the intestine continuously renews itself every 3 to 5 days. In the crypts of the murine small intestine, long-lived, label-retaining stem cells in the +4 position marked by *Bmi1*, *Tert* and *Hopx* interact with and interconvert with more rapidly proliferating, radiation resistant, crypt base columnar cells that express markers including *Lgr5*, *Olfm4*, *Lrig1* and *Ascl2* (Barker et al., 2008; Buczacki et al., 2013; Powell and Saada, 2012; Takeda et al., 2011; van der Flier et al., 2009; Wong et al., 2012; Yan et al., 2012). This stem cell compartment gives rise to committed progenitor cells that proliferate rapidly and produce the diverse differentiated progeny that migrate up the villi before being shed into the lumen.

Wnt/β-catenin signaling plays a crucial role in maintaining normal proliferation in the intestinal crypt of the adult mouse. Secreted Wnts bind to LRP5/6 and Frizzled co-receptors present on epithelial crypt cells, leading to an increase in β-catenin protein (Clevers and Nusse, 2012). Activated β-catenin binds to the nuclear transcription factor TCF4 to drive a gene expression program that supports stem cell maintenance, proliferation and differentiation. Disruption of the Wnt/β-catenin pathway blocks intestinal proliferation. Embryonic knockout of *TCF4* in intestinal epithelial cells leads to lack of proliferation in the inter-villus region of the neonatal small intestine, whereas inducible knockout of *TCF4* and β-catenin in adults blocks proliferation in the crypt compartment (Fevr et al., 2007; Korinek et al., 1998; van Es et al., 2012). Conversely, stabilization of β-catenin by expression of constitutive active β-catenin or mutation of *APC* stimulates proliferation. Surprisingly, the evidence that secreted Wnt ligands regulate intestinal homeostasis in adult mice remains indirect. The strongest evidence comes from studies inhibiting or knocking out the Wnt ligand co-receptors *Lrp5* and *Lrp6*, leading to a near total loss of epithelial proliferation (Kuhnert et al., 2004; Zhong et al., 2012).

The identity and cellular source of the Wnts that regulate the intestinal stem cell niche is not clear. Many of the 19 different Wnt genes are expressed in the small intestine, each with a distinct pattern of expression in diverse cell types of the epithelium and stroma (Gregorieff et al., 2005). These multiple Wnts may regulate diverse processes beyond the stem cell niche, including innate and adaptive immunity, injury repair, and intermediary metabolism (Cervantes et al., 2009; Davies et al., 2008; Zeve et al., 2012). The limited number of studies knocking out Wnts in mouse intestine have not identified defects in *in vivo* crypt stem cell proliferation (Cervantes et al., 2009; Farin et al., 2012). Paneth cells in the crypt base are a potential source of the Wnts that regulate stem cell proliferation. They express several Wnts, including *Wnt3*. In purified epithelial cell preparations, WNT3 from Paneth cells is required for the growth in culture of organoids derived from *Lgr5*-expressing intestine stem cells, although supplementation with R-Spondin 1 (RSPO1) is also required (Farin et al., 2012; Ootani et al., 2009; Sato et al., 2011, 2009). Based on these data, it has been proposed that Paneth cells form the niche for the isolated intestinal stem cells (Sato et al., 2011). However, depletion of Paneth cells or knockout of *Wnt3* in the epithelial cells of the intestine did not show an obvious *in vivo* phenotype (Durand et al., 2012; Farin et al., 2012), indicating that other functionally important Wnts are made by epithelial or stromal niche cells. Several groups have demonstrated that stromal cells can

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support the growth of intestinal epithelium in culture (Farin et al., 2012; Lahar et al., 2011). Intestinal stromal cells express multiple Wnts (Farin et al., 2012; Gregorieff et al., 2005). Farin et al. demonstrated that purified stromal cells could support organoid formation from *Wnt3* knockout epithelial cells, but the question of whether other epithelial-produced Wnts are essential *ex vivo* and more importantly, *in vivo*, remains unanswered.

One approach to address the functionally relevant source of Wnts in the small intestine is to globally target their secretion. This can be achieved by knockout or inhibition of either of two genes, *Porcn* and *Wls*, that are indispensable parts of the core Wnt secretion machinery (Najdi et al., 2012; Proffitt and Virshup, 2012). The PORCN protein is a membrane bound O-acyl transferase that resides in the endoplasmic reticulum. PORCN palmitoleates all Wnts as they are synthesized (Biechele et al., 2011; Kadowaki et al., 1996; Tanaka et al., 2000). Palmitoleation is required for Wnts to bind to WLS, an integral membrane carrier protein that is essential for the secretion of all known vertebrate Wnts (Coombs et al., 2012; Najdi et al., 2012). Wnt palmitoleation is also required for secreted Wnt ligands to interact with Frizzled receptors at the cell membrane (Janda et al., 2012). *Porcn* is encoded by a single copy gene on the X chromosome and has no closely related homologs in the genome. Zygotic *Porcn* mutants exhibit gastrulation failure leading to early embryonic lethality (Barrott et al., 2011; Biechele et al., 2011, 2013). Several potent small molecule inhibitors of PORCN have been developed that phenocopy the biochemical effects of *Porcn* knockout, and may be of value in the treatment of Wnt-high diseases, including cancer and fibrotic disorders (Chen et al., 2009; Proffitt et al., 2013). Indeed, one PORCN inhibitor, LGK974, is currently in phase 1 trials in humans (Liu et al., 2013). However, due to potential detrimental effects on adult stem cell self-renewal, the role of pharmacologic inhibition of PORCN remains to be determined.

To dissect the role of epithelial and stromal Wnts in intestinal homeostasis and assess the potential toxicity of PORCN inhibition, we used genetic and pharmacological approaches to block Wnt production in the gut. We find that epithelial Wnts are dispensable for normal proliferation in the mouse intestine. By contrast, pharmacologic inhibition of Wnt production produces a graded decrease in proliferation. At intermediate doses, Wnt-responsive Lgr5 expression is markedly reduced, accompanied by impaired recovery from radiation injury, but without a decrease in the proliferation of transit amplifying cells. At higher doses of the PORCN inhibitor, global intestinal proliferation ceases. A stromal fraction enriched for myofibroblasts that endogenously expresses both Wnts and *Rspo3* supports $Porcn^{Del}$ organoid proliferation in the absence of supplemental RSPO1. These studies suggest that a stromal Wnt/RSPO3-producing niche is sufficient for normal and stressed intestinal homeostasis and support the role of the Lgr5 cell population in recovery from radiation injury.

RESULTS

Normal intestine homeostasis after complete inhibition of epithelial Wnt secretion

Multiple studies have demonstrated a role for Wnt/β-catenin signaling in intestinal homeostasis, but whether intestinal epithelial cells provide any Wnts important in the niche remains an open question. To assess the role of the epithelial Wnts in intestinal homeostasis, we used a floxed allele of *Porcn* that is null for Wnt secretion after Cre-mediated excision (Biechele et al., 2013; Proffitt and Virshup, 2012). $Porcn^{flox}$ and $Porcn^{WT}$ mice were crossed with *Villin-Cre* mice to generate $Porcn^{Del}$/*Villin-Cre* or $Porcn^{WT}$/*Villin-Cre* mice, respectively. *Villin* expression begins in late embryogenesis in all epithelial cells of the intestine. Our hypothesis was that embryonic inactivation of *Porcn* in the intestinal epithelium would be lethal in the neonatal period, similar to what was observed in the *Tcf4* or *Lrp5/Lrp6* double knockout (Korinek et al., 1998; Zhong et al., 2012). Unexpectedly, $Porcn^{Del}$/*Villin-Cre* pups were viable, appeared phenotypically normal, and suckled and weaned without difficulty. We confirmed by genomic PCR and RT-qPCR that purified intestinal epithelial cells from $Porcn^{Del}$/*Villin-Cre* mice had complete excision of the *Porcn* gene (Fig. 1A,B). To confirm functional excision of *Porcn*, we took advantage of the observation that Wnt secretion from Paneth cells or other epithelial cells is required for purified crypts to form organoids in culture (Ootani et al., 2009; Sato et al., 2009). Indeed, purified isolated crypts from $Porcn^{Del}$/*Villin-Cre* mice did not form organoids *in vitro* (Fig. 1C,E). This result phenocopies both knockout of *Wnt3* (Farin et al., 2012) and the effect of small molecule PORCN inhibition (Sato et al., 2011), confirming the functional inactivation of epithelial *Porcn* in $Porcn^{Del}$/*Villin-Cre* mice and supporting the central role of epithelial Wnts in *ex vivo* culture of purified intestinal stem cells.

To confirm that the defect in organoid formation was due to loss of secreted Wnts and not to a non-Wnt consequence of *Porcn* knockout (Covey et al., 2012), we employed two approaches. First organoid formation from $Porcn^{Del}$/*Villin-Cre* crypts was rescued by co-culture with WNT3A-secreting mouse L (L3A) or human HEK293 (STF3A) cells (Fig. 1D,E). Second, organoid formation was also partially rescued by co-culture with mouse embryo fibroblasts (MEFs) that we found express endogenous WNT3 (Fig. 1C,E; supplementary material Fig. S1A). However, $Porcn^{Del}$ MEFs failed to rescue (Proffitt and Virshup, 2012). These findings are consistent with existing data that intestinal epithelial stem cells require a source of palmitoleated Wnts to proliferate and form organoids, and that *ex vivo* the Wnts can be supplied by co-culture with WNT3A-secreting cells.

We assessed the phenotype of the intestine in the $Porcn^{Del}$/*Villin-Cre* mice. The small and large intestines were grossly and microscopically normal (Fig. 2A). In addition, epithelial lineages including enteroendocrine, Paneth and goblet cells were not detectably altered in the absence of epithelial Wnt secretion, and there was no increase in apoptotic cells (Fig. 2B). Loss of epithelial *Porcn* did not alter the committed progenitor (also called transit amplifying) compartment, as assessed by short term EdU incorporation (Fig. 2C; supplementary material Fig. S1B). Importantly, β-catenin was observed in the nuclei of crypt cells of $Porcn^{Del}$/*Villin-Cre* mice, indistinguishable from controls (Fig. 2C). Consistent with intact β-catenin signaling, the expression of *Axin2*, *Lgr5* and *Olfm4* did not differ significantly between jejunum samples of control and *Porcn*-inactivated mice (Fig. 2D). These data demonstrate that Wnt/β-catenin/LGR5 signaling is ongoing in intestinal crypt cells in the absence of epithelial Wnt secretion. This strongly suggests that in $Porcn^{Del}$/*Villin-Cre* mice the Wnts important in intestinal proliferation are adequately supplied from the stroma.

We tested whether the unaltered proliferation and differentiation after epithelial loss of Wnt secretion resulted from compensatory changes in other signaling pathways regulating intestinal stem cell renewal. To assess this, gene expression profiling was performed on isolated epithelium and stroma from wild-type and $Porcn^{Del}$ mouse intestine. As supplementary material Fig. S1C shows, there were only minimal changes in global gene expression detected, and there were no statistically significant alterations in known Wnt/β-catenin pathway regulators. There were no significant changes in expression of genes associated with the Notch and EGF pathways (supplementary material Fig. S1D,E). We conclude that epithelial Wnt secretion is not essential

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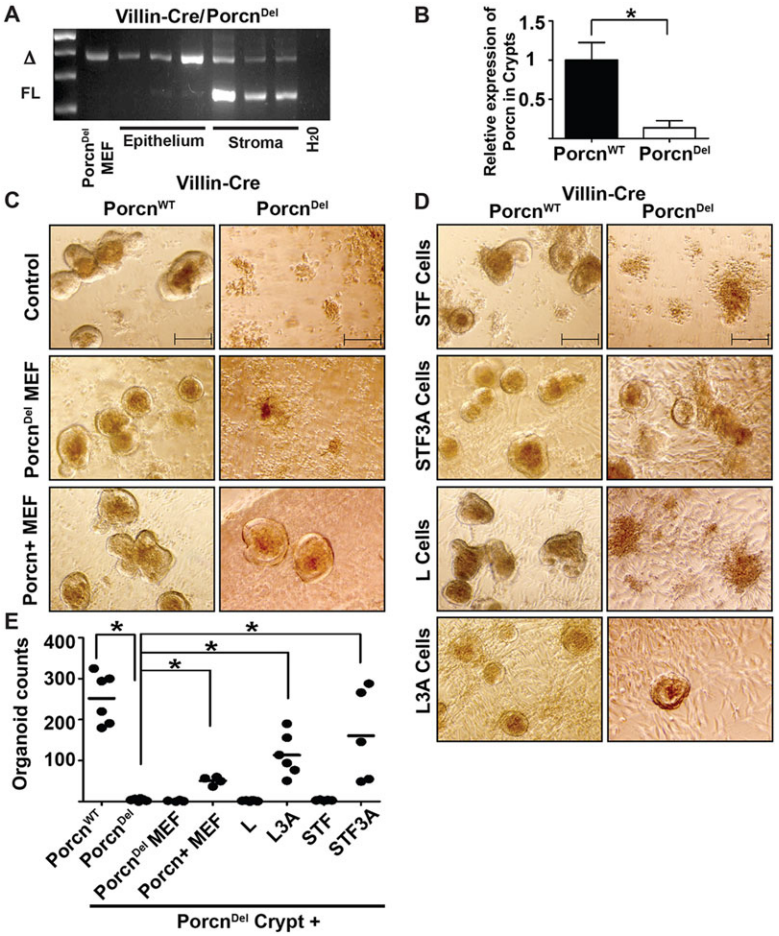

Fig. 1. *Porcn* deletion renders intestinal epithelial crypts dependent on exogenous Wnts. (A) *Porcn* deletion in purified intestinal epithelial cells from $Porcn^{Del}$/*Villin-Cre* mice is efficient. PCR was performed using gDNA from the epithelial or stromal fractions of individual mice, or knockout MEFs. The small amount of unexcised *PORCN* allele present in two of the epithelial fractions most likely represents residual stromal contamination visible after 40 PCR cycles. (B) Purified crypt cells were analyzed for *Porcn* mRNA expression using RT-qPCR. Samples were normalized for housekeeping genes (*Pgk* and β-actin). Data represent different epithelial preps from age- and gender-matched mice (n=5 in each group). Error bars indicate s.d. (C) Crypts from $Porcn^{Del}$/*Villin-Cre* mice, either alone or co-cultured with $Porcn^{Del}$ MEFs, did not form organoids (top and middle panels, respectively). By contrast, co-culture with $Porcn^{Del}$ MEFs stably expressing $Porcn^{WT}$($Porcn^{+}$ MEFs) rescued organoid formation (bottom panels). 5000 MEFs were plated with 6000 crypts per well. (D) Organoid formation from $Porcn^{Del}$ crypts is rescued by Wnt-secreting cells. No organoids form in co-cultures of crypt cells from $Porcn^{Del}$/*Villin-Cre* mice with L cells or STF cells, whereas the same cell lines expressing Wnt3A (L3A and STF3A cells, respectively) restore organoid formation. 5000 feeder cells were plated with 6000 crypts per well. (E) Quantification of experiments in panel C and D. Each point represents an independent biological replicate. *P<0.001, two-tailed t-test. Scale bars: 200 μm. Δ, excised *Porcn* allele; FL, floxed allele.

for normal murine small intestinal homeostasis *in vivo*, and its loss causes no readily detectable compensatory alterations in gene expression.

Stroma expresses Wnts and *Rspo3* and forms a niche for epithelial stem cells

A critical role for intestinal stroma in the self-renewal of epithelial stem cells has previously been suggested by Farin et al. (2012). To test this, we isolated $Porcn^{Del}$ crypts and $Porcn^{flox}$ unfractionated stromal cells ('fresh stroma') from $Porcn^{Del}$/*Villin-Cre* mice and cultured them separately or together. Stroma added to $Porcn^{Del}$ crypts supported cystic organoid formation (Fig. 3A). Crypts depleted of stroma require supplementation with RSPO1 to form organoids in culture. We reasoned that the added RSPO1 might be required to amplify the WNT3 signal from the Paneth cells. Remarkably, we found that $Porcn^{Del}$ crypts supplied with stroma did not require exogenous RSPO1 (Fig. 3A).

Wnts signal at short range, and myofibroblasts are anatomically adjacent to the epithelial crypt, making them a candidate source of stem-cell supporting factors. We therefore prepared cultured adherent stromal cells highly enriched for myofibroblasts [vimentin positive (~100%), alpha-smooth muscle actin (α-SMA) positive (>50%), desmin positive (4%)] (Fig. 3C). Notably, these cells supported organoid formation better than unfractionated stromal cells even in the absence of supplemental RSPO1 (Fig. 3A). The myofibroblast-enriched population expresses multiple Wnts (supplementary material Fig. S2C). In addition, these cells express abundant *Rspo3* (Fig. 3B; supplementary material Fig. S2D). By contrast, the epithelium was a poor source of RSPOs, as assessed by both microarray and qPCR (Fig. 3B; supplementary material Fig. S2D). Thus, the myofibroblast-enriched stromal fraction can provide both Wnts and R-Spondin and support intestinal stem cell proliferation.

To assess the contribution of immune and/or hematopoietic cells as a Wnt source for intestinal homeostasis, we crossed $Porcn^{flox}$/*Villin-Cre* mice to *Vav-Cre* mice to generate *Porcn* knockout in intestinal epithelial cells as well as hematopoietic and immune cells. These mice, which will be described in more detail elsewhere, were viable and their cultured adherent stroma also fully supported organoid formation from $Porcn^{Del}$ epithelial cells (supplementary material Fig. S2A). Taken together, the data are most consistent with stromal myofibroblasts as a significant source of Wnts and *Rspo3* both *in vivo* and in culture, although we cannot exclude the possibility that rare cell types form the niche.

Wls knockout resembles the *Porcn* knockout

As loss of Wnt secretion in the intestinal epithelium was so strongly predicted to produce a phenotype, we asked if knockout of *Wls* phenocopied the *Porcn* knockout. *Wls* is a dedicated Wnt carrier that is required to transport palmitoleated Wnts to the cell membrane. *Wls*, like *Porcn*, is required for the activity of all human Wnts (Najdi et al., 2012). Similar to the *Porcn* deletion, Wls^{Del}/*Villin-Cre* pups were viable and had normal development and growth. Histological appearance, proliferation, and β-catenin nuclear localization were identical in the intestines of one-year-old control (Wls^{WT}/*Villin-Cre*) and *Wls*-deleted (Wls^{Del}/*Villin-Cre*) mice (supplementary material Fig. S2B). These data confirm that inhibition of epithelial Wnt secretion by targeting either *Porcn* or *Wls* does not significantly impact intestinal development and homeostasis.

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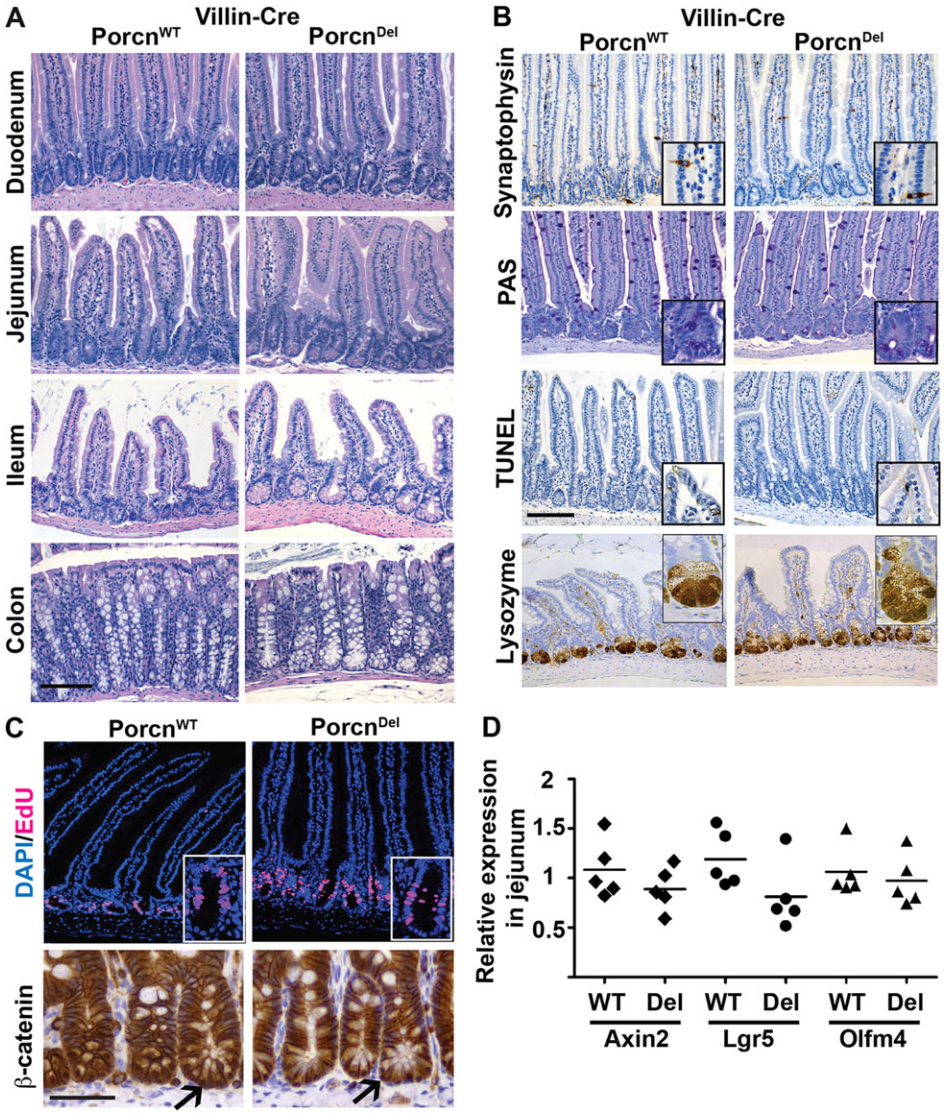

Fig. 2. Epithelial Wnt secretion is dispensable for intestinal homeostasis in mice. (A) Representative histology of small intestine and colon from *Porcn*^{WT}/*Villin-Cre* (*n*=6) and *Porcn*^{Del}/*Villin-Cre* (*n*=6). Mice were sacrificed between 3 and 12 months of age. (B) Normal epithelial differentiation in *Porcn*^{WT}/*Villin-Cre* (*n*=3) and *Porcn*^{Del}/*Villin-Cre* (*n*=3) mice. Enteroendocrine cells labeled by synaptophysin-specific staining, goblet and Paneth cells labeled by PAS staining, apoptotic cells labeled by TUNEL staining and Paneth cell differentiation was assessed by lysozyme immunohistochemistry. Insets show higher magnification. (C) Normal proliferation and nuclear β-catenin in *Porcn*^{WT}/*Villin-Cre* (*n*=3) and *Porcn*^{Del}/*Villin-Cre* (*n*=3). Mice were injected with EdU 2 h before sacrifice. Arrows indicate nuclear β-catenin. (D) No significant changes in expression of Wnt/β-catenin target genes *Axin2*, *Lgr5* and *Olfm4* between jejunum of *Porcn*^{WT}/*Villin-Cre* and *Porcn*^{Del}/*Villin-Cre* mice. Expression levels were assessed by qPCR and normalized to *Pgk* and β-actin (*n*=5 mice, $P>0.05$, two-tailed *t*-test). Scale bars: 200 μm in B; 50 μm in C.

The role of stromal Wnts *in vivo*

Substantial data indicate that Wnt signaling is essential for intestinal stem cell regulation, but the targeted inhibition of Wnt secretion from the intestinal epithelium produced no phenotype. Either, against all expectations, Wnts are not required, or the intestinal stroma is sufficient as the niche providing the Wnts regulating stem cell proliferation. To differentiate between these possibilities, we took advantage of a recently validated pharmacological inhibitor of *Porcn*, C59. This drug is a readily absorbed, bioavailable inhibitor of *Porcn* with a sub-nanomolar IC50 that inhibits Wnt/β-catenin signaling and blocks the growth of WNT1-dependent mammary tumors in mice (Proffitt et al., 2013). An added benefit of using a drug is the ability to titrate the dose to dissect intermediate phenotypes that are difficult to detect in gene deletion models. We reasoned that C59 should inhibit stromal as well as epithelial PORCN activity and, taken together with the results of epithelial *Porcn* knockout, be a good test of the role of stromal Wnt secretion.

We confirmed that C59 prevented the formation of intestinal organoids in culture, similar to the activity of IWP (supplementary material Fig. S3A) (Sato et al., 2011). We previously reported that orally administered C59 at 5-10 mg/kg/day inhibited the growth of MMTV-Wnt1 mammary tumors without intestinal toxicity (Proffitt et al., 2013). To identify a C59 dose that inhibited Wnt/β-catenin activity in the intestine, mice were administered various amounts of C59 daily for 2 days by gavage and their small intestines were harvested 20-24 h after the second dose. There was a dose-dependent reduction in the expression of the Wnt/β-catenin target gene *Lgr5* and the crypt base columnar (CBC) cell marker *Olfm4* (supplementary material Fig. S3B).

Mice receiving C59 50 mg/kg/day for 6 days maintained body weight and had normal intestinal structure and proliferation (Fig. 4A,C). This was despite the systemic C59 treatment causing a rapid, significant and persistent reduction in expression of the β-catenin target genes *Lgr5*, *Axin2* and *Ascl2*, as well as the CBC cell marker *Olfm4*, but not *Bmi1* in the small intestine (Fig. 4B; supplementary material Fig. S3C). As an independent approach to confirm that *Lgr5* expression was reduced by C59 treatment, we treated *Lgr5-IRES-EGFP-CreERT2* mice that express EGFP from the endogenous *Lgr5* promoter with 50 mg/kg/day C59 for 6 days (Barker et al., 2007). We observed a marked decrease in EGFP expression in the C59-treated intestine (Fig. 4C). We confirmed that the C59-dependent decrease in Wnt/β-catenin target gene expression was also seen in isolated intestinal crypts (supplementary material Fig. S3D). Wnt signaling can regulate Paneth cell differentiation, and we observed that the Paneth markers lysozyme and MMP7, but not

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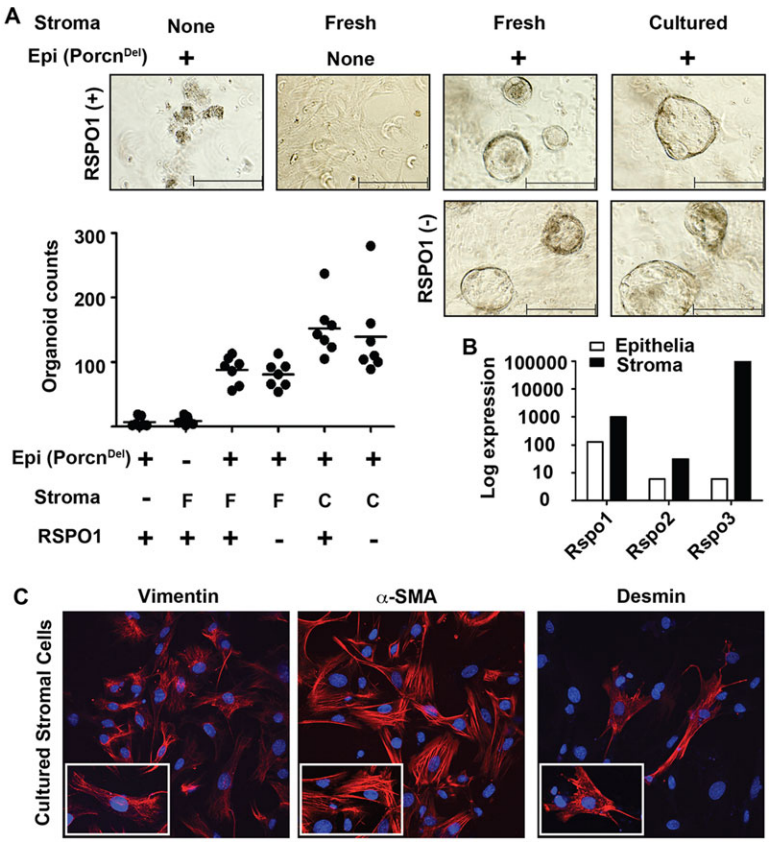

Fig. 3. *Porcn*-deficient crypts form organoids in the presence of stromal cells. (A) *Porcn*^{Del}/*Villin-Cre* crypts [Epi (*Porcn*^{Del})] do not form organoids (top left panel), unless supplemented with stromal cells (top right panels). Stromal and epithelial cells were prepared as described and cultured alone or in combination. Where indicated, 6000 crypts were combined with freshly prepared or cultured adherent stromal cells (5×10^4 and 2.5×10^4, respectively) and then cultured for 5 days in the presence or absence of supplemental RSPO1. Below, organoid counts from multiple wells from four mouse samples. All samples containing stromal cells had significantly higher organoid counts than group containing epithelial cells alone or stromal cells alone ($P<0.005$, Mann–Whitney U-test). (B) R-spondin 3 (*Rspo3*) is highly expressed in intestinal stroma. Epithelial crypts and cultured stromal cells were analyzed for Rspo gene expression using RT-qPCR. Relative expression of the various Rspo genes is calculated as $2^{(-\Delta Ct)}$ using *Pgk* expression level as a reference, $n=3$. (C) Cultured stroma is enriched in myofibroblasts. Stromal cells were cultured for 8 days and then assessed for vimentin, desmin or smooth muscle actin (α-SMA) by immunofluorescence confocal microscopy. Specific staining for indicated differentiation markers is shown in red. DAPI-labeled nuclei are shown in blue. Each picture is representative of three biological replicates. Scale bar: 200 μm.

cryptdin nor the goblet cell marker MUC2, were also decreased after 1 week of treatment (supplementary material Fig. S4A,B). Thus, systemic, but not isolated epithelial inhibition of PORCN-dependent Wnt secretion suppresses Wnt/β-catenin target gene expression in the intestine, consistent with an important role for stroma. Unexpectedly, this suppression of Wnt/β-catenin signaling was not essential for short-term intestinal homeostasis.

Lgr5-expressing CBC cells are relatively radiation resistant and proliferate after radiation injury (Hua et al., 2012). We reasoned that suppression of Wnt/β-catenin signaling by PORCN inhibition might impair the response to injury. C57BL/6 mice were therefore treated with 50 mg/kg/day C59 for 6 days. Twenty hours following the final dose of C59, mice were irradiated with a single dose of 12 Gy, and sacrificed when they became ill 5 days later. In the C59 treated mice, the duodenum was dilated and the small intestine was markedly shortened (supplementary material Fig. S4C). Histologic examination demonstrated a marked loss of crypts and villi throughout the small intestine in Wnt-suppressed compared with control mice (Fig. 5A). Proliferation of the epithelial layer was assessed by EdU incorporation 5 days after radiation. As expected, increased proliferation was observed in the epithelium of the control mice due to tissue regeneration. However, the C59-treated mice showed a marked reduction in EdU incorporation in the small intestine (Fig. 5B). Thus, intermediate inhibition of Wnt/β-catenin signaling by C59 significantly reduced *Lgr5* expression in the CBC cells and impaired recovery from radiation injury. This is consistent with a recently proposed role for *Lgr5*-expressing cells in the response to radiation damage (Hua et al., 2012; Metcalfe et al., 2014).

We tested whether epithelial Wnts are required for recovery from radiation injury. *Porcn*^{WT} and *Porcn*^{Del}/*Villin-Cre* mice were treated with the same radiation protocol as above. Three out of five wild-type, and five out of five *Porcn*^{Del}/*Villin-Cre* mice survived 8 days after radiation. All the mice were ill and had significant weight loss. Histologic examination of the intestines of surviving mice showed successful ongoing epithelial regeneration regardless of genotype, with no detectable differences between wild-type and knockout mice (Fig. 5C). Hence, epithelial Wnt production is not required for intestinal regeneration after radiation injury. The radiation sensitivity seen in C59-treated, but not *Porcn* knockout, mice is consistent with functionally important Wnts coming from the stromal niche.

High-dose PORCN inhibition impaired intestinal homeostasis

C59 given once daily at 50 mg/kg significantly reduced expression of the CBC cell markers *Lgr5*, *Ascl2*, and *Olfm4*, decreased expression of Paneth cell markers and prevented an effective response to radiation injury. However, the persistence of EdU incorporation and nuclear β-catenin (supplementary material Fig. S3E) in these mice suggested residual Wnt activity might be sufficient to maintain a stem cell population, as has been suggested for the related compound LGK974 (Liu et al., 2013). To test this, we increased the C59 dose to twice daily to increase trough drug levels. Mice treated with 50 mg/kg per dose, twice daily, were moribund within 6 days. At this dose intensity, intestinal crypts began to disappear in the duodenum and jejunum on the second and fourth day of treatment, respectively (Fig. 6A).

After 6 days of high dose treatment, there was a global reduction in proliferation, as indicated by loss of all crypts in the small intestine and absence of EdU incorporation (Fig. 6A,B). This toxicity is unlikely to be nonspecific, as there was a decrease in proliferation rather than an increase in apoptosis (Fig. 6B; supplementary material Fig. S5A). These dramatic findings resemble the proliferation defect observed after total loss of Wnt/β-catenin signaling in the epithelium in mice with conditionally deleted β-catenin or *TCF4* alleles (Fevr et al., 2007; van Es et al., 2012). We conclude that full inhibition of

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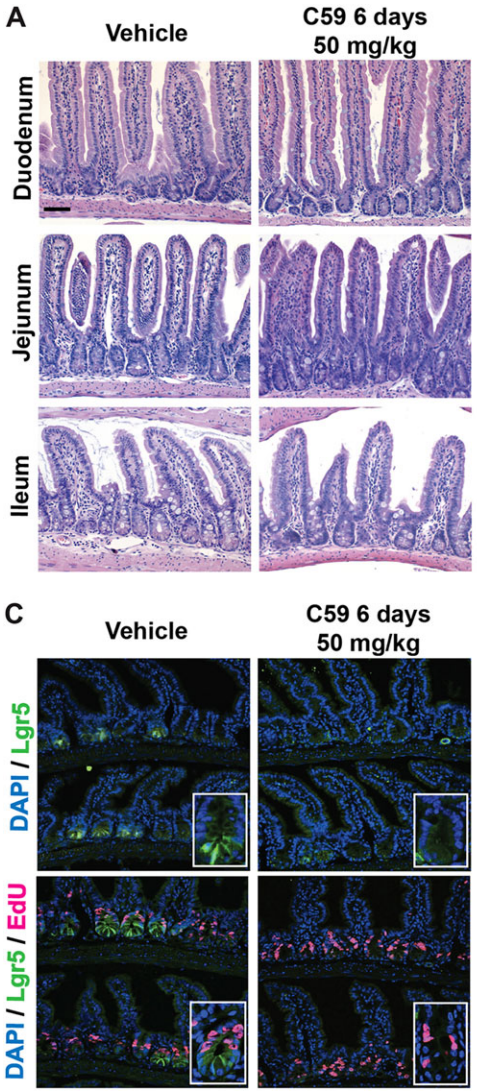

Fig. 4. Pharmacological inhibition of PORCN decreases intestinal stem cell marker expression, but does not affect normal proliferation of gut. (A) Moderate PORCN inhibition does not alter intestinal structure. Histology of different regions of small intestine following vehicle or C59 treatment (50 mg/kg/day) for 6 days, *n*=5 in each group. (B) Systemic PORCN inhibition leads to decreased expression of intestinal stem cell markers. C59, 50 mg/kg/day, was administered for the indicated period and mice sacrificed 20-24 h after the last dose. Expression levels of *Lgr5* and *Olfm4* were normalized to *Pgk* and β-actin. Mean values of respective vehicle-treated groups were set to 1 and compared to individual measurements within the same group. ***P*<0.01 and ****P*<0.001, Mann–Whitney U-test. (C) Suppression of Wnt/β-catenin target genes including *Lgr5* does not affect short-term proliferation. *Lgr5-IRES-EGFP-CreER*^{T2} mice were treated with vehicle (*n*=6) or C59 (50 mg/kg/day, for 6 days, *n*=6). *Lgr5*-expressing cells are labeled by EGFP. Nuclei of cells are labeled by DAPI. Proliferative cells were labeled with EdU given 2 h before sacrifice (bottom panels). Insets show higher magnification of single crypt. Scale bar: 100 μm.

PORCN activity in both epithelium and stroma of the intestine blocks proliferation and intestinal homeostasis.

DISCUSSION

Our study indicates that stromal Wnts are sufficient to maintain mouse small intestinal homeostasis. Tissue-specific knockout of *Porcn* and *Wls* shows that epithelial Wnt production is dispensable for normal intestinal stem cell development, self-renewal, proliferation, and the response to radiation-induced injury. Conversely, using a small molecule inhibitor of Wnt production we find that varying levels of systemic PORCN inhibition produce distinct phenotypes in the gut. Moderate global inhibition of Wnt secretion markedly reduced *Lgr5* expression and impaired intestinal homeostasis after radiation injury, whereas more complete inhibition of Wnt secretion immediately affected stem cell function, similar to results seen after genetic knockout of key Wnt/β-catenin pathway components. The ability to moderately inhibit PORCN function and not impair short-term intestinal homeostasis suggests that drugs inhibiting PORCN will have a therapeutic index allowing clinical use.

Purified epithelial stem cells can form organoids and expand *ex vivo* in the presence of Wnt3-producing Paneth cells and exogenous RSPO1. Here, we found epithelial Wnts supplemented with recombinant RSPO1 can be replaced by an intestinal myofibroblast-enriched stromal fraction that endogenously produces Wnts and RSPO3. Taken together, the data are consistent with the hypothesis that stromal cells can form a Wnt- and RSPO3-producing niche for intestinal epithelial stem cells in the absence of epithelial Wnt production.

Is there a single essential source of Wnts in the small intestine? We found that both epithelial and hematopoietic *Porcn* function are dispensable for intestinal homeostasis. Our data on stroma as a source of both Wnts and RSPO3 *ex vivo* are consistent with myofibroblasts in close proximity to the epithelial stem cells forming the Wnt-producing niche. However, this stands in contrast to a paper published while this work was in revision (San Roman et al., 2014). The authors of that paper reported that inducible short-term double deletion of *Porcn* in both intestinal epithelium (in *Porcn*^{flox}*/villin-creER*^{T2} mice) and subepithelial myofibroblasts (in *Porcn*^{flox}*/Myh11-creER*^{T2} mice) did not alter intestinal homeostasis. However, that study did not determine the efficiency of *Porcn* deletion in the myofibroblasts and whether the *Myh11-creER*^{T2} driven excision in fact abrogated the organoid-supporting ability of the stroma.

Several publications have suggested that there are redundant sources of Wnts supporting the intestine (Farin et al., 2012; San Roman et al., 2014). Indeed, the intestinal stroma contains multiple cell types capable of making Wnts, including endothelial cells, macrophages, neurons, fibroblasts, and myofibroblasts. Wnts

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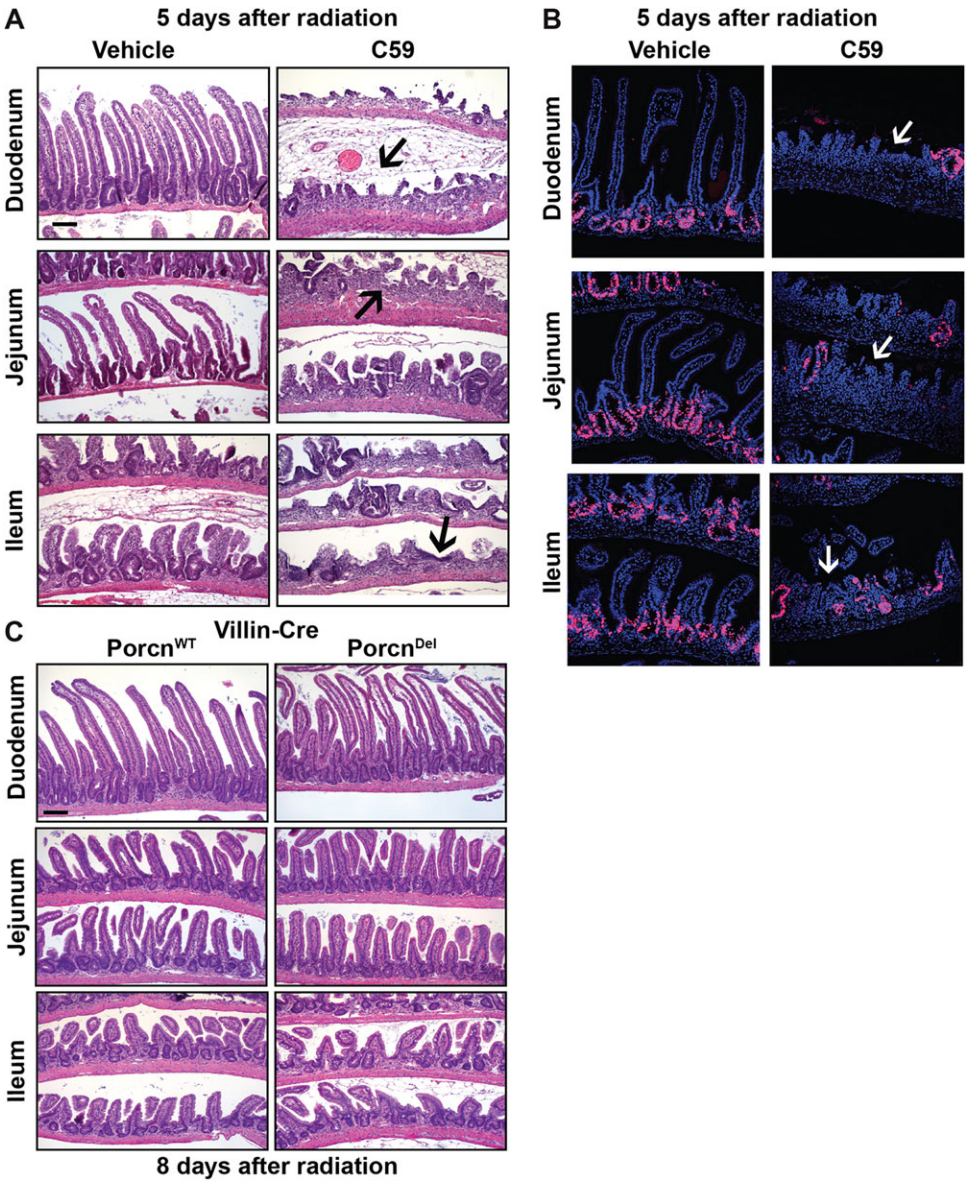

Fig. 5. Stromal Wnts support epithelial regeneration in response to radiation-induced injury. (A) Stromal Wnts support a cell population required for epithelial regeneration in response to radiation-induced injury. *Lgr5* and *Olfm4* were suppressed by pretreatment of C57BL/6 mice with 50 mg/kg/day of C59 for 6 days followed by irradiation (12 Gy) 20 h after the last dose. Architecture of the small intestine was analyzed 5 days after irradiation (n=5 for each group). Note the markedly impaired crypt regeneration (indicated by arrows) in the C59-treated group (bottom panels). (B) Proliferation 5 days after radiation damage was assessed by EdU incorporation assay. Although proliferation was high in the epithelium of the controls (left panels), the response in C59-treated mice (right panels) was dramatically compromised. Nuclei of proliferative cells were labeled with EdU (red), and nuclei of all cells were labeled by DAPI (blue). (C) Epithelial Wnt secretion is not required for epithelial regeneration after radiation injury. Architecture of different parts of small intestine was analyzed 8 days after irradiation (n=3 and n=5 for $Porcn^{WT}$/*Villin-Cre* and $Porcn^{Del}$/*Villin-Cre*, respectively). Scale bars: 200 μm.

produced in combinations of these cells could produce a cocktail of redundant Wnt ligands maintaining intestinal homeostasis *in vivo*. Alternatively, our data are also consistent with the niche being a specific myofibroblast population adjacent to the crypts that produces both Wnts and RSPO3.

Our study complemented the genetic knockout of PORCN with pharmacologic inhibition. One unexpected finding was the broad therapeutic range for pharmacologic PORCN inhibition. We previously reported that as little as 5 mg/kg/day of C59 blocked proliferation of a Wnt1-dependent mammary tumor, yet here, also in C57BL/6 mice, a 20-fold higher dose was required for inhibition of intestinal stem cell proliferation. Our data suggest that even small amounts of Wnt secretion can maintain an intestinal stem cell niche in the absence of external stress. This robust network of stem cell regulators suggests that inhibition of Wnt production may be effective for diseases with pathological Wnt elevation at doses that do not perturb normal stem cell niches.

We noted that *Lgr5*/*Ascl2*/*Olfm4* expression could be reduced by C59 without immediate effect on overall intestinal architecture, similar to genetic knockout of Lgr5 cells with diphtheria toxin (Metcalfe et al., 2014). However, several of the mice treated at the intermediate dose of C59 become ill after 18 days. Examination of the small intestine revealed patchy loss of proliferation and lack of crypts in the proximal small intestine (supplementary material Fig. S5B,C). This may be due to differential sensitivity of two distinct populations of stem cells in small intestine. Buczacki et al. recently demonstrated that label retaining cells (LRCs) in the +4 position of the crypt are secretory precursors of Lgr5 cells and serve as a reserve pool of stem cells after intestinal damage (Buczacki et al., 2013). We speculate that long-lived LRCs are relatively insensitive to C59-mediated *Porcn* inhibition and sustain intestinal homeostasis in the absence of Lgr5 stem cells. Impaired crypt homeostasis would slowly occur as the LRCs were depleted at the intermediate dose, or rapidly if they are completely deprived of Wnts at the high dose of C59.

The role of Lgr5+ and Paneth cells in the response to radiation damage is of great recent interest (Buczacki et al., 2013; Hua et al., 2012; Metcalfe et al., 2014; Roth et al., 2012). We addressed the role of Wnt production in the radiation response and found that global pharmacologic, but not epithelial-specific, inhibition of PORCN caused markedly increased sensitivity to radiation stress in the intestine. Although PORCN inhibition both reduced *Lgr5* expression and modified Paneth cell differentiation, Paneth cell depletion

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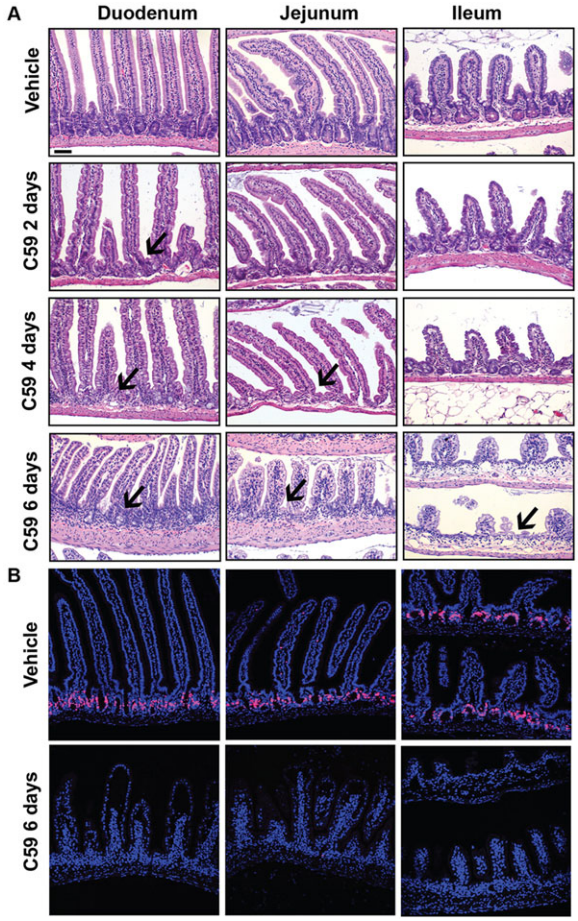

Fig. 6. Systemic inhibition of Wnt signaling leads to impaired intestinal homeostasis. (A) Twice daily oral administration of 50 mg/kg/dose (100 mg/kg/day) C59 leads to inhibition of gut proliferation, as shown by absence of crypts and shrinkage of villi. Histology of the small intestine following treatment for 2, 4 and 6 days is shown. Data represent results derived from at least three mice per group. (B) High dose C59 treatment for 6 days blocks proliferation in all regions of the small intestine. Nuclei of proliferative cells were labeled with EdU (red), nuclei of all cells were labeled by DAPI (blue). Note lack of EdU staining in the C59-treated group, bottom panels.

reportedly does not affect recovery from radiation (Metcalfe et al., 2014). Our data are therefore most consistent with the model that PORCN-dependent Lgr5+ cells are required for recovery from radiation, and suggest the possibility of synergistic toxicity in clinical settings.

In conclusion, taking advantage of the essential roles of *Porcn* and *Wls* in Wnt secretion, we have demonstrated that epithelial Wnts are not vital for intestinal homeostasis or recovery from radiation injury, while confirming that they are required for *ex vivo* cultures. In addition, we provide strong evidence that stromal Wnts play a crucial role in the maintenance of small intestine homeostasis *in vivo*.

MATERIALS AND METHODS

Mouse strains and drug administration

Porcn^{flox} mice (Biechele et al., 2013) were backcrossed to C57BL/6 mice at least for six generations. *Porcn*^{flox} mice were crossed with BL/6 *Villin-Cre* mice. Age- and gender-matched mice were used as controls for all experiments. *Lgr5*-IRES-*CreER*^{T2}-*EGFP* mice were obtained from Jackson Laboratories (Barker et al., 2007). *Wls*^{flox} mice, studied at the Van Andel Research Institute, were from Richard Lang (Carpenter et al., 2010). All mouse procedures were approved by the respective Institutional Care and Use Committees (IACUC). Genotyping and PCR are described in Table S1 and the Materials and Methods in the supplementary material. PORCN inhibitor C59 was suspended in a mixture of 0.5% methylcellulose and 0.1% Tween 80 by sonication for 30 min and then administrated by gavage as described (Proffitt et al., 2013).

Crypt isolation and culture

Intestine was harvested, cut longitudinally, washed, and macerated in pieces not exceeding 2 mm in size. Fragments were incubated in ice-cold PBS containing 2 mM EDTA for 40 min with gentle shaking every 10 min. The solution was pipetted up and down for 40 times followed by 3 min of gravity sedimentation. Supernatant fractions containing released cells were collected after each sedimentation step. This procedure was repeated three times, resulting in three collected fractions. After the first round, intestinal pellets were washed with PBS three times. Fraction 3 was used for subsequent crypt isolation. Crypts were enriched by centrifugation at 200 ***g*** for 2 min and counted using a phase contrast microscope. As judged by microscopy, this routinely yielded >90% pure crypts. All the procedures were performed at 4°C. Crypt culture was performed in 48-well plates using 6000 crypts per well closely following conditions described by Sato et al. (2009).

Stromal isolation for culture

Tissues remaining after crypt isolation were subjected to an additional round of pipetting for 40 times to remove most of the remaining epithelial cells, and washed once with PBS and with serum-free DMEM containing 1% Glutamax and 1% Penicillin-Streptomycin (both reagents from Life Technologies). Thereafter, tissues were digested for 3 h in 6 ml of serum-free DMEM containing 1% Glutamax, 1% Penicillin-Streptomycin and 2 mg/ml of Collagenase/Dispase (Roche). The digestion solution was replaced with fresh digestion solution every 60 min. At the end of this digestion step, tissues were further dissociated by vigorous pipetting. To inhibit proteolytic activity and cellular aggregation, at this step suspensions were supplemented with 5% fetal calf serum. Thereafter, samples were passed through a 70 µm cell strainer, centrifuged at 400 ***g*** for 4 min, washed once in PBS and counted. At this point, stromal cells were either directly mixed with epithelial cells ('fresh stroma') or cultured for 5 days in RPMI1640 containing 10% FCS, 1% Penicillin-Streptomycin and 1% Glutamax ('cultured stroma').

To test the ability of stroma to support epithelial crypt proliferation, fresh or cultured stroma (50,000 or 25,000 cells, respectively) was mixed with epithelial crypts (generally 6000 crypts) in 15 ml tubes and pelleted via centrifugation at 400 ***g*** for 4 min. Following centrifugation, supernatant was carefully removed and then pellets were re-suspended in 50 µl of Matrigel per well equivalent and then directly distributed into 48-well plates. The Matrigel was allowed to gel for 30 min at 37°C and then each well was supplemented with complete crypt culture medium. Each experiment included wells containing stroma alone to estimate amounts of contaminating epithelial stem cells.

Tissue preparation for staining

Intestine was harvested immediately after sacrifice and washed extensively with PBS. The small intestine was cut in two identical lengths, small fragments were collected for RNA isolation, and the remaining small intestine was flushed with 4% formalin and prepared for formalin fixation and paraffin embedding as a Swiss Roll.

For confocal imaging of EGFP, the small intestine was washed with PBS and then perfused with cold 4% paraformaldehyde, prepared as a Swiss Roll, and fixed for an additional 2 h. Samples were then incubated in 15% sucrose solution for 24 h, followed by 30% sucrose for another 24 h, all at 4°C. Samples were then embedded in OCT and stored at −80°C.

Antibodies and staining condition

Synaptophysin (Lifespan Biosciences, Cat. #LS-C49473), β-catenin [Becton Dickinson, Cat. #(421)610154] and lysozyme (Abcam, Cat. #ab108508) antibodies were used at dilution of 1:50, 1:150 and 1:5000, respectively. Antigens in formaldehyde-fixed and paraffin embedded intestinal tissues were retrieved by boiling in citrate buffer, pH 6, for 10 min. Thereafter, samples were blocked in 1% BSA for 60 min. Tissues were incubated with primary antibodies for 60 min, washed and subsequently incubated for 60 min with

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secondary antibody diluted 1:200. Sections were mounted in DPX medium and analyzed using a Leica DM2000 microscope. Apoptotic cells were detected by TUNEL assay, using ApopTag Plus Peroxidase In Situ (Millipore, Cat. #S7101). Ki67 and β-catenin staining in Wls^{flox} samples were performed as described (Zhong et al., 2012). Stromal cells cultured for 6 days were then cultured on glass coverslips for 2 days, fixed in 2% PFA in PBS for 15 min and permeabilized with 0.2% Triton X-100 for 10 min. Thereafter, samples were washed once in PBS followed by the staining procedures described above using primary antibodies to Vimentin (Cell Signaling, Cat. #5741), Desmin (Cell Signaling, Cat. #5332) and Smooth muscle actin (α-SMA; Abcam, Cat. #ab-7817) diluted 1:100 in PBS containing 1% BSA. Secondary ant-rabbit and anti-mouse antibodies (Invitrogen Alexa Fluor 594 goat anti-rabbit and mouse (Cat. #A1102, #A11005) were diluted 1:500. After staining, samples were mounted in Vectashield medium containing DAPI and analyzed using a LSM710 Carl Zeiss confocal microscope.

Cell proliferation assay

Cell proliferation *in vivo* was assessed by EdU incorporation. EdU and Click-iT EdU Alexa Fluor 555 Imaging Kit, were purchased from Life Technologies (Cat. #A10044 and #C10338, respectively). Two hours before sacrifice mice were injected with 0.5 mg EdU in 150 µl PBS (~16.66 mg/kg). Incorporated EdU was visualized following the manufacturer's instructions and mounted in fluorescent mounting media with DAPI (VectaShield, Cat. #H-1200). When EdU staining was performed on OCT embedded samples, incubation time for the Click iT reaction cocktail was reduced to 1 min.

Microarray

Epithelial (crypt cells) and stromal cells from $Porcn^{Del}$/*Villin-Cre* and $Porcn^{WT}$/*Villin-Cre* mice were harvested as described in the Materials and Methods in the supplementary material. RNA was isolated using the RNeasy purification kit from Qiagen. Labeled cRNA was prepared and hybridized to MouseWG-6 v2.0 Expression BeadChip Kit (Illumina) according to the manufacturer's protocols. The gene expression data were extracted by GenomeStudio (v1.7.0) software. After normalization by median centering, significance analysis of microarrays (SAM) with less than 10% false discovery rate (FDR) was used to compare the samples. Signaling pathways are downloaded from Molecular Signatures database (MsigDB 2.5, http://www.broadinstitute.org/gsea/msigdb/). The genes in each pathway were centered using Cluster software and their heatmaps were generated with TreeView software. Microarray data are available at Gene Expression Omnibus with accession number GSE56911.

Statistical analysis

Data were analyzed using Prism 5 software and Excel. A two-tailed *t*-test was performed in Excel for Mac 2011 version 14.3.2.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Z.K. and G.G.: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript. B.M.: analysis and interpretation of data and manuscript. S.B. and J.R.: developed key reagent, analysis of data and manuscript. Z.Z. and B.O.W.: study design, acquisition and analysis of data. E., H.Z. and J.A.: acquisition of data, technical support. Y.W.: analysis of microarray data. R.B.: mouse pathology analysis. D.M.V.: analysis and interpretation of data, drafting manuscript, study supervision.

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Supplementary material

Supplementary material available online at
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