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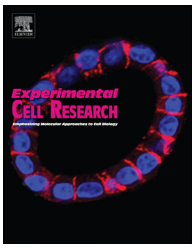

Research Article

Modulation of stemness in a human normal intestinal epithelial crypt cell line by activation of the WNT signaling pathway

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ABSTRACT

The small intestine consists of two histological compartments composed of the crypts and the villi. The function of the adult small intestinal epithelium is mediated by four different types of mature cells: enterocytes, goblet, enteroendocrine and Paneth. Undifferentiated cells reside in the crypts and produce these four types of mature cells. The niche-related Wnt and Bmp signaling pathways have been suggested to be involved in the regulation and maintenance of the stem cell microenvironment. In our laboratory, we isolated the first normal human intestinal epithelial crypt (HIEC) cell model from the human fetal intestine and in this study we investigated the expression of a panel of intestinal stem cell markers in HIEC cells under normal culture parameters as well as under conditions that mimic the stem cell microenvironment. The results showed that short term stimulation of HIEC cells with R-spondin 1 and Wnt-3a ± SB-216763, a glycogen synthase kinase 3β (GSK3β) inhibitor, induced β-catenin/TCF activity and expression of the WNT target genes, cyclin D2 and LGR5. Treatment of HIEC cells with noggin, an antagonist of BMP signaling, abolished SMAD2/5/8 phosphorylation. Inducing a switch from inactive WNT/active BMP toward active WNT/inactive BMP pathways was sufficient to trigger a robust intestinal primordial stem-like cell signature with predominant LGR5, PHLDA1, PROM1, SMOC2 and OLFM4 expression. These findings demonstrate that even fully established cultures of intestinal cells can be prompted toward a CBC stem cell-like phenotype. This model should be useful for studying the regulation of human intestinal stem cell self-renewal and differentiation.

Abbreviations: BMP, bone morphogenetic protein; BMPR, BMP receptor; BrdU, bromodeoxyuridine; CBC, crypt-base columnar; GSK3β, glycogen synthase kinase 3β; HIEC, human intestinal epithelial crypt; LGR5, leucine-rich-repeat-containing G-protein-coupled receptor-5; OLFM4, olfactomedin-4; PHLDA1, pleckstrin homology-like domain family member-1; qPCR, quantitative polymerase chain reaction; SMOC2, secreted modular calcium-binding protein-2; TCF4, T-cell factor-4

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Introduction

The small intestinal epithelium is characterized by rapid and constant renewal; this very dynamic system involves cell generation and migration from the crypt cell population located at the bottom of the crypt to extrusion of senescent cells at the tip of the villus [1–4]. The intestinal crypt is a highly hierarchized structure. Its lower third contains the slow growing stem cell population as well as Paneth cells [1,2,5–9]. The middle third contains the rapidly growing transit cells, which are committed toward secretory or absorptive lineages, while remaining poorly differentiated [1,9] being under the influence of repressive mechanisms [10,11]. Except for Paneth cell precursors which down-migrate to complete their differentiation in the crypt base, terminal differentiation of the other epithelial cell types occurs in the upper third of the crypt so that all cells reaching the base of the villi are fully functional [3,12–14].

Characterization of the crypt cell microenvironment [15] has provided key information on molecular components of the intestinal stem cell niche in which the Wnt and Bmp signaling pathways play a major role [2,4–6]. The Wnt pathway is a main regulator of cell proliferation. As reviewed recently [16], this signaling pathway is based on autocrine and paracrine interactions of secreted Wnts, a family of cysteine-rich glycoproteins, with their receptor Frizzled and co-receptor lipoprotein related protein. Activation of the canonical pathway leads to stabilization and nuclear translocation of β-catenin, which interacts with the T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors, inducing the expression of a series of downstream target genes such as c-Myc and cyclin D [5]. In the absence of binding of Wnt to its receptor, free cytosolic β-catenin is targeted to the destruction complex, consisting of scaffolding proteins such as axin and adenomatous polyposis coli as well as kinases such as casein kinase1 and glycogen synthase kinase 3β (GSK3β), which phosphorylate β-catenin for its ubiquitination leading to proteosomal degradation [16]. Ligands responsible for canonical Wnt signaling, e.g. Wnt-3a, are expressed only by epithelial crypt cells [17] including Paneth cells [18]. Epithelial crypt cells also express the corresponding Wnt receptor frizzled and co-receptor Lrp as well as Wnt inhibitors such as Dkk1 [19]. Members of the R-spondin family of proteins have arisen as major regulators of Wnt signaling. R-spondins enhance Wnt signaling in the presence of canonical Wnt ligands [20]. Bmp signaling, on the other hand, is a negative regulator of crypt cell proliferation [21]. In the intestine, Bmps such as Bmp4 are mainly expressed by stromal cells although some appear to be of epithelial origin [21–23]. They bind type I and type II Bmp receptors in villus and crypt epithelial cells which leads to the phosphorylation of the Smads [21,22]. Once activated, Smads translocate to the nucleus where, in cooperation with other transcription factors, they activate the expression of Bmp-specific genes [22]. Interestingly, the potent Bmp antagonist, noggin, is expressed by intestinal myofibroblasts at the crypt base [22].

Over the years, two populations of stem cells have been recognized in the small intestine. The so-called crypt base columnar (CBC) cells that were identified between the Paneth cells at the bottom of the crypts by Cheng and Leblond four decades ago [1,24] and the DNA-label-retaining cells (LRC) identified just above the Paneth cells [7]. The identification of the CBC cell specific marker leucine-rich-repeat-containing G-protein-coupled receptor-5 (Lgr5), a Wnt downstream target, and its monitoring in lineage-tracing experiments provided evidence that CBC-Lgr5 positive cells represent the primordial stem cells [25]. Interestingly, recent studies have shown that LRCs are quite distinct from CBCs and appear to represent a quiescent reserve stem cell population that can regenerate the primordial stem cell population in case of injury [26–28]. Additional candidate markers for CBC cells have been identified through gene expression profiling of LGR5+ cells, with validation of OLFM4 (olfactomedin-4) in the small and large intestine [29]. Lineage-tracing studies of the transmembrane glycoprotein prominin-1 (PROM1) also known as CD133 have shown that a proportion of Prom1-positive cells located at the base of the intestinal crypts also express Lgr5 and have the potential to generate the entire intestinal epithelium, and thus appear to be CBC cells as well [30,31]. PHLDA1 (Pleckstrin homology-like domain family member 1) has also been identified as a putative epithelial stem cell marker in the human small and large intestine [32]. Finally, SMOC2 has recently been identified as a specific CBC stem cell marker [33].

Despite substantial advances in investigating stemness and cellular hierarchy in the mouse, limited progress has been made regarding the human intestinal crypt due to limited access to valid human intestinal cell models. In our laboratory, we isolated the first normal non-transformed non-immortalized human intestinal epithelial crypt (HIEC) cell model from the immature intestine [34]. The epithelial origin of the HIEC has been confirmed by identification of the intestinal keratins 8, 18, 19, and 20/21 [34]. These cells retain the ability to express the crypt cell-specific marker MIM-1/39, the integrins α6β4, α8β1 and α9β1 and an intestine specific truncated crypt-form of the integrin β4 subunit [13,34–37], as well as detectable levels of progenitor crypt cell markers such as Musashi-1, BMI1, DCAMKL1, EpCAM and CD44 [10,38]. Under standard culture conditions, HIEC cells hold their characteristics up to 30 passages. Analysis of the expression of intestinal functional markers has confirmed the undifferentiated nature of HIEC cells by demonstrating basal levels of expression of the brush border hydrolases aminopeptidase N and dipeptidylpeptidase IV, but not sucrase-isomaltase [34]. The lack of sucrase-isomaltase at both protein and transcript levels is indicative of the non-committed nature of these crypt-like cells [13,39,40]. HIEC cells have been proven useful in studying human crypt cell functions [13,40] such as proliferation [35,41], apoptosis [42–44], cell–matrix interactions [35,36,45], metabolism [46] and the inflammatory response [42,43]. Interestingly, ectopic expression of pro-differentiation transcription factors such as CDX2 and HNF-1α has been found to trigger the enterocytic differentiation program in HIEC cells including sucrase-isomaltase expression [38] while abolition of PRC2 epigenetic regulation further promoted terminal differentiation [10] indicating that cultured HIEC cells maintain the capacity to undertake an intestinal cell differentiation program.

In the present study, considering the undifferentiated/non-committed nature of HIEC cells and their expression of progenitor/stem cell markers such as Musashi-1, BMI1 and DCAMKL1 [38] as well as EpCAM and CD44 [10], we sought to evaluate their stemness potential. Our data revealed that HIEC cells exhibit, consistent with their low level of LGR5 expression [38], weak WNT/β-catenin activity. However, switching the activation statuses of the WNT/BMP pathways with a combination of Wnt-3a, R-spondin 1 and noggin triggered a conversion of HIEC cells toward a robust CBC-like cell signature including predominant LGR5, PHLDA1, PROM1, SMOC2 and OLFM4 expression.

Materials and methods

Cell culture

The human intestinal epithelial crypt-like (HIEC) cells were generated and grown as described previously [34,40]. HIEC cells exhibit all the morphological and functional characteristics of normal human proliferative crypt cells and are considered to be undifferentiated crypt-like progenitor cells [13,39,41,47].

The colorectal cancer cell lines Caco-2/15, HT-29, HCT-116 and SW480, obtained and cultured as previously described [47–50] were used as positive controls for luciferase assays and qPCR expression studies.

Antibodies, mediators and inhibitors

The mouse primary antibodies used in this study were anti-β-actin (Millipore, MAB1501) and anti-active-β-catenin (Millipore, 05–665). The rabbit primary antibodies used in this study were anti-LGR5 (Origene, TA301323), anti-cyclin D2 (Santa Cruz Biotechnology, sc-181), anti-PHLDA1/TDAG51 (Santa Cruz Biotechnology, sc-23866) and anti pSMAD2/5/8 (Cell Signaling, 9511). Secondary antibodies were AlexaFluor 488 or 594 goat anti-mouse (Molecular Probes, A11017, A11020) and AlexaFluor 488 or 594 goat-anti rabbit (Molecular Probes, A11070, A11072). The human recombinant molecules Wnt-3a (5 ng/ml; 5036-WN), R-spondin 1 (10 ng/ml; 4645-RS/CF) and noggin (100 ng/ml; 3344-NG) were purchased from R&D Systems and the GSK3β inhibitor, SB-216763, was from Sigma-Aldrich (20 μM, S3442).

Western blot analysis

Western blot analyses were performed on SDS-PAGE gels under denaturing conditions as previously described [41]. Total protein (50 μg/ml) preparations were separated on 12% or 15% gels and electrotransferred onto nitrocellulose membranes (BioRad, 162-0115). Nonspecific protein binding was blocked using 10% Blotto–0.1% Tween followed by incubation with primary antibodies diluted in the blocking solution, overnight at 4 °C. After washing with PBS, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse, NA931V and anti-rabbit, NA934V, Amersham) and developed using the Immobilon Western Kit (Millipore, WBKLS0100).

Transfection and luciferase assay measurement

Equal numbers of HIEC cells were seeded in 12-well plates (BD Biosciences, 353043) and grown to 40–60% confluence before being transiently transfected in serum-free medium using Effectene Transfection Reagent (Qiagen, 301425). Cells were treated with R-spondin 1/Wnt-3a ± SB-216763 for 48 h. All transfections were performed as co-transfections using a renilla luciferase expression plasmid to establish an internal control for transfection efficiency thus allowing for normalization of cell number and viability. The β-catenin/TCF4 responsive luciferase reporter plasmid used was TOPflash (Millipore, 21–170). Firefly and renilla luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, PRE1980) as described previously [51].

Cell proliferation and BrdU incorporation assays

HIEC cells were seeded in 35-mm dishes at 2×10^5 cells/dish in OptiMEM medium. A first 24 h resting period was allowed before starting the treatment. Twenty four and forty eight hours after treatment, three plates of each cell line were washed with PBS and counted with a BioRad TC10 Automated Cell Counter.

BrdU incorporation and staining was performed according to the manufacturer's (Roche, 11 810 740 001) instructions. Cells were prepared as above. At 20 and 44 h post-treatment, cells were incubated for 4 h with normal medium containing BrdU then immediately subjected to anti-BrdU and DAPI staining as described previously [37].

RNA extraction and quantitative polymerase chain reaction

RNA extraction, reverse transcription and quantitative polymerase chain reaction (qPCR) assays were performed as described previously [49]. Human LGR5 and RPLPO were evaluated by qPCR as previously described [38]. For Phlda1, the forward 5′-CCTCCAACTCTGCCTGAAAG-3′ and reverse 5′-TTCCTGCATCGTGATGAAAA-3′ primers, for SMOC2, the forward 5′–CAGCCGAAATGTGACAACAC-3′ and reverse 5′-GTTCTGAGAGCCTGCCTGAC-3′ primers, for PROM1, the forward 5′-TTTGGTGCAAATGTGGAAAA-3′ and reverse 5′-TTGAAGCTGTTCTGCAGGTG-3′ primers, for OLFM4, the forward 5′-CCAGCTGGAGGTGGAGATAA-3′ and reverse 5′-GCTGATGTTCACC ACACCAC-3′ primers and for cyclin D2, the forward 5′-TGGGGAAGTTGAAGTGGAAC-3′ and reverse 5′-ATCATCGAC GGTGGGTACAT-3′ primers were used. For quantitative RT-PCR, the threshold cycle values were converted into relative expression values compared with a QPCR Human Reference Total RNA standard (Stratagene, 750500) before normalization of gene expression against the normalizing gene RPLPO as described previously [49]. The annealing temperature of the reactions was 55 °C and the amplification efficiencies of the reactions were between 96% and 112% as determined by standard curve analysis.

Indirect immunofluorescence staining

Cells were seeded onto glass cover slips. Confluent cells (40–60%) were treated or not with the R-spondin 1/Wnt-3a/SB-216763/noggin cocktail for 48 h. Cells were fixed with MeOH for 20 min at −20 °C and processed as described previously [34] for the immunodetection of LGR5. Slides were viewed with a DMRXA microscope (Leica, Concord, ON) equipped for epifluorescence and digital imaging (RTE/CCD Y/Hz-1300 cooled camera). Images were acquired using MetaMorph software (Universal Imaging Corporation) under identical conditions and exposure time.

Statistical analysis

All experiments were performed in at least triplicate. Student's unpaired *t*-test and ANOVA using Bonferroni's Multiple Comparison Test were used to analyze the results. Data were considered to be significantly relevant at $p<0.05$ and are presented respectively as, mean ± SD and mean ± SEM. Statistical calculations were performed using Prism 3.0 software (GraphPad Software).

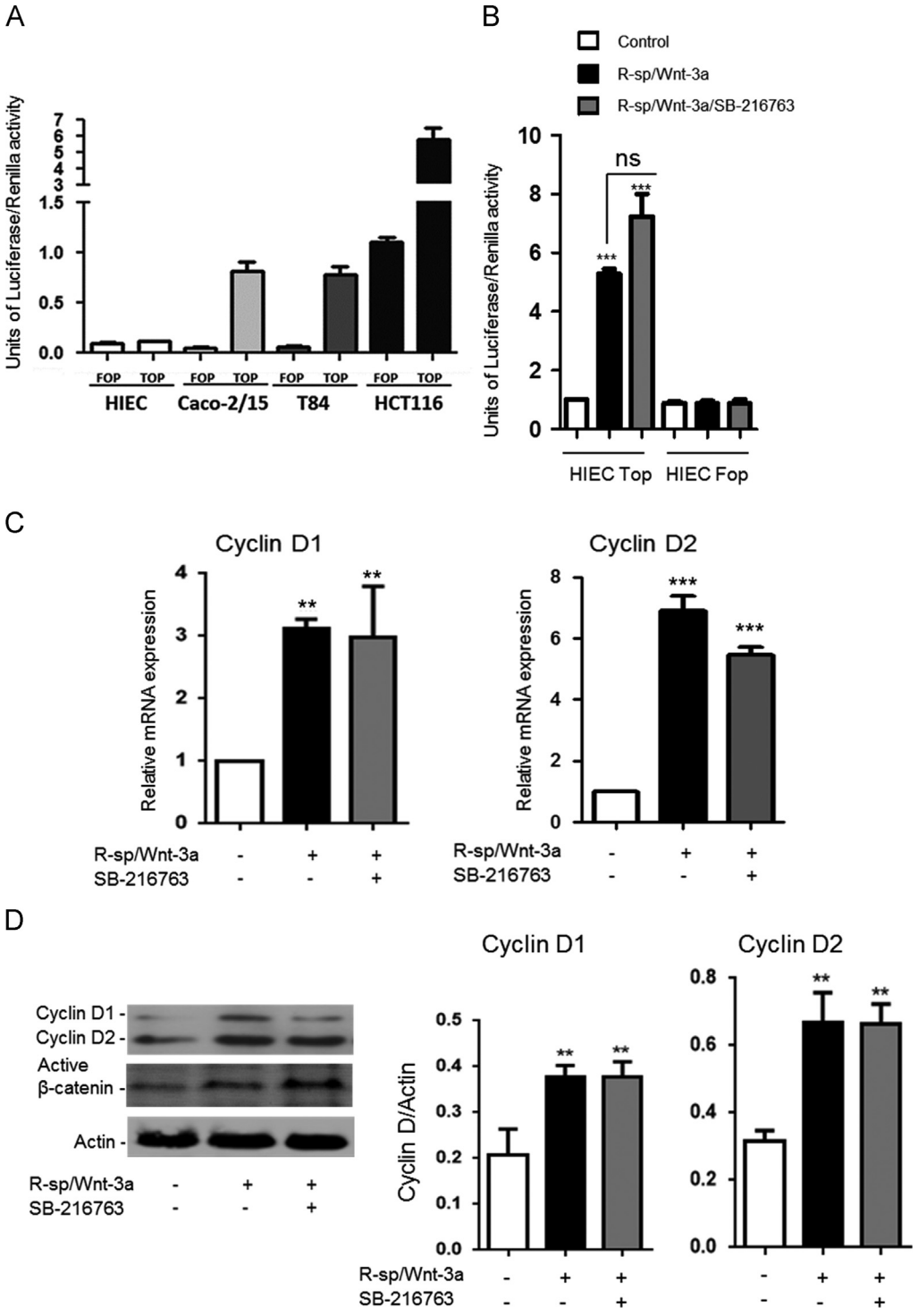

Fig. 1 – The WNT pathway is inactive but inducible in HIEC cells. (A) HIEC cells and CRC cell lines (Caco-2/15, T84, HCT-116) were transiently co-transfected with TOPflash or FOPflash plasmid and pRL-TK renilla luciferase plasmid DNA. Activity of the WNT/β-catenin signaling pathway was quantified by measuring relative firefly luciferase activity units (RLUs) normalized to renilla luciferase. (B) HIEC cells were transiently transfected with the β-catenin responsive luciferase plasmid, TOPflash or inactive FOPflash, and stimulated with R-spondin 1/Wnt-3a / SB-216763 for 48 h ($n = 3$, $^{***}p < 0.0001$). (C) Quantitative PCR of cyclin D1 and D2 expression in HIEC control and treated with R-spondin 1/Wnt-3a/SB-216763 for 48 h. Unstimulated HIEC cells were used as control. Results were normalized to the RPLP0 housekeeping gene ($n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$). (D) Representative western blot analysis of HIEC cell lysates for the detection of cyclin D1/D2, active β-catenin and actin in control and cells treated with R-spondin 1/Wnt-3a/SB-216763 for 48 h and corresponding densitometries for both cyclin D1 and D2 bands (mean ± SEM, $^{**}p \leq 0.01$, $n = 3$, one way ANOVA test).

Results and discussion

The WNT pathway is inactive but inducible in HIEC cells

Characterization of the crypt cell microenvironment has provided key information on molecular components of the intestinal stem cell niche [15]. Considering its central role on the regulation of the intestinal stem cell system, we first verified the status of activation of the WNT pathway in HIEC cell lines using the TOPflash/FOPflash luciferase assay to evaluate WNT downstream β-catenin/TCF transactivation activity. As positive controls for the normal HIEC cells, the human colorectal cancer cell lines Caco-2/15, T84 and HCT-116 were also tested. As expected [52], Caco-2/15 and T84 cells, both possessing a mutated APC, displayed moderate β-catenin activity while HCT-116 which has a mutated β-catenin that is constitutively active, displayed high β-catenin activity (Fig. 1A). In comparison with the colorectal cancer cell lines, β-catenin/TCF transactivation activity in HIEC was found to be very low (Fig. 1A).

To test whether the WNT pathway was nevertheless functional, HIEC cells were stimulated with activators of the WNT canonical pathway. We first tested R-spondin 1 and Wnt-3a. R spondin 1 alone (10 ng/ml) did not activate WNT signaling by itself (not shown), and its activity on HIEC cells was found to depend on the presence of Wnt-3a (5 ng/ml). Indeed in combination, a statistically significant 5 times increase in β-catenin/TCF activity was observed (Fig. 1B). The synergistic effect of R-spondin 1 and Wnt-3a on β-catenin/TCF activation has been previously described [20], a complex phenomenon that appears to involve the participation of specific R-spondin 1 receptors [53–55]. The effect of the selective GSK3β inhibitor SB-216763 was also investigated on normal human HIEC cells. This GSK3β inhibitor has been commonly used to mimic the action of Wnt molecules to activate β-catenin/TCF signaling [56]. Used alone, SB-216763 induced a significant increase in β-catenin/TCF transactivation activity (not shown) but its additive effect when used in combination with R-spondin 1 and Wnt-3a was found to be negligible (Fig. 1B).

As a classical downstream effector of WNT signaling [5], cyclin D expression was then evaluated to confirm the previous observations with TOP/FOPflash activity. Using qPCR and western blot analyses, a significant increase in both cyclin D1 and D2 expression was observed at both transcript (Fig. 1C) and protein levels (Fig. 1D). Co-treatment of R-spondin 1/Wnt-3a and SB-216763 also significantly increased active β-catenin levels (Fig. 1D). Taken together, these results indicate that the WNT canonical pathway is inactive in normal HIEC cells but remains fully functional.

The BMP pathway is active in HIEC cells

The BMP signaling pathway is a well characterized negative regulator of intestinal cell proliferation. BMPs can be secreted by both subepithelial stromal and epithelial cells [21–23] while the expression of noggin, an antagonist of BMP signaling, is expressed by intestinal myofibroblasts at the crypt base [21,22]. The BMP pathway appears to be active in normal HIEC cells as noted by the significant levels of phosphorylation of SMAD2/5/8 as evaluated by western blot (Fig. 2). Treatment of HIEC cells with 100 ng/ml of noggin for 48 h abolished SMAD2/5/8 phosphorylation (Fig. 2) confirming that BMP signaling can be efficiently inhibited in HIEC.

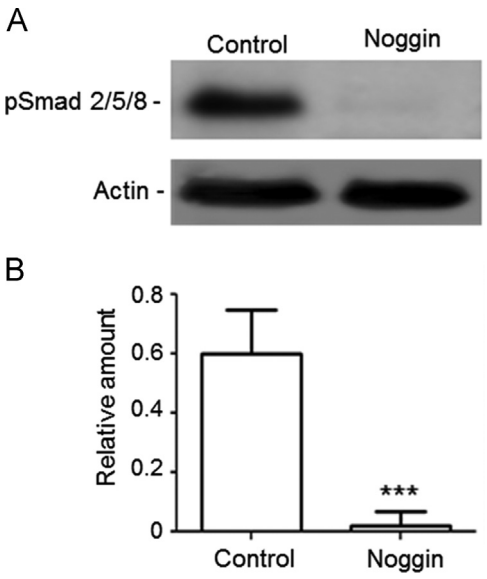

Fig. 2 – The BMP pathway is active in HIEC cells. (A) Representative western blot showing the expression levels of pSMAD2/5/8 protein in control and treated HIEC cells with the BMP pathway inhibitor, noggin, for 48 h. Actin was used as loading control. (B) Representative graph showing relative amounts of pSMAD2/5/8 determined by optical densitometry. Data were normalized with actin. Mean ± SEM, $^{***}p \leq 0.001$, $n = 3$, paired *t*-test.

Switching the activation status of the WNT/BMP pathways triggers conversion of crypt cells toward a CBC-like cell phenotype

Consistent with their typical non-CBC cell-like signature (inactive WNT/active BMP pathways) as shown above, wild-type HIEC cells have been previously shown to express low levels of the specific CBC stem cell marker LGR5 although other progenitor crypt cell markers such as Musashi-1, BMI1 and DCAMKL1 were detected [38]. To test the hypothesis that inducing a switch from inactive WNT/active BMP toward active WNT/inactive BMP may be sufficient to trigger a CBC-like cell signature, HIEC cells were treated with various combinations of R spondin 1/Wnt-3a, SB-216763 and noggin for 48 h and analyzed for the expression of CBC cell specific markers. As positive control for expression of stem cell markers, we used a pool of Caco-2/15, HT-29, HCT-116 and SW480 colorectal cancer (CRC) cell lines since recent studies have reported the presence of cancer stem cells within CRC cell line populations [57,58]. Untreated HIEC cells were used as control cells.

The CBC cell-specific markers LGR5 [25] and PHLDA1 [32] were both detectable in control cells at transcript and protein levels (Fig. 3) consistent with the fact that the detection of these markers in non-CBC crypt cells was recently reported in the intact intestine [29,33]. Stimulation of HIEC cells with R-spondin 1/Wnt-3a with or without SB-216763 as well as noggin alone had no significant effect on *LGR5* or *PHLDA1* expression while a sharp increase in the expression of the two transcripts was observed in the presence of R-spondin 1/Wnt-3a ± SB-216763 with noggin (Fig. 3A). Similarly at the protein level, a significant induction of

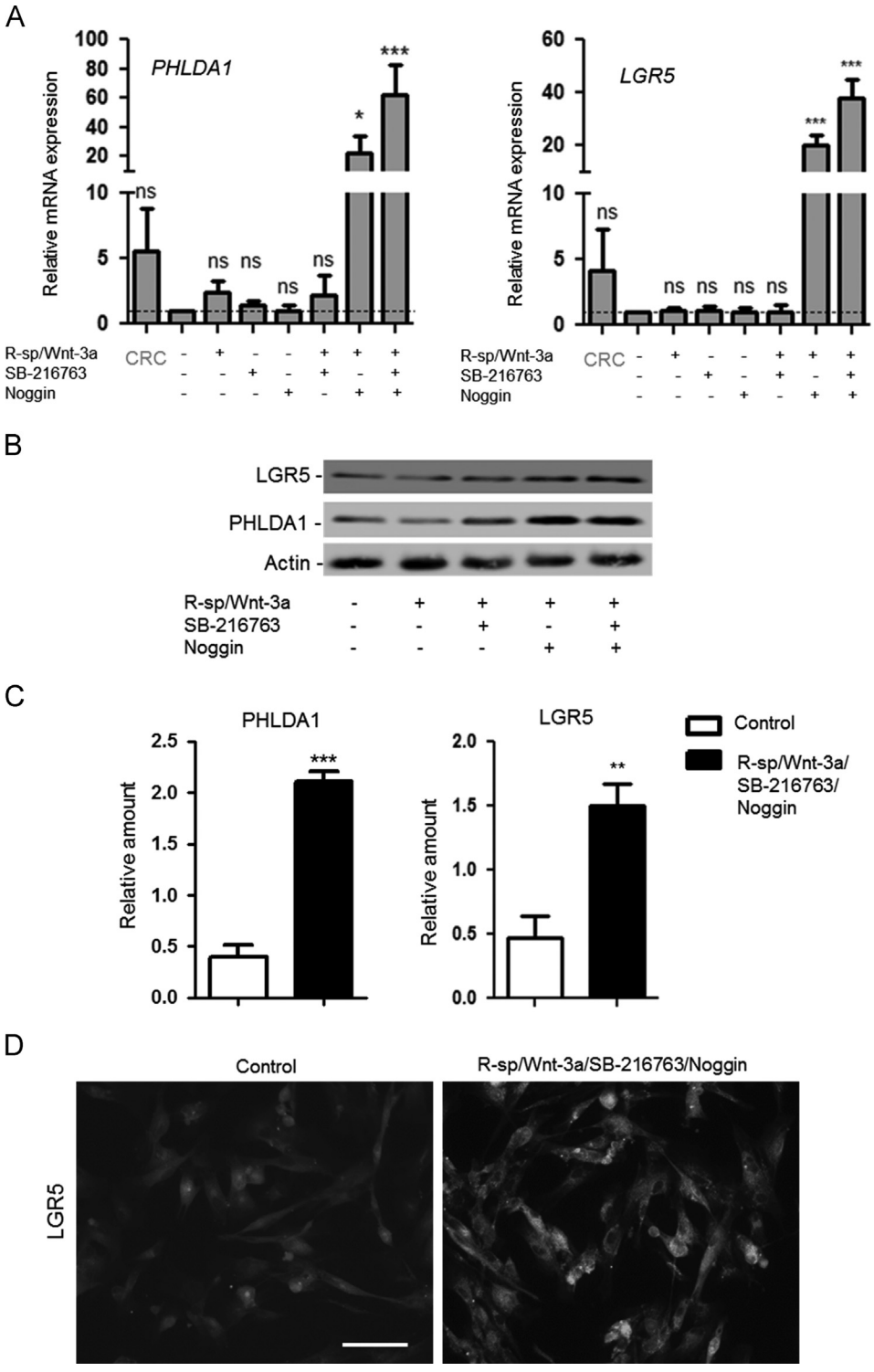

Fig. 3 – Altering the activation status of the BMP and WNT pathways affects the expression profile of the CBC cell markers PHLDA1 and LGR5. HIEC cells were treated with R-spondin 1/Wnt-3a ± SB-216763 with and without noggin for 48 h. Gene and protein expression were determined by quantitative PCR and western blotting, respectively. (A) Levels of mRNAs for *LGR5* and *PHLDA1* were analyzed by qPCR. The data are expressed relative to untreated control HIEC cells. A pool of CRC cell lines was used as positive control for expression of stem cell markers. Mean ± SEM, $^{***}p \leq 0.001$, $n = 3$, one way ANOVA test. (B) Representative western blot analyses of LGR5 and PHLDA1 expression in HIEC cells treated with R-spondin 1/Wnt-3a ± SB-216763 ± noggin. (C) Graph showing relative amounts of LGR5 and PHLDA1 as determined by optical densitometry of western blot membranes. Data were normalized relative to actin. Mean ± SEM, $^{***}p \leq 0.001$, $n = 3$, paired *t*-test. (D) Representative images of indirect immunofluorescence for the detection of LGR5 on control and treated HIEC cells with the R-spondin 1/Wnt-3a/SB-216763/noggin cocktail for 48 h. Scale bar = 50 μm.

LGR5 and PHLDA1 was observed after 48 h in the presence of the R-spondin 1/Wnt-3a, SB-216763 and noggin combination (Fig. 3B, C) confirming a good agreement between mRNA and protein induction even after only 48 h induction. The synergistic effect of these factors on LGR5 and PHLDA1 expression is consistent with the concept that CBC cells exhibit a WNT/on BMP/off signature [2,6]. Furthermore, immunodetection of the CBC cell marker LGR5 confirmed that the increased expression occurred in a majority of the cells treated with the R-spondin 1/Wnt-3a/SB-216763/noggin cocktail (Fig. 3D). Taken together, the current study represents a clear demonstration that the CBC-like cell phenotype requires both an active WNT pathway and an inhibited BMP pathway for its triggering.

Because LGR5 is a direct WNT-target gene [59] and PHLDA1 expression is also related to this pathway although not a direct WNT-target gene [32], we investigated the expression of the 3 additional CBC-like cell markers PROM1 [30,31], SMOC2 [33] and OLFM4 [29], including at least one, OLFM4, which is not under the control of WNT [60]. In control HIEC cells, *PROM1*, *SMOC2* and *OLFM4* expressions were detectable although at approximately 10% of the levels detected in the colorectal cancer cell pool (Fig. 4). Stimulation with the R-spondin 1/Wnt-3a+noggin ± SB-216763 combination resulted in a 15–40 times increase while no significant effect was observed in the presence of the factors tested individually (Fig. 4) confirming the synergistic effect of a dual WNT pathway activation/BMP pathway inhibition for CBC cell marker expression. The comparable pattern of OLFM4 expression with the other four CBC cell markers supports the occurrence of an overall conversion of HIEC cells to CBC-like cells.

It is noteworthy that the gene products of both *SMOC2* and *OLFM4* gene families have been reported to act as potential antagonists of the BMP signaling pathway [61,62] suggesting the existence of an autocrine way of inhibiting BMP signaling in the stem cell niche. In the context in which another specific CBC-cell marker, LGR5, can serve as a R-spondin 1 receptor, cooperative signaling appears to be a landmark of CBC cell homeostasis

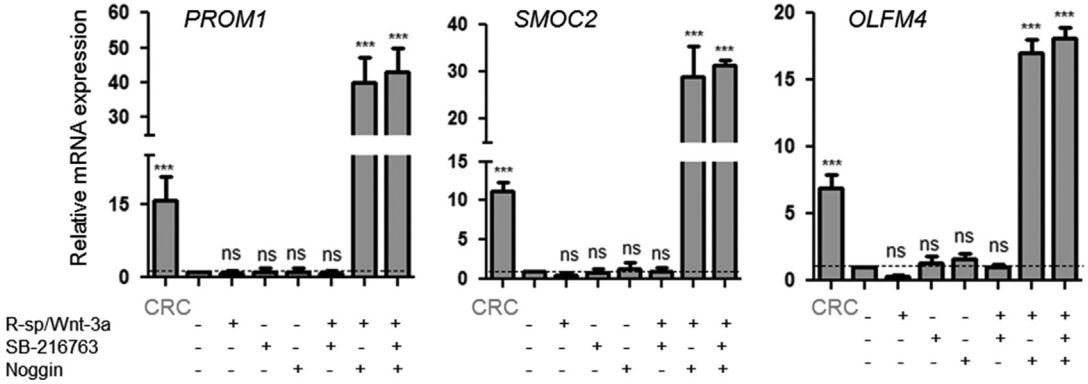

Fig. 4 – (A) Altering the activation status of the BMP and WNT pathways affects the expression profile of the CBC cell markers *PROM1*, *SMOC2* and *OLFM4*. HIEC cells were treated with R-spondin 1/Wnt-3a ± SB-216763 with and without noggin for 48 h. Levels of *PROM1*, *SMOC2* and *OLFM4* transcripts were evaluated by qPCR. The data were expressed relative to control untreated HIEC cells. A pool of CRC cell lines was used as positive control for expression of stem cell markers. Mean ± SEM, $^{***}p \leq 0.001$, $n = 3$, one way ANOVA test.

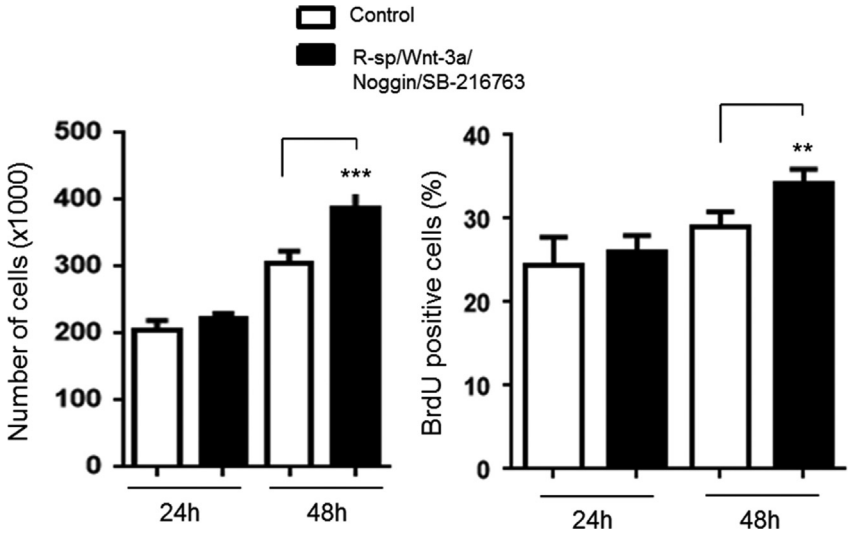

Fig. 5 – Activation of the WNT pathway does not affect cell-cycle progression. (A) Subconfluent HIEC cells untreated (control) or treated with a cocktail of R-spondin 1/Wnt-3a, SB-216763 and noggin were counted after 24 and 48 h. (B) Control and treated HIEC cell proliferation was also evaluated by assessing BrdU incorporation. Mean ± SEM, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $n = 3$, one way ANOVA test).

and reinforces the notion that Wnt signaling and intestinal stem cell biology are closely linked [54,55]. The lack of significant modulation of other crypt cell markers such as *SMOC1*, *DCKL1* and *MSI1* by HIEC cell stimulation with the R-spondin 1/Wnt-3a/SB-216763/noggin cocktail (data not shown) confirmed the specificity of WNT pathway activation/BMP pathway inhibition for the induction of the CBC cell-like phenotype.

Conversion of HIEC cells toward a CBC-like cell phenotype modulates cell-cycle progression

Considering that both the WNT and BMP pathways can regulate cell-cycle progression and considering our observations showing an increase in active β-catenin and cyclin D levels in R-spondin 1/Wnt-3a/SB-216763/noggin stimulated cells, we assessed the proliferation rate of HIEC control and treated cells by BrdU incorporation experiments. Treatment of HIEC cells with the R-spondin 1/Wnt-3a/SB-216763/noggin cocktail exerted a statistically significant effect on proliferation over a 48 h period (Fig. 5). HIEC cells are normal human proliferative/crypt stem cells, and it is known that in normal cells, the cell cycle is subject to very complex mechanisms of regulation to avoid excessive proliferation of cells. Interestingly, as mentioned above, even if cycling Lgr5+ stem cells at the bottom of the crypts are in a rich Wnt microenvironment, the average cycling time of CBC cells is relatively long (in the order of 24 h) in comparison with TA cells that cycle every 12 h under a Wnt-independent environment [6,9,14]. The data presented herein showing a statistically significant ~20% increase in cell proliferation after 48 h of treatment is consistent with the existence of an intrinsic mechanism of regulation. Although highly controlled in normal CBC cells, the deregulation of the Wnt/β-catenin signaling pathway by genetic alterations of β-catenin or APC results in a strong increase in the rate of intestinal epithelial cell proliferation leading to colorectal cancer [63]. Therefore, while Wnt/β-catenin signaling supports proliferation and self-renewal of CBC cells in the normal intestinal epithelium, its alteration can result in cancer formation [64].

Conclusions

In cellulo models should allow for a more direct approach to the investigation of cellular and molecular mechanisms controlling stem cell maintenance and deregulation, which are difficult aspects to approach in vivo [1,12,65–69]. Recent efforts in this direction have shown that single LGR5 positive cells can grow in vitro in a laminin-containing substrate supplemented with EGF, R-spondin 1 and the BMP inhibitor noggin [70]. Differentiation of pluripotent stem cells into intestinal tissue in vitro using laminin gel, Wnt-3a and FGF4 has also recently been achieved [71]. Efforts have also targeted the isolation of intestinal stem cell populations [72–74]. In this context, the present finding demonstrating that fully established cultures of normal human intestinal epithelial crypt cells can be prompted toward a CBC stem cell-like phenotype as evaluated by LGR5, PHLDA1, PROM1, SMOC2 and OLFM4 expression and maintenance of cell proliferation dynamics is very promising as a relatively straightforward tool to further investigate human intestinal crypt cell self-renewal and differentiation.

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