Molecules and Cells

Indole-3-Carbinol Promotes Goblet-Cell Differentiation Regulating Wnt and Notch Signaling Pathways AhR-Dependently

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http://dx.doi.org/10.14348/molcells.2018.2167
www.molcells.org

Using an *in vitro* model of intestinal organoids derived from intestinal crypts, we examined effects of indole-3-carbinol (I3C), a phytochemical that has anticancer and aryl hydrocarbon receptor (AhR)-activating abilities and thus is sold as a dietary supplement, on the development of intestinal organoids and investigated the underlying mechanisms. I3C inhibited the *in vitro* development of mouse intestinal organoids. Addition of α-naphthoflavone, an AhR antagonist or AhR siRNA transfection, suppressed I3C function, suggesting that I3C-mediated interference with organoid development is AhR-dependent. I3C increased the expression of Muc2 and lysozyme, lineage-specific genes for goblet cells and Paneth cells, respectively, but inhibits the expression of IAP, a marker gene for enterocytes. In the intestines of mice treated with I3C, the number of goblet cells was reduced, but the number of Paneth cells and the depth and length of crypts and villi were not changed. I3C increased the level of active nonphosphorylated β-catenin, but suppressed the Notch signal. As a result, expression of Hes1, a Notch target gene and a transcriptional repressor that plays a key role in enterocyte differentiation, was reduced, whereas expression of Math1, involved in the differentiation of secretory lineages, was increased. These results provide direct evidence for the role of AhR in the regulation of the development of intestinal stem cells and indicate that such regulation is likely mediated by regulation of Wnt and Notch signals.

Keywords: AhR, goblet, intestinal organoid, I3C, Notch

INTRODUCTION

The intestinal epithelium plays a central role in immune homeostasis in the intestine, not only by acting as a physical barrier to commensal bacteria and foreign antigens, but also by being actively involved in antigen capture and processing and regulation of various immune cells (Peterson and Artis, 2014). The epithelial monolayer is folded into many villous protrusions into the lumen and crypts of Lieberkühn, invasions into the underlying connective tissue. The crypts are an important site of intestinal epithelial-cell (IEC) differentiation and are where the four IEC types (goblet, enteroendocrine, absorptive, and Paneth cells) are derived from $Lgr5^+$ intestinal stem cells (ISC) (Barker et al., 2007; van der Flier and Clevers, 2009). The mouse intestinal epithelium constantly undergoes homeostatic renewal every 4-5 days as it endures continual mechanical and chemical stress (Cheng and Leblond, 1974), making it one of the favorite tissues in which to study stem-cell maintenance and differentiation. ISCs residing in the bottom of the crypts can differentiate into the four types of IEC by complex interactions among four signaling pathways (Wnt, Notch, bone morphogenic proteins (BMP) and Hedgehog) (Medema and Vermeulen, 2011).

Received 11 August, 2017; revised 18 December, 2017; accepted 8 January, 2018; published online 21 March, 2018

eISSN: 0219-1032

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the basic region-helix-loop-helix (bHLH) superfamily of DNA binding proteins (Burbach et al., 1992). Some structurally diverse low-molecular-weight chemicals have been identified as naturally occurring exogenous and endogenous AhR ligands (Denison and Nagy, 2003; Nguyen and Bradfield, 2008). In addition to its role in detoxification of xenobiotics, AhR is intimately involved in diverse physiological processes (Barouki et al., 2007). In mice and humans, AhR is expressed in several organs, including the lung, the heart, the liver, the thymus, and the kidney (Abbott et al., 1995).

The intestines of mice express AhR (Chmill et al., 2010). The intestines can be exposed to various AhR ligands by foods contaminated with toxic chemicals, phytochemicals including indole-3-carbinol (I3C), and metabolites produced by commensal bacteria, such as indole-3-acetic acid (Celloto et al., 2012; Perdew and Babbs, 1991; Yaktine et al., 2006). AhR is involved in maintaining intraepithelial lymphocytes (IEL) and innate lymphoid cells (ILC) (Li et al., 2011; Qiu et al., 2012) and regulates organogenesis of intestinal lymphoid follicles (Kiss et al., 2011). We previously reported that AhR activation by 6-formylindolo[3,2-b]carbazole (FICZ) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inhibits the *in vitro* differentiation of mouse intestinal epithelial cells (Park et al., 2016). However, which cell type of IEC is affected by AhR and how AhR works remain unknown. In addition, although AhR is activated by several different ligands, giving rise to similar effects on diverse physiological activities, accumulating evidence suggests that AhR ligands may act differentially, giving rise to different effects (Hammerschmidt-Kamper et al., 2017; Quintana et al., 2008; Veldhoen et al., 2008).

Indole-3-carbinol (I3C), which is a breakdown product of glucobrassicin (3-indolylmethyl glucosinolate), a sulfur-containing compound that is rich in cruciferous vegetables such as broccoli and cabbage, can be converted into 3,3'-diindolylmethane (DIM) and indole[3,2-b]carbazole (ICZ) in the acidic environment of the stomach (Bjeldanes et al., 1991). I3C and DIM have gained considerable attention because of their anticancer properties, which are attributable to its ability to target signaling pathways governing apoptosis and cell cycle progression (Ahmad et al., 2010; Maruthanila et al., 2014; Weng et al., 2008). I3C and DIM are ligands for AhR (Bjeldanes et al., 1991). In mice fed with defined diets nearly free of phytochemicals, such as polyphenols and glucosinolates, I3C supplementation induced AhR activity within RORγt+ innate lymphoid cells (ILC) and intraepithelial lymphocytes (IEL), promoting organogenesis of intestinal lymphoid follicles and helping to maintain IELs (Kiss et al., 2011; Li et al., 2011). In addition, certain plant-derived AhR ligands such as I3C are sold as dietary supplements in an uncontrolled market.

The aim of this study was to find out if diet-supplement I3C can affect the development of mouse intestinal organoids *in vitro* and how it works. We observed that I3C inhibits intestinal organoid development in an AhR-dependent manner. I3C upregulated the expression of Muc2 and lysozyme but inhibited the expression of IAP. In the intestines of mice treated with I3C, the number of goblet cells was increased. I3C increased the level of active nonphosphorylated β-catenin but suppressed the Notch signal. In addition, expression of Hes1 and Math1, which are involved in lineage determination of IEC, was differently regulated: Hes1 was downregulated, but Math1 expression was upregulated by I3C. Thus, we provide evidence that I3C promoted the differentiation of goblet cells, regulating Wnt and Notch signals and expression of their downstream target genes, Hes1 and Math1.

MATERIALS AND METHODS

Mice

C57BL/6 female mice, 6-12 weeks of age, were purchased from the Korean Institute for Chemistry (Korea) and acclimatized for two weeks before use in experiments. The animals were housed, 5 mice per cage, in a laminar air-flow room maintained at 22 ± 2℃ with a relative humidity of 55 ± 5%. Mice were cared for and treated in accordance with the guidelines established by the Changwon National University public health service policy on the use of laboratory animals. The animal study was performed in the immunology laboratory, Department of Biology, Changwon National University.

Chemicals and reagents

Matrigel was from Corning Life Sciences (USA). Jaggged-1 peptide, murine noggin, and R-Spondin1 were purchased from AnaSpec (USA), Peprotech (USA), and ACROBiosystems (USA), respectively. EGF, TrypLE express, and N2 and B-27 supplements were from Life Technologies (USA). I3C, a periodic acid-Schiff kit, *N*-acetylcysteine, and Harris' Hematoxylin were from Sigma-Aldrich (USA). Anti-activated Notch1 Ab was from Abcam (USA) and anti-non-phospho (active) β-catenin (Ser33/37/Thr41) Ab and anti-β-actin Ab were from Cell Signaling Technologies (USA).

Intestinal organoid development in vitro

In vitro intestinal organoid development from crypts and $Lgr5^+$ stem cells was previously described (Park et al., 2016). Briefly, crypts isolated from the mouse small intestine were resuspended with Matrigel™ (250 crypts/50 μl/well) and applied into a 24-well plate. Then, 0.5 ml complete culture medium [DMEM/F12 medium supplemented with mouse EGF (50 ng/ml), mouse Noggin (100 ng/ml), human R-spondin1 (500 ng/ml), N2 and B-27 supplements, and 1 mM *N*-acetylcysteine] was added to the solidified Matrigel™. Culture medium was changed every 4 days. For *in vitro* intestinal organoid development from $Lgr5^+$ stem cells, mouse crypts were resuspended in single-cell dissociation medium consisting of 3 ml DMEM/F12 medium with N2 and B-27 supplements, 1 mM *N*-acetylcysteine, 1 ml TrypLE express, and DNase I (2,000 units/ml), and were incubated in a 37℃ water bath with intermittent pipetting every 8 min for 30 min. Then, crypts were mechanically pipetted with a fire-polished Pasteur pipette 10 times. The sample was passed through a 40-μm cell strainer, followed by a 20-μm cell strainer. Cells containing $Lgr5^+$ cells were resuspended with Matrigel™ (200 cells/25 μl) with 1 μM Jagged-1 peptide and

applied into a 48-well plate. Then, 0.25 ml of single-cell culture medium [DMEM/F12 medium supplemented with mouse EGF (50 ng/ml), mouse Noggin (100 ng/ml), human R-spondin1 (500 ng/ml), 10 μM Y-27632, 1 μM Jagged-1 peptide, N2 and B-27 supplements, and 1 mM *N*-acetylcysteine] was added to the plate. EGF, R-spondin 1, and Noggin were added to the culture every 2 days and the culture medium was changed every 4 days.

siRNA transfection

Crypt cells containing Lgr5+ stem cells were electroporated using the Cell Line Nucleofector Kit V and Nucleofector II/2b device from Amaxa (Lonza, USA) according to the manufacturer's protocol. In brief, 1 x 10^3 crypt cells were resuspended in 100 μl of Nucleofector solution and 5 μl of siRNA against mouse AhR (Santa Cruz Biotechnology, USA, catalogue number sc-29655) or control siRNA (sc-37007) was added to the cell suspension at a final concentration of 0.2 μM. Cell suspension was transferred into a cuvette and electroporated with program U-031. After electroporation, 0.5 ml of single-cell culture medium [DMEM/F12 medium supplemented with mouse EGF (50 ng/ml), mouse Noggin (100 ng/ml), human R-spondin1 (500 ng/ml), 10 μM Y-27632, 1 μM Jagged-1 peptide, N2 and B-27 supplements, and 1 mM N-acetylcysteine] was added to the cuvette, cultured for 24 h, and assayed for RNA analyses.

Administration of AhR ligands

I3C dissolved in DMSO was administered to 6-week-old female mice (on average, weighing 20 g) after being mixed with 140 μl corn oil (2 mg of I3C per mouse per injection or 100 mg/kg body weight/injection, 5 mice per group) by gavage five times on days 1, 3, 5, 7, and 9; the mice were sacrificed on day 10 for tissue fixation and RNA analysis. For control, 10 μl DMSO or 10 μl PBS in 140 μl corn oil was administered to mice as for I3C. For *in vitro* experiments, AhR ligands (0.1-1 μM; for vector control 0.1% DMSO v/v) were added 1.5 h before the addition of growth and differentiation factors.

Histochemical analyses of the intestine

Intestinal tissues dissected from mice were fixed overnight in 4% PBS-buffered formalin, paraffin-embedded, sectioned at 5 μm, and stained with Hematoxylin and Eosin. Paneth cells and goblet cells are microscopically identified by large eosinophilic refractile granules and the "empty" roundish appearance caused by their being poorly stained cytoplasm, respectively. For comparison, sections were also stained with a Periodic acid-Schiff (PAS) staining kit for goblet-cell analyses. Paneth cells in the small intestine were counted in three adjacent crypts in ten discrete loci, and the number of Paneth cells was presented as an average number of Paneth cells per the three crypts. Goblet cells in the small intestine and the colon were counted in three adjacent villi and crypts, respectively, in ten discrete loci, and the number of goblet cells was presented as an average number of goblet cells per the three villi or crypts. Depth of crypts and height of villi were measured in three adjacent crypts or villi from 10 discrete loci and presented as the average depth of crypts or average height of villi.

RNA preparation and RT-PCR

Total cellular RNA was extracted from cells using the RNAzol method (TEL-TEST, INC., USA). For PCR analysis, RNA was used after contaminating DNA was completely removed by DNase I treatment. RT-PCR analysis was performed using pairs of oligonucleotide primers. The PCR products were confirmed to correspond to their original sequence by DNA sequencing. Gene-specific primers, the number of cycles of amplification, annealing temperature, and expected size of the PCR product are listed in Table 1.

Western blotting

Cells or tissues were homogenized in lysis buffer containing 20 mM Tris-HCl (*p*H 7.4), 1 mM EDTA (*p*H 8.0), 50 μM sodium vanadate, 20 mM *p*-nitrophenylphosphate, 50 mM sodium fluoride, leupeptin (0.5 μg/ml), aprotinin (10 μg/ml), and soybean trypsin inhibitor (10 μg/ml). Proteins size-fractionated on SDS/PAGE were transferred to polyvinylidene difluoride (PVDF) membranes, and the blots were blocked with 3% bovine serum albumin in TBS buffer (20 mM Tris-HCl, *p*H 7.5/137 mM NaCl). The blots were sequentially treated with primary and secondary antibodies in TBST [20 mM Tris-HCl (*p*H 7.5)/137 mM NaCl/0.1% Tween 20] with intermittent washing with TBST. Immunodetection was performed with the EzWestLumi plus kit (ATTO, Japan). Densitometer analysis was performed using the Image Master 2-D Platinum software (Amersham Biosciences, Piscataway, USA) according to the protocols provided by the manufacturer.

Cell proliferation assay

The CellTiter96^{R}AQ One Solution Cell Proliferation Assay System (Promega, USA) was used to assay cell proliferation. Crypts isolated from the mouse small intestine were resuspended with MatrigelTM (50 crypts/10 μl/well) and applied into a 96-well plate. Then, 0.1 ml complete culture medium was added to the solidified MatrigelTM. After 4 days of incubation, 20 μl of CellTiter96^{R}AQ One Solution was added to each well, and the plates were incubated for 4 h at 37℃. The optical density was measured at 490 nm using a 96-well plate reader.

Cell viability assay

Cell viability was measured using a quantitative colorimetric MTT assay. Crypts isolated from the mouse small intestine were resuspended with MatrigelTM (50 crypts/10 μl/well) and applied into a 96-well plate. Then, 0.1 ml complete culture medium was added to the solidified MatrigelTM. After 4 days of incubation, 10 μl of MTT solution (5 mg/ml) was added to each well, and the plates were incubated for 2 h at 37℃. After removal of culture medium, 20 μl of 2% SDS solution was added to each well and incubated at 37℃ for 2 h to dissolve the Matrigel. Finally, 100 μl of DMSO was placed in each well and incubated at 37℃ for 1 h to dissolve the internalized purple formazan crystals. The optical density was measured at 562 nm using a 96-well plate reader.

Table 1. Primers used in RT- PCR

Name	Nucleotide sequence	Annealing temperature (℃)	Cycles[a]	Size[b] (bp)
mumuc2fw	5'-ggcaaccagaccacatgtga-3'	58	35	949
mumuc2rv	5'-ggcacatgggtacaggagat-3'			
mulyzfw	5'-ctaccgtggtgtcaagctgg-3'	58	30	569
mulyzrv	5'-ggcaatcactgtgttggctg-3'			
mualpifw	5'-gcatgctcagcaggaatcca-3'	58	30	510
mualpirv	5'-agcctgcgaatgccatgacg-3'			
muchgafw	5'-atgccaagcacagagacgca-3'	58	35	683
muchgarv	5'-ccatcctgctccatcgcttg-3'			
muhgprtfw	5'-gctggtgaaaaggacctctc-3'	60	30	248
muhgprtrv	5'-caggactagaacacctgc-3'			
muhes1fw	5'-gcctatcatggagaagaggc-3'	58	35	688
muhes1rv	5'-agccactggaaggtgacact-3'			
mumath1fw	5'-tggctgactcccactttgca-3'	58	35	819
mumath1rv	5'-cacttctgtgggatctggga-3'			
mutcf4fw	5'-ttcagccagcactgccgact-3'	58	35	888
mutcf4rv	5'-agttgcagactggaccggaa-3'			
mulrp6fw	5'-gtagttggaggcttggagga-3'	58	35	849
mulrp6rv	5'-cagtggcaccatctttgcag-3'			
mulgr5fw	5'-gcctctgcttcctagaagag-3'	58	35	560
mulgr5rv	5'-gctccggtattgacctgatg-3'			
muwnt3fw	5'-tgcgcttctgccgcaattac-3'	58	30	542
muwnt3rv	5'-gtgcatgtggtccaggatgg-3'			
mudelta4fw	5'-aaccctctgcagttgcccctt-3'	58	35	409
mudelta4rv	5'-tctggcttgctgcagtaacc-3'			
munotch1fw	5'-ccttctactgcgaatgtccg-3'	58	35	549
munotch1rv	5'-ttcttgcatggtgtgctggc-3'			
mucyp1a1fw	5'-caccatcccccacagcac-3'	58	35	125
mucyp1a1rv	5'-tcgtttgggtcaccccacag-3'			
muahrfw	5'-ggccaagagcttctttgatg-3'	59	35	305
muahrrv	5'-ctgggtttagagcccagtga-3'			

a: indicates the number of cycles of amplification
b: indicates the expected size of PCR products

Statistical analysis

All experiments were performed three to five times, and a representative experiment is shown. Data are presented as the mean ± SD and analyzed by the paired Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

I3C inhibits the development of intestinal organoids from crypts in an AhR-dependent way

As described earlier, I3C works as an AhR ligand in addition to its anti-oxidative and anticancer activities. We previously reported that AhR is expressed in crypts, particularly highly in $Lgr5^+$ stem cells, and that intestinal organoids consisting of a central lumen lined by villus-like epithelium and crypts started to form 3 days after incubation (Park et al., 2016; Fig. 1A). Thus, we investigated whether I3C regulated the development of intestinal organoids. On average, 20% of crypts grew into organoids (51 ± 5 organoids out of 250 crypts for PBS) 4 days after incubation. I3C concentration-dependently inhibited organoid development by ~22% and ~32% compared with PBS control (40 ± 4 and 35 ± 3 organoids for 0.1 μM and 1 μM I3C, respectively) (Fig. 1B). DMSO showed negligible effect. The effects of I3C on organoid development were remarkably suppressed by α-naphthoflavone, an AhR antagonist. Expression of the Cyp1A1 gene, a target gene of AhR, was induced by I3C (Fig. 1C). When α-naphthoflavone was added to the cell culture, Cyp1A1 expression was significantly reduced compared to I3C alone. To further clarify the role of AhR in the I3C-mediated inhibition

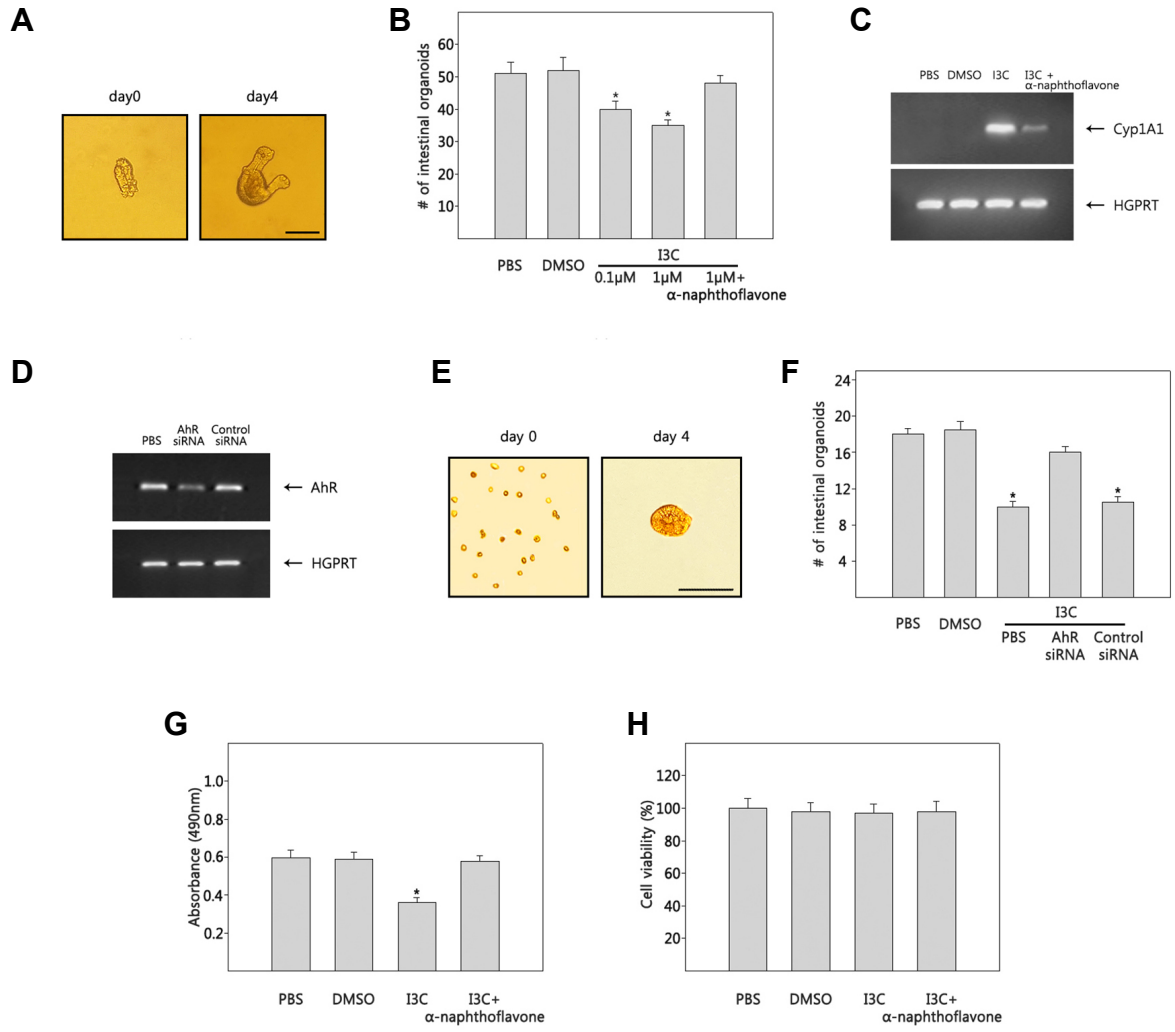

Fig. 1. I3C inhibits the development of intestinal organoids from crypts or Lgr5$^+$ stem cells AhR-dependently. Crypts, isolated from the small intestine, were resuspended with Matrigel and applied into a 24-well plate and then cultured in complete culture medium for 4 days. I3C (0.1-1 μM), α-naphthoflavone (1 μM), and DMSO (0.1% v/v) were added to the culture medium 1.5 h before the addition of growth and differentiation factors. Then, organoids were observed (A) and counted (B) under the inverted microscope, and assayed for RNA analysis (C), cell proliferation (G), or cell viability assay (H). For siRNA nucleofection, 1 x 10^3 crypt cells were suspended in 0.1 ml of Nucleofector solution and 5 μl of siRNA against mouse AhR or control siRNA and electroporated. Cells were cultured and harvested 24 h later and assayed for AhR expression (D). Crypt cells containing Lgr5$^+$ cells, which were nucleofected with siRNA, were mixed with Matrigel (200 cells/25 μl) with 1 μM Jagged-1 peptide, subjected to organoid development, and observed and counted under the inverted microscope (E, F). Results are presented as mean ± SD of 5 (B, G, and H) or 3 (F) independent experiments. Statistical significance was analyzed using the paired Student's *t*-test. * $p < 0.05$ compared with PBS control. Scale bar, 50 μm.

of organoid development, Lgr5$^+$ cells were separated from crypts, transfected with siRNA against mouse AhR, and subjected to organoid development. One day after culture, developing organoids were harvested and assayed for AhR expression. Because of the low frequency of Lgr5$^+$ stem cells (~5% of a crypt), we could not detect AhR signals in the Western blotting assays. The level of AhR mRNA was significantly reduced in the culture transfected with the AhR siRNA but not that with the control siRNA (Fig. 1D). Thus, we examined siRNA effects on organoid development. Four days after culture when developing organoids started to form (Fig. 1E), organoids were counted. The number of organoids was decreased by I3C (10.2 ± 2.5) compared with PBS or DMSO control (18.3 ± 2.7 or 18.6 ± 3.0, respectively). I3C effects were remarkably reduced when AhR siRNA (16.5 ± 2.7), but not control siRNA (10.2 ± 2.2), was transfected into stem cells (Fig. 1F). These results suggest that I3C inhibits organoid development AhR-dependently.

Next, we examined if I3C inhibits organoid development by interfering with proliferation of Lgr5+ stem cells and transit amplifying (TA) cells, two types of cells which actively proliferate in the developing crypts, or alternatively by de-

creasing cell viability. Cell proliferation was examined using the CellTiter96RAQ One Solution Cell Proliferation Assay System. The optical density was decreased by ~40% by I3C, suggesting that I3C might inhibit the formation of intestinal organoids, probably by inhibiting proliferation of stem cells and transit-amplifying cells. Considering the lower yield of organoids in cultures treated with 1 μM I3C (~32% compared with the PBS control), it appeared that I3C slightly inhibited proliferation of organoids. When cell viability was tested using the MTT assay, there was no significant difference between the samples tested (Fig. 1H).

I3C increases expression of Muc2 and lysozyme genes but inhibits expression of IAP genes

As described earlier, there are four types of IEC. It is possible that I3C regulates the development of intestinal organoids by working on certain types of IEC. Thus, we examined which type of IEC is affected during differentiation by I3C in two different but mutually complementary ways. First, intestinal organoids cultured for 4 days were analyzed for expression of lineage-specific genes: Muc2 for goblet cells, intestinal alkaline phosphatase (IAP) for enterocytes, lysozyme for Paneth cells, and chromogranin A (ChgA) for enteroendocrine cells. Muc2 expression, which was of a background level in PBS and DMSO control, was greatly increased by I3C (Fig. 2), whereas IAP expression was remarkably reduced by I3C (Fig. 2). Expression of lysozyme and ChgA was slightly increased (lysozyme) or not affected (ChgA) by I3C. Next, we examined the effect of I3C on the histology of the intestine. Adult mice were administered I3C or DMSO and sacrificed for histological examination on day 10. I3C-treatment

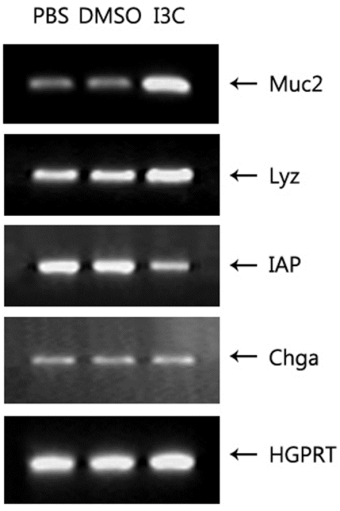

Fig. 2. I3C promotes the differentiation of goblet and Paneth cells but inhibits enterocyte differentiation. Crypts cultured in complete culture medium in the presence of I3C (1 μM) or DMSO (0.1% v/v) for 4 days were harvested and analyzed for expression of Muc2, lysozyme, IAP, and ChgA genes by RT-PCR. Results are representative of three separate experiments that showed similar results.

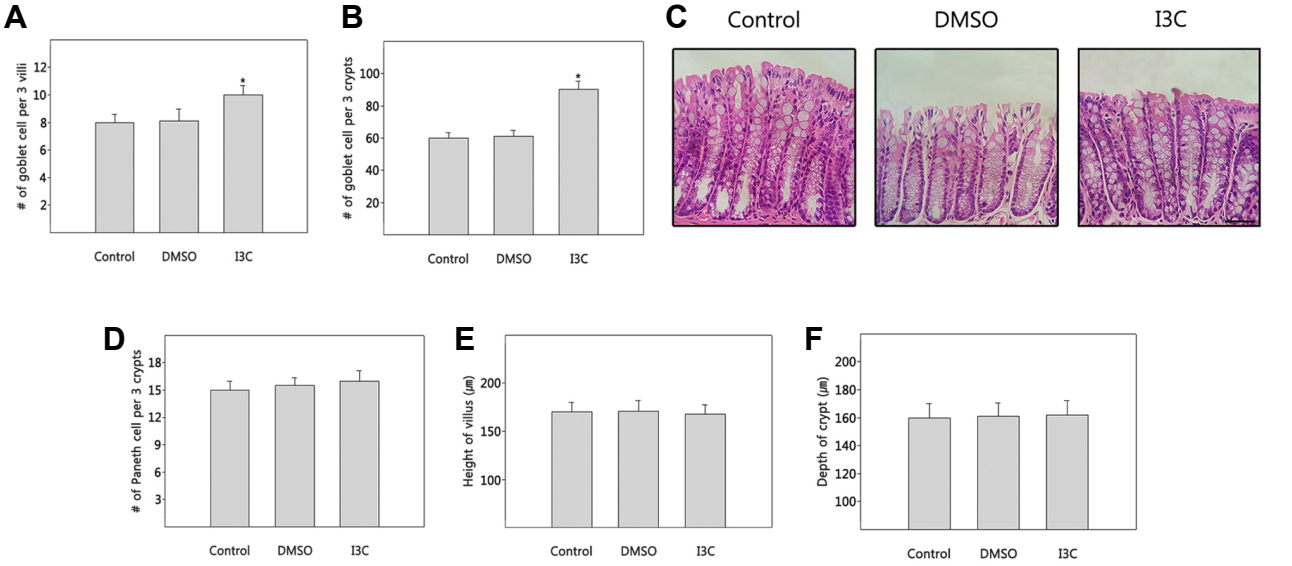

Fig. 3. The number of goblet cells in the intestine was increased in mice treated with I3C. Mice (8-week-old females weighing an average of 20 g) were administered with AhR ligands mixed with 140 μl corn oil (PBS control, 10 μl PBS; DMSO control, 10 μl DMSO; I3C, 2 mg in 10 μl) by gavage 5 times on days 1, 3, 5, 7, and 9 and sacrificed on day 10. Tissues were fixed in 4% formaldehyde, embedded in paraffin, and stained with Hematoxylin & Eosin. Paneth cells and goblet cells were counted in three adjacent crypts from ten discrete loci and the number of each cell type was presented as average number of each cell type per three crypts. Depth of crypts and length of villi were measured in three adjacent crypts and villi from ten discrete loci and presented as the average depth of crypts and length of villi, respectively. Results are presented as mean ± SD of data for five mice per group. (A, D, and E) for small intestine; (B, C, and F) for colon. Statistical significance was analyzed using the paired Student's *t*-test. * $p < 0.05$ compared with PBS control. Scale bar, 50 μm.

of mice produced no significant histological abnormalities except for remarkably increasing the frequency of goblet cells in the small intestine and colon (Figs. 3A and 3B). In the small intestine, the number of goblet cells per three villi was higher in I3C-treated mice (10 ± 1.3) than in PBS control (8.1 ± 1.5) and DMSO-treated mice (8.1 ± 1.5) (Fig. 3A). I3C-mediated increase in the frequency of goblet cells was more pronounced in the colon, where the number of goblet cells was significantly high (90 ± 8) compared to PBS (60 ± 5) or DMSO control (61 ± 6) (Figs. 3B and 3C). However, I3C showed little effect on the number of Paneth cells and the height of villi of the small intestine (Figs. 3D and 3E), or on the depth of crypts of the colon (Fig. 3F). These results appear to be somewhat different from the results reported in a previous study done with FICZ, where the number of Paneth cells and the depth of crypts in the small intestine and colon were decreased in FICZ-treated mice, but FICZ had little effect on the frequency of goblet cells in both intestines (Park et al., 2016).

I3C promotes Wnt3 signaling but inhibits Notch1 signaling

The Wnt3/β-catenin signaling pathway plays a key role in the proliferation of intestinal stem cells (Reya and Clevers, 2005). In the intestine, Notch has dual functions in the crypt: it directs proliferation of Lgr5^{+} stem cells in concert with Wnt (Fre et al., 2005) and induces enterocyte differentiation when Wnt signals are low (van Es et al., 2005a). Inhibition of the Notch pathway results in a massive increase in goblet cells, whereas its activation results in goblet-cell depletion (van Es et al., 2005b). Thus, we examined whether the Wnt and Notch pathways are modulated by I3C. Canonical Wnt signaling leads to stabilization of cytoplasmic β-catenin by inhibiting phosphorylation events that otherwise target the protein for proteosomal degradation (MacDonald et al., 2009). To see the effects of I3C on Wnt activity, we measured the level of nonphosphorylated (active) (Ser33/37/Thr41) β-catenin 4 days after the start of organoid development. The level of active β-catenin was increased by I3C to three times that in the PBS or DMSO control (Fig. 4A).

In mouse intestine, Notch1 and Notch2 are specifically expressed in crypt stem cells (Fre et al., 2011). The Notch pathway is activated by a cascade of proteolytic cleavages, resulting in the translocation of Notch intracytoplasmic domain (NICD) into the nucleus to induce target-gene transcription by interactions with other DNA binding proteins (Bray, 2006; Kopan and Ilagan, 2009). Thus, the level of NICD was measured 2 h after the addition of Jagged-1 peptide, a Notch ligand. The level of NICD was decreased by I3C to half that in the PBS or DMSO control (Fig. 4A). Next, we examined if I3C regulates expression of genes associated with Wnt3/β-catenin and Notch signals. Expression of low-density lipoprotein receptor protein 6 (LRP6) and Lgr5, which are involved in Wnt signaling, was increased by I3C, whereas expression of Wnt3, Delta, and Notch1 was not modulated by I3C (Fig. 4B). Whether the upregulated expression of Lgr5 by I3C results in the increase of stem cells remains to be tested.

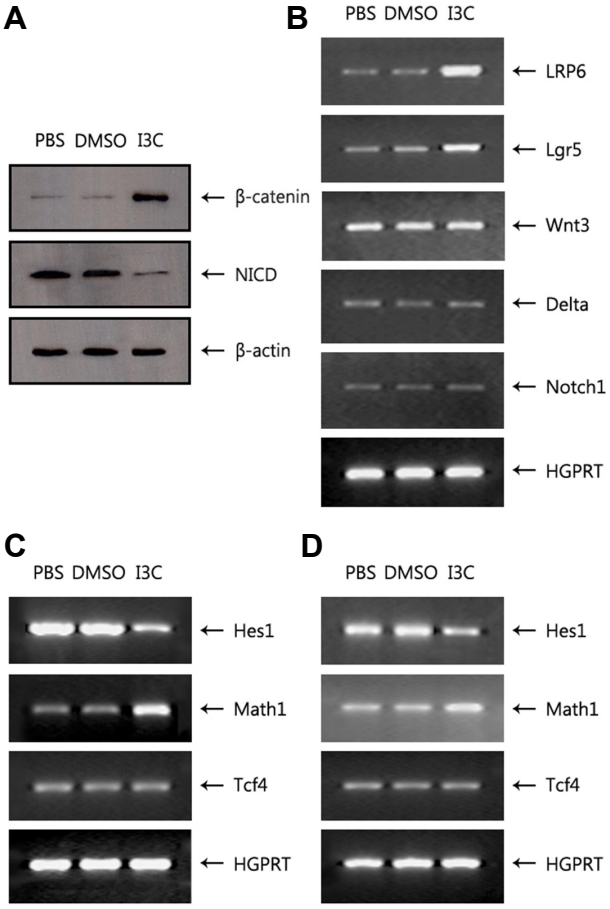

Fig. 4. I3C stimulates Wnt signaling but inhibits Notch signaling. Crypts cultured in complete culture medium in the presence of I3C (1 μM) or DMSO (0.1% v/v) for 4 days were harvested and analyzed for activation of Wnt and Notch signals by Western blotting (A) and for RNA expression of several genes by RT-PCR (B, C). For Notch activation, organoids cultured for 4 days were subsequently treated with Jagged-1 peptide (1 μM) for 2 h and subjected to Western blotting. For expression analysis in the small intestine, mice (8-week-old females weighing an average of 20 g) were administered with AhR ligands mixed with 140 μl corn oil (PBS control, 10 μl PBS; DMSO control, 10 μl DMSO; I3C, 2 mg in 10 μl) by gavage 5 times on days 1, 3, 5, 7, and 9 and sacrificed on day 10. Then, the small intestine was analyzed for RNA expression by RT-PCR. Results are representative of three separate experiments, which showed similar results.

Next, we examined if I3C modulates expression of Hes1, a Notch target gene (Ohtsuka et al., 1999), and transcription factor 4 (Tcf4), a transcriptional partner with activated β-catenin. Hes1 is a transcriptional repressor that represses transcription of the transcription factor Math1 (Jensen et al., 2000). Intestinal Math1 expression is required for commitment toward the secretory lineage, including goblet cells and Paneth cells (Yang et al., 2001). Thus, we analyzed the expression of Hes1, Math1, and TCF4 both in organoids and in mice. Organoids cultured for 4 days and mice treated with

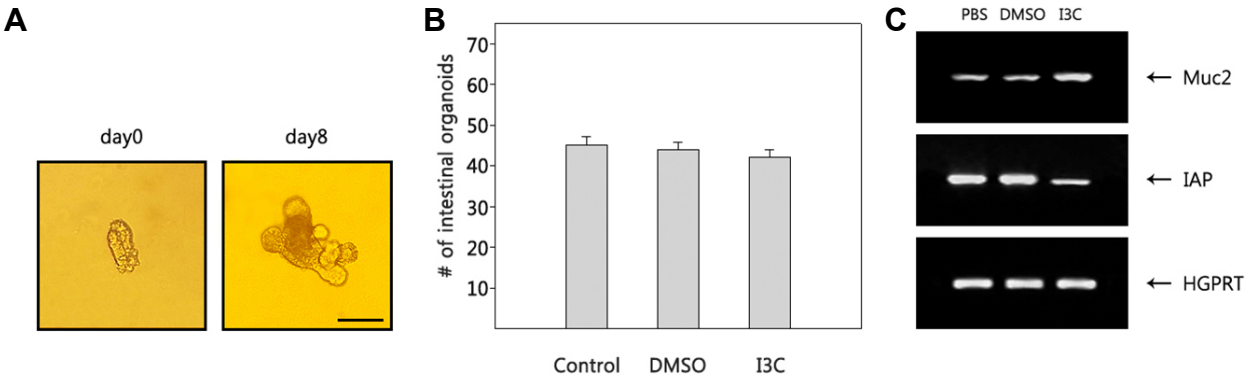

Fig. 5. I3C does not inhibit further development of 4-day-old organoids but increases Muc2 expression while inhibiting IAP expression. Crypts cultured in the complete culture medium for 4 days (~50 organoids/plate) were treated with I3C (1 μM) or DMSO (0.1% v/v) and cultured for 4 additional days. Then, organoids were observed (A), counted (B), and assayed for RNA expression (C). Results are presented as mean ± SD of 3 independent experiments. Statistical significance was analyzed using the paired Student's *t*-test. Scale bar, 50 μm.

AhR ligands for 10 days were used for this assay. Hes1 expression was downregulated by I3C, whereas Math1 expression was upregulated by I3C, both in organoids (Fig. 4C) and in mice (Fig. 4D). Tcf4 expression was not changed by I3C.

I3C did not appear to impair further development of 4-day old organoids, but increases Muc2 expression while inhibiting IAP expression

Next, we examined if I3C affects the development of developing organoids. Crypts cultured in the complete culture medium for 4 days start to show the morphology of intestinal organoids. Thus, we treated these developing organoids (~50 organoids/plate) with I3C to see if I3C has any effect on the development of organoids for an additional 4 days (Fig. 5A). I3C did not appear to inhibit the development of organoids. Almost all of the organoids tested developed further in the presence or absence of I3C (Fig. 5B). Then, organoids were harvested and analyzed for Muc2 and IAP RNA expression. I3C increased Muc2 expression but inhibited IAP (Fig. 5C).

DISCUSSION

Previously we reported that AhR activation by TCDD and FICZ inhibited the development of intestinal organoids (Park et al., 2016). In the present study, we demonstrated that I3C also inhibited intestinal organoid development AhR-dependently, promoting the expression of Muc2 and lysozyme genes but inhibiting IAP gene expression. In mice treated with I3C, proportions of goblet cells in the intestines were remarkably increased, confirming the *in vitro* results. I3C showed opposing effects on Wnt and Notch signals, stimulating Wnt signaling but inhibiting Notch activation, and on the expression of two transcription factors involved in intestinal cell differentiation, inhibiting Hes1 but promoting Math1. These results suggest that I3C regulates IEC development controlling Wnt and notch signals, promoting goblet-cell differentiation.

In this study, we observed that I3C inhibited organoid development by 32% at 1 μM. When crypts were isolated from the small intestine, they varied in depth, some being 4-5 cells deep, some being 9-10 cells deep, and some others in between. Crypts are composed of two compartments: a stem-cell compartment at the base of the crypt, which is 4-5 cells deep, and a transit-amplifying (TA) cell compartment at the top of the crypt which is 5-20 cells deep (Barker et al., 2008). At the base of each crypt, stem cells can self-renew while generating early progenitors, TA cells, which then divide 4-5 times before they terminally differentiate into the specialized intestinal epithelial-cell types. We noticed that longer crypts were less susceptible to I3C than shorter ones, suggesting that I3C inhibits the transition from stem cells into TA cells. In this study, it was shown that I3C did not appear to inhibit the development of 4-day old developing organoids, but did appear to affect the differentiation of stem cells, promoting Muc2 expression but inhibiting IAP expression. Four-day-old organoids may have a longer TA zone, which might be resistant to I3C action.

Inhibitor of DNA binding I (ID1), which plays an essential role in the self-renewal of stem cells (Lasorella et al., 2014), is expressed in stem cells and TA cells (Zhang et al., 2014). c-Myc promotes differentiation of human epidermal stem cells into TA cells (Gandarillas and Watt, 1997). In organoids and the small intestine treated with I3C, expression of ID-1 but not of c-Myc was decreased (data not shown), suggesting that reduced expression of ID-1 may contribute to a lower yield of organoids by I3C. Immunohistochemical analyses of intestinal tissues with anti-ID1 Ab may address this issue.

In this study, we observed that I3C inhibited Notch activation. How I3C regulates Notch signals is unknown. We observed in this study that expression of Delta and Notch1 was not modulated by I3C, making it unlikely that AhR transcriptionally regulates Notch-related genes. I3C has an antioxidant function inhibiting NF-κB-mediated oxidative stress (Li et al., 2013). NADPH oxidase 1 (NOX1), a reactive oxygen species (ROS)-producing oxidase, plays a key role in cell fate

decisions in the colon (Coant et al., 2010). The number of goblet cell was increased in NOX1-deficient mice, in which Notch1 signals were inhibited because of reduced expression of γ-secretase and metalloproteinases involved in Notch activation. Thus, it is possible that I3C may inhibit the expression of γ-secretase by reducing oxidative stress, resulting in the inhibition of Notch activation. In addition to direct transcriptional regulation, activated AhR also has E3 ubiquitin ligase activity, resulting in ubiquitination and degradation of certain proteins, including androgen receptor and estrogen receptor alpha. Thus, it will be of great interest to study the interaction between AhR and Notch.

AhR activation can alter Wnt signaling. AhR promotes Wnt signaling in a mouse embryonal carcinoma cell line and a human prostate cancer cell line but inhibits Wnt signaling in mouse intestinal tissue and a rat stem cell-like cell line (Schneider et al., 2014). Oxidative-stress-induced NF-κB activates protein kinase B (Akt), which phosphorylates glycogen synthase kinase-3-beta (GSK-3β), inhibiting phosphorylation of β-catenin and subsequent degradation of β-catenin (McManus et al., 2005). I3C has an antioxidant function inhibiting NF-κB-mediated oxidative stress (Li et al., 2013), suggesting that I3C may promote the degradation of β-catenin by inhibiting GSK-3β phosphorylation. However, in this study, we observed that β-catenin expression was remarkably increased by I3C. Thus, it is unlikely that AhR activation by I3C activates Wnt signaling in an oxidation-related way. In this study, we observed that expression of Wnt receptors LRP6 and Lgr5 was increased by I3C. Upregulation of LRP6 and Lgr5 in human hepatocytes and breast-cancer stem cells promotes Wnt signaling, increasing the level of β-catenin (Tung et al., 2012; Yang et al., 2015). Thus, it is possible that AhR promotes Wnt activation by upregulating expression of Wnt receptors LRP6 and Lgr5.

Wnt signals are associated with the maintenance of intestinal stem cells (Reya and Clevers, 2005). In this study, it was shown that, although I3C stimulated Wnt signals by increasing the level of active β-catenin, the development of organoids was inhibited, which is in contrast with the expected role of Wnt signals. As described in the result section 'I3C promotes Wnt3 signaling but inhibits Notch1 signaling', blocking Notch cascade impairs the maintenance of undifferentiated proliferative cells in crypts, indicating that concerted activation of the Wnt and Notch signals is necessary for the proliferation of intestinal stem cells (Fre et al., 2005; van Es et al., 2005a). Thus, it is likely that diminished activation of the Notch signals by I3C could explain the I3C-mediated inhibition of the development and proliferation of organoids.

We observed that the expression of lysozyme in organoids was increased by I3C (Fig. 2), but the number of Paneth cells in the small intestine was not changed by I3C treatment (Fig. 1D). Possible explanations for this discrepancy are as follows. Paneth cells have a life expectancy of at least three weeks (Bjerknes and Cheng, 1981), much longer than that (several days) of their terminally differentiated villus counterparts (Simon-Assmann et al., 2007). We treated mice with I3C for 10 days. Thus, a longer treatment may be effective to see I3C effects on the number of Paneth cells. Active Wnt signaling promotes the maturation of Paneth cells but not their proliferation (van Es et al., 2005a). In addition to Wnt signals, Lgr4 and nonreceptor tyrosine phosphates Shp2/MAK2 signals are also necessary for Paneth cell differentiation (Heuberger et al., 2014; Mustata et al., 2011). These remain to be examined.

Although AhR is activated by several different ligands, giving rise to similar effects on diverse physiological activities, accumulating evidence suggests that AhR ligands may act differentially. FICZ exacerbated experimental autoimmune encephalomyelitis (EAE), but TCDD ameliorated disease progression (Quintana et al., 2008; Veldhoen et al., 2008). TCDD induced regulatory T (Treg) cell differentiation, but FICZ both interfered with Treg development and promoted Th17 cell differentiation. TCDD impaired oral tolerance against ovalbumin in mice, but I3C boosted the oral tolerance (Hammerschmidt-Kamper et al, 2017). Previously, we reported that FICZ inhibited intestinal organoid development (Park et al., 2016). Although FICZ (Park et al., 2016) and I3C in this study similarly inhibited intestinal organoid development, it acts differently in Wnt signaling and in the development of the intestine. FICZ inhibits Wnt signaling, but I3C stimulates it. The number of Paneth cells and the depth of crypts in both intestines were reduced in FICZ-treated mice but not in I3C-treated mice. The number of goblet cells was increased in I3C-treated mice but not in FICZ-treated mice. It remains unknown how FICZ and I3C act differently, perhaps because of the difference in AhR affinity; FICZ has a higher affinity for AhR, 2 or more orders of magnitude higher than for I3C (Bjeldanes et al., 1991; Wincent et al., 2009), or perhaps because of differential interaction with other transcription factors (Hankinson et al., 2005; Lee et al., 2012). Furthermore, in contrast to I3C, which has an antioxidant function, FICZ induced oxidative stress in mast cells and epidermal keratinocytes (Park et al., 2015; Wang et al., 2017).

In conclusion, the results obtained in this study provide evidence that I3C, a phytochemical with anticancer activity, regulates the development of the intestine, promoting goblet-cell differentiation in an Ahr-dependent manner. This activity of I3C is mediated by a concerted regulation of Wnt and Notch signals, resulting in differential expression of the transcription factors Hes1 and Math1, which are involved in lineage decisions of IEC. Several issues remain to be addressed:

(1) How is Notch activation suppressed by I3C-activated AhR?
(2) How is Wnt activation stimulated by AhR activated by I3C?
(3) How do different AhR ligands work differently under the same environment?
(4) Do other phytochemicals with AhR-activating ability, including quercetin and kaempferol, and metabolites produced by commensal bacteria, such as indole-3-acetic acid, show similar effects on intestinal development?

ACKNOWLEDGMENTS

This work was supported by the Basic Science Research Pro-

gram through the National Research Foundation of Korea funded by the Ministry of Education (2016R1D1A1B03 931275).

REFERENCES

Abbott, B.D., Birnbaum, L.S., and Perdew, G.H. (1995). Developmental expression of two members of a new class of transcription factors: I. Expression of aryl hydrocarbon receptor in the C57BL/6N mouse embryo. Dev. Dyn. *204*, 133-143.

Ahmad, A., Sakr, W.A., and Rahman, K.M. (2010). Anticancer properties of indole compounds: mechanism of apoptosis induction and role in chemotherapy. Curr Drug Targets *11*, 652-666.

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J., et al. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003-1007.

Barker, N., van de Wetering, M., and Clevers, H. (2008). The intestinal stem cell. Genes Dev. *22*, 1856-1864.

Barouki, R., Coumoul, X., and Fernandez-Salguero, P.M. (2007). The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. FEBS Lett. *581*, 3608-3615.

Bjeldanes, L.F., Kim, J.Y., Grose, K.R., Bartholomew, J.C., and Bradfield, C.A. (1991). Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*: comparisons with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Proc. Natl. Acad. Sci. USA *88*, 9543-9547.

Bjerknes, M., and Cheng, H. (1981). The stem-cell zone of the small intestinal epithelium. I. Evidence from Paneth cells in the adult mouse. Am. J. Anat. *160*, 51-63.

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nat. Rev. Mol. Cell Biol. 7, 678-689.

Burbach, K.M., Poland, A., and Bradfield, C.A. (1992). Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proc. Natl. Acad. Sci. USA *89*, 8185-8189.

Celloto, V.R., Oliveira, A.J., Goncalves, J.E., Watanabe, C.S., Matioli, G., and Goncalves, R.A. (2012). Biosynthesis of indole-3-acetic acid by new Klebsiella oxytoca free and immobilized cells on inorganic matrices. ScientificWorldJournal *2012*, 1-7.

Cheng, H., and Leblond, C.P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. Am. J. Anat. *141*, 461-479.

Chmill, S., Kadow, S., Winter, M., Weighardt, H., and Esser, C. (2010). 2,3,7,8,-tetrachlorodibenzo-p-dioxin impairs stable establishment of oral tolerance in mice. Toxicol. Sci. *118*, 98-107.

Coant, N., Ben Mkaddem, S., Pedruzzi, E., Guichard, C., Tréton, X., Ducroc, R., Freund, J.N., Cazals-Hatem, D., Bouhnik, Y., Woerther, P.L., et al. (2010). NADPH oxidase 1 modulates WNT and NOTCH1 signaling to control the fate of proliferative progenitor cells in the colon. Mol. Cell Biol. *30*, 2636-2650.

Denison, M.S., and Nagy, S.R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Ann. Rev. Pharmacol. Toxicol. *43*, 309-334.

Fre, S., Hannezo, E., Sale, S., Huyghe, M., Lafkas, D., Kissel, H., Louvi, A., Greve, J., Louvard, D., and Artavanis-Tsakonas, S. (2011). Notch lineages and activity in intestinal stem cells determined by a new set of knock-in mice. PLoS One *6*, e25785.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., and Artavanis-Tsakonas, S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. Nature *435*, 964-968.

Gandarillas, A., and Watt, F.M. (1997). c-Myc promotes differentiation of human epidermal stem cells. Genes Dev. *11*, 2869-2882.

Hammerschmidt-Kamper, C., Biljes, D., Merches, K., Steiner, I., Daldrup, T., Bol-Schoenmakers, M., Pieters, R.H.H., and Esser, C. (2017). Indole-3-carbinol, a plant nutrient and AhR-Ligand precursor, supports oral tolerance against OVA and improves peanut allergy symptoms in mice. PLoS One *12*, e0180321.

Hankinson, O. (2005). Role of coactivators in transcriptional activation by the aryl hydrocarbon receptor. Arch. Biochem. Biophys. *433*, 379-386.

Heuberger, J., Kosel, F., Qi, J., Grossmann, K.S., Rajewsky, K., and Birchmeier, W. (2014). Shp2/MAPK signaling controls goblet/paneth cell fate decisions in the intestine. Proc. Natl. Acad. Sci. USA *111*, 3472-3477.

Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., and Madsen, O.D. (2000). Control of endodermal endocrine development by Hes-1. Nat. Genet. *24*, 36-44.

Kiss, E.A., Vonarbourg, C., Kopfmann, S., Hobeika, E., Finke, D., Esser, C., and Diefenbach, A. (2011). Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. Science *334*, 1561-1565.

Kopan, R., and Ilagan, M.X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. Cell *137*, 216-233.

Lasorella, A., Benezra, R., and Iavarone, A. (2014). The ID proteins: master regulators of cancer stem cells and tumour aggressiveness. Nat. Rev. Cancer *14*, 77-91.

Lee, D.M., Lee, S.H., Jeong, K.T., Hwang, S.J., and Park, J.H. (2012). SDS3 interacts with ARNT in an AhR ligand-specific manner regulating expression of cKrox and S100A4 in CD4+CD8+ DPK thymocytes differentiation. Environ. Toxicol. Pharmacol. *34*, 858-868.

Li, Y., Innocentin, S., Withers, D.R., Roberts, N.A., Gallagher, A.R., Grigorieva, E.F., Wilhelm, C., and Veldhoen, M. (2011). Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. Cell *147*, 629-640.

Li, Y., Kong, D., Ahmad, A., Bao, B., and Sarkar, F.H. (2013). Antioxidant function of isoflavone and 3,3'-diindolylmethane: are they important for cancer prevention and therapy? Antioxid. Redox. Signal. *19*, 139-150.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev. Cell *17*, 9-26.

Maruthanila, V.L., Poornima, J., and Mirunalini, S. (2014). Attenuation of carcinogenesis and the mechanism underlying by the influence of indole-3-carbinol and its metabolite 3,3'-diindolylmethane: a therapeutic marvel. Adv. Pharmacol. Sci. *2014*, 832161.

McManus, E.J., Sakamoto, K., Armit, L.J., Ronaldson, L., Shpiro, N., Marquez, R., and Alessi, D.R. (2005). Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. EMBO J. *24*, 1571-1583.

Medema, J.P., and Vermeulen, L. (2011). Microenvironmental regulation of stem cells in intestinal homeostasis and cancer. Nature *474*, 318-326.

Mustata, R.C., Van Loy, T., Lefort, A., Libert, F., Strollo, S., Vassart, G., and Garcia, M.I. (2011). Lgr4 is required for Paneth cell differentiation and maintenance of intestinal stem cells ex vivo. EMBO Rep. *12*, 558-564.

Nguyen, L.P., and Bradfield, C.A. (2008). The search for endogenous activators of the aryl hydrocarbon receptor. Chem. Res. Toxicol. *21*, 102-116.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., and Kageyama, R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J. *18*, 2196-2207.

Park, S.L., Justiniano, R., Williams, J.D., Cabello, C.M., Qiao, S., and Wondrak, G.T. (2015). The tryptophan-derived endogenous Aryl hydrocarbon receptor ligand 6-formylindolo[3,2-b]carbazole is a nanomolar UVA photosensitizer in epidermal keratinocytes. J. Invest. Dermatol. *135*, 1649-1658.

Park, J.H., Choi, A.J., Kim, S.J., Cheong, S.W., and Jeong, S.Y. (2016). AhR activation by 6-formylindolo[3,2-b]carbazole and 2,3,7,8-tetrachlorodibenzo-p-dioxin inhibit the development of mouse intestinal epithelial cells. Environ. Toxicol. Pharmacol. *43*, 44-53.

Perdew, G.H., and Babbs, C.F. (1991). Production of Ah receptor ligands in rat fecal suspensions containing tryptophan or indole-3-carbinol. Nutr. Cancer *16*, 209-218.

Peterson, L.W., and Artis, D. (2014). Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat. Rev. Immunol. *14*, 141-153.

Qiu, J., Heller, J.J., Guo, X., Chen, Z.M., Fish, K., Fu, Y.X., and Zhou, L. (2012). The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity *36*, 92-104.

Quintana, F.J., Basso, A.S., Iglesias, A.H., Korn, T., Farez, M.F., Bettelli, E., Caccamo, M., Oukka, M., and Weiner, H.L. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature *453*, 65-71.

Schneider, A.J., Branam, A.M., P and eterson, R.E. (2014). Intersection of AHR and Wnt signaling in development, health, and disease. Int. J. Mol. Sci. *15*, 17852-17885.

Simon-Assmann, P., Turck, N., Sidhoum-Jenny, M., Gradwohl, G., and Kedinger, M. (2007). In vitro models of intestinal epithelial cell differentiation. Cell Biol Toxicol. *23*, 241-256.

Tung, E.K., Wong, B.Y., Yau, T.O., and Ng, I.O. (2012). Upregulation of the Wnt co-receptor LRP6 promotes hepatocarcinogenesis and enhances cell invasion. PLoS One *7*, e36565.

van der Flier, L.G., and Clevers, H. (2009). Stem cells, self-renewal, and differentiation in the intestinal epithelium. Ann. Rev. Physiol. *71*, 241-260.

van Es, J.H., Jay, P., Gregorieff, A., van Gijn, M.E., Jonkheer, S., Hatzis, P., Thiele, A., van den Born, M., Begthel, H., Brabletz, T., et al. (2005a). Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nat. Cell Biol. *7*, 381-386.

van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., et al. (2005b). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature *435*, 959-963.

Veldhoen, M., Hirota, K., Westendorf, A.M., Buer, J., Dumoutier L, Renauld, J.C., and Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature *453*, 106-109.

Wang, H.C., Zhou, Y., and Huang, S.K. (2017). SHP-2 phosphatase controls aryl hydrocarbon receptor-mediated ER stress response in mast cells. Arch. Toxicol. *91*, 1739-1748.

Weng, J.R., Tsai, C.H., Kulp, S.K., and Chen, C.S. (2008). Indole-3-carbinol as a chemopreventive and anti-cancer agent. Cancer Lett. *262*, 153-163.

Wincent, E., Amini, N., Luecke, S., Glatt, H., Bergman, J., Crescenzi, C., Rannug, A., and Rannug, U. (2009). The suggested physiologic aryl hydrocarbon receptor activator and cytochrome P4501 substrate 6-formylindolo[3,2-b]carbazole is present in humans. J. Biol. Chem. *284*, 2690-2696.

Yaktine, A.L., Harrison, G.G., and Lawrence, R.S. (2006). Reducing exposure to dioxins and related compounds through foods in the next generation. Nutr. Rev. *64*, 403-409.

Yang, Q., Bermingham, N.A., Finegold, M.J., and Zoghbi, H.Y. (2001). Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science *294*, 2155-2158.

Yang, L., Tang, H., Kong, Y., Xie, X., Chen, J., Song, C., Liu, X., Ye, F, Li, N., Wang, N., et al. (2015). LGR5 promotes breast cancer progression and maintains stem-like cells through activation of Wnt/β-catenin signaling. Stem Cells *33*, 2913-2924.

Zhang, N., Yantiss, R.K., Nam, H.S., Chin, Y., Zhou, X.K., Scherl, E.J., Bosworth, B.P., Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843-850.