PhIP/DSS-Induced Colon Carcinogenesis in CYP1A-Humanized Mice and the Possible Role of Lgr5+ Stem Cells

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ABSTRACT

In the past decades, experimental rodent models developed to study the pathogenesis of human colorectal cancer (CRC) generally employed synthetic chemical carcinogens or genetic manipulation. Our lab, in order to establish a more physiologically relevant CRC model, recently developed a colon carcinogenesis model induced by the meat-derived dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and promoted by dextran sodium sulfate (DSS)-induced colitis in the cytochrome P450 1A-humanized (hCYP1A) mice. The resulting colon tumors shared many histologic and molecular features of human colon cancer. In this study, we characterized the early stages of PhIP/DSS-induced colon carcinogenesis. We found that PhIP/DSS treatments caused rapid destruction of the colon mucosa with severe inflammation, followed by the presence of reactive changes and low-grade dysplastic lesions, and then manifestation of high-grade dysplastic lesions and finally adenocarcinomas. Molecular analysis of the early time-points (ie, days 1, 3, 7, 11, 14, and 21 after DSS exposure) indicates *Ctnnb1*/ β -catenin mutations and β -catenin nuclear accumulation in the high-grade dysplastic lesions, but not low-grade dysplastic lesions or adjacent normal tissues. In addition, we investigated the role of Lgr5+ colon stem cells in the PhIP/DSS-induced colon carcinogenesis and found the presence of Lgr5-enhance green fluorescent protein-expressing cells amidst some ulcerated mucosa, high-grade dysplastic lesions and adenocarcinomas, suggesting a possible role of Lgr5+ stem cells in this dietary carcinogen-induced, inflammation-promoted colon carcinogenesis model. Overall, the findings suggest that PhIP/DSS-induced colon carcinogenesis is likely initiated by dominant active *Ctnnb1*/ β -catenin mutation in residual epithelial cells, which when promoted by colitis, developed into high-grade dysplasia and adenocarcinoma.

Key words: Colon carcinogenesis; PhIP; DSS; Lgr5+; β -catenin.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and a leading cause of cancer mortality among men and women in the United States (Siegel *et al.*, 2015). Although some genetic events underlining CRC have been identified (Fearon, 2011; Wood *et al.*, 2007), the etiology of the major nonhereditary

form of the disease (sporadic CRC) remains unclear. Epidemiologic studies have identified a number of lifestyle and dietary risk factors associated with sporadic CRC, including obesity, smoking, physical inactivity, moderate-to-heavy alcohol consumption, and diets that are high in red and/or

processed meat (American Cancer Society, 2014; Chan et al., 2011; Cross et al., 2010; Huxley et al., 2009). It is also one of the most serious complications of inflammatory bowel disease, such as ulcerative colitis and Crohn's disease (Eaden et al., 2001; van Hogezaand et al., 2002).

In the past decades, dozens of experimental rodent models have been developed to investigate the pathogenesis of CRC (De Robertis et al., 2011; Rosenberg et al., 2009). However, the most popular and widely studied of these models employed genetic manipulation (eg, *Apc*^{min} mutants) or synthetic chemicals, such as azoxymethane (AOM) and/or dextran sodium sulfate (DSS). In order to establish a more physiologically relevant CRC model, our lab recently developed a novel colon carcinogenesis model induced by the meat-derived dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and promoted by DSS-induced colitis in cytochrome P450 1A-humanized (hCYP1A) mice (Cheung et al., 2011). PhIP, a carcinogen generated from high-temperature cooking of meat, is mainly metabolically activated by the human CYP1A2 enzyme (Cheung et al., 2005), but detoxified by the murine *cyp1a2*. We used the hCYP1A mice, with the human CYP1A2 replacing the murine *cyp1a2* ortholog, to mimic human metabolism of PhIP. We also used DSS to induced colitis, which is also commonly associated with CRC development.

In a previous study, a single dose of PhIP (200 mg/kg, i.g.), followed by 1 week of DSS exposure (1.5% in the drinking water) effectively induced colonic tumors in hCYP1A mice, but not in wild-type C57BL/6 mice (Cheung et al., 2011). Histopathologic and biochemical evaluations revealed, at as early as 10 weeks after PhIP/DSS treatments, tubular adenocarcinomas with marked overexpression of key proteins in Wnt signaling pathway (ie, β -catenin), cell regulation (ie, c-Myc and cyclin D1), and inflammation (ie, iNOS and COX-2). In addition, the majority of the PhIP/DSS-induced colon tumors were shown to carry dominant active mutations in the *Ctnnb1*/ β -catenin gene, specifically at the key recognition site for ubiquitin-mediated proteasome degradation (Wang et al., 2015).

In this study, we investigated the early stages of PhIP/DSS-induced colon carcinogenesis in hCYP1A mice to further characterize this model. Colon of PhIP/DSS-treated mice was evaluated for histopathologic and molecular changes at multiple early time-points and a later end-point. Colon tissues at selected early time-points were also analyzed for *Ctnnb1*/ β -catenin hot-spot mutations. In addition, we also explored the role of Lgr5+ colon stem cells in the PhIP/DSS colon carcinogenesis. The results suggest that rapid induction of colon carcinogenesis by PhIP/DSS is initiated by dominant active *Ctnnb1*/ β -catenin mutation in residual epithelial cells (possibly Lgr5+ colon stem cells), which when promoted by colitis, developed into precancerous lesion, high-grade dysplasia and finally adenocarcinoma.

MATERIALS AND METHODS

Chemicals. PhIP was obtained from Wako Pure Chemicals (Osaka, Japan) and dissolved in deionized water before administration. DSS (molecular weight 35,000–44,000) was purchased from MP Biomedicals (Solon, OH) and dissolved in deionized water to 1.5% (w/v) before given.

hCYP1A mice study. All animal experiments were conducted in accordance to the protocol (no. 02-027) approved by the Rutgers University Institutional Animal Care and Use Committee. Female hCYP1A [*Cyp1a2/Cyp1a1*^{tm2Dwn} Tg (CYP1A1, CYP1A2)1Dwn/Dwn] mice were previously purchased from Jackson Laboratories (Bar Harbor, Maine) and used as founders

to establish homozygous breeding colonies in-house. The mice (on a C57BL/6 background) were homozygous for the human CYP1A1/2 transgene and homozygous for the mouse *Cyp1a1/2* null allele, as confirmed by PCR genotyping using vender's protocol. Throughout the study, mice were maintained in a sterile room of animal facility at room temperature ($20 \pm 2^\circ\text{C}$) and on a standard 12 h light/dark cycle with water and diet provided *ad libitum* or otherwise specified.

At approximately 5 week of age, the mice were permanently switched from a normal chow diet to the AIN-93M control diet (Research Diets, New Brunswick, NJ). To induce colon carcinogenesis, ~6 week olds mice were administered two doses of PhIP (100 mg/kg b.w.) via intragastric gavage at 3 days apart and then followed by DSS treatment (1.5% in drinking water) for 4 days at 1 week after the first PhIP administration (Figure 1A). The health and body weight of mice were monitored daily during and after PhIP/DSS treatments until sacrificed at days 1, 3, 7, 11, 14, and 21 post-treatments (4 mice at each time-point); mice sacrificed at week 10 were monitored weekly after day 21. CO₂ asphyxiation was used as the method of euthanasia. Mouse colon was carefully excised and cleansed with phosphate-buffered saline before thoroughly evaluated for tumors and colon length. After evaluation, the colons were either fixed in 10% buffered formalin overnight or stored in RNAlater solution (Qiagen, Valencia, CA) at -80°C .

Histopathology. Formalin-fixed colon tissues were processed into Swiss rolls, embedded in paraffin, and then serially sectioned at 4 μm thickness. Histopathologic analysis of the colon was conducted on two step sections stained with hematoxylin and eosin. Colon crypts were evaluated as: (1) normal—well-defined crypts with normal intact epithelium, (2) hyperplastic—thickened crypts with enlarged epithelial cells, increased basophilia, and nuclei that may be vesicular or elongated with prominent nucleoli, (3) low-grade dysplastic—elongated or branched crypts with irregular epithelial cells, reduced interglandular stroma, and fewer goblet cells, or iv) high-grade dysplastic—atypical crypts with cribriform (sieve-like) structures and back-to-back glands, epithelial cells with architectural and cytologic changes, marked reduction of interglandular stroma, and few or no goblet cells (Boivin et al., 2003; Whiteley et al., 1996). Adenomas were diagnosed with grossly visible dysplastic epithelial lesions characterized by hypercellularity and abnormal morphology, including enlarged spindle-shaped nuclei with hyperchromasia, nuclear stratification and loss of nuclear polarity, and increased nucleus to cytoplasm ratio and mitotic activity. Adenocarcinoma were diagnosed based on the extent of cellular abnormality, including largely increased nucleus to cytoplasm ratio, enlarged pleomorphic and hyperchromatic nuclei with prominent nucleoli, frequent mitotic figures, distorted glandular structure, and cell necrosis.

Immunohistochemistry. Immunohistochemical analysis was carried out using 4 μm paraffin-embedded sections from formalin-fixed Swiss-rolled colons. A standard avidin-biotin peroxidase complex method was employed. In brief, sections were deparaffinized and rehydrated before treated with antigen unmasking solution (Vector Laboratories, Burlingame, California) heated in a microwave oven for antigen retrieval. After immersed in 3% hydrogen peroxide to quench endogenous peroxidase, sections were blocked with normal serum and then incubated with primary antibody β -catenin (1:200) (Santa Cruz Biotechnology, Santa Cruz, California) or Ki-67 (1:500) (Abcam, Cambridge, Massachusetts) overnight at 4°C . Next day, sections were then incubated with biotinylated secondary antibody (1:200), followed by streptavidin-biotin peroxidase conjugate (Vector

FIG. 1. PhIP/DSS treatment procedures and the effects on hCYP1A mice. A, hCYP1A mice were treated with PhIP and DSS, and sacrificed at day 1 (D1), D3, D7, D11, D14, and D21 as well as week 10 (W10) after DSS cessation. B, Mice body weight changes after PhIP administrations (approximately 5% reduction), DSS treatment (approximately 10% reduction), and throughout the early time-points. Data presented in mean $\pm$ SEM ($n = 4$). C, Representative colons from vehicle- and PhIP/DSS-treated mice show dramatic shortening at the early time-points and the presence of colonic tumors (arrow) at D21. D, Significant reductions in colon length were found in PhIP/DSS-treated mice as compared with vehicle-treated control mice (VC). Box-and-whisker plot presented in mean with max/min ($n = 4$). Statistical analysis was done using ANOVA-Dunnett (*** $P < .001$, ** $P < 0.01$, * $P < .05$).

Laboratories, Burlingame, California). Proteins were visualized using 3, 3'-diaminobenzidine before counterstained with hematoxylin and mounted with Permount. The total number of cells and infiltrated positive-stained cells in mucosa and submucosa was performed using the Image-Pro Plus Image Processing System (Media Cybernetics, Bethesda, Maryland).

Laser capture microdissection and *Cttnb1*/ β -catenin mutation analysis. Tissue microdissection was performed using an Axiovert 200 microscope fitted with a Zeiss P.A.L.M. MicroBeam (Carl Zeiss Microscopy GmbH, Jena, Germany). Formalin-fixed Swiss-rolled colons were serially sectioned at 8 μ m onto RNase free P.A.L.M. membrane slides (Carl Zeiss Microscopy LLC, Thornwood, New York) and stained with hematoxylin according to manufacturer's protocol. Areas of normal or dysplastic epithelial cells were identified by morphology and matched with hematoxylin- and eosin-stained slides before microdissected using focused laser microbeam pulse and catapulted onto specialized adhesive cap (Carl Zeiss Microscopy LLC, Thornwood, New York). Additionally, all high-grade dysplastic lesions selected for tissue microdissection were positive for β -catenin staining (data not shown). After incubation in proteinase K buffer at 56°C overnight, genomic DNA was extracted from microdissected tissues of 2–3 serial sections using QIAamp DNA micro kit (Qiagen, Valencia, California) according to the manufacturer's protocol.

Analysis for *Cttnb1*/ β -catenin mutation hot spots was performed using Sanger sequencing method as described previously in Wang et al. (2015). *Cttnb1*/ β -catenin exon 3 was directly amplified from extracted DNAs using by Advantage 2 PCR kit (Clontech Laboratories, Inc, Mountain View, California) and primers, CTAACATACTGTGTTTTACAGCTGAC and CAGCTACTTGCTCTT GCGTGA (250bp product size). The PCR-amplified products were the separated by electrophoresis and purified using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). The purified products were sequenced from both directions with the aforementioned PCR primers by Genewiz (South Plainfield, New Jersey).

hCYP1A/*Lgr5*-EGFP mice breeding and study. Mice that harbor human CYP1A2 gene and express enhance green fluorescent protein (EGFP) from the *Lgr5* promoter/enhancer elements (henceforth as "hCYP1A/*Lgr5*-EGFP mice") were generated from breeding homozygous hCYP1A mice with heterozygous *Lgr5*-EGFP-IRES-CreERT2 mice obtained from Jackson Laboratories (Bar Harbor, Maine) (Supplementary Figure S2A). In brief, hCYP1A (+/+) *mcp1a*(-/-) mice was mated with *Lgr5*-EGFP-IRES-CreERT2(+/-) to produce the hCYP1A(+/-) *mcp1a*(+/-) *Lgr5*-EGFP-IRES-CreERT2(+/-) F1 mice. Then, the F1 mice were then mated together to produce the hCYP1A(+/-) *mcp1a*(-/-) *Lgr5*-EGFP-IRES-CreERT2(+/-) F2 mice. Finally, the F2 mice were mated together to produce the hCYP1A(+/-) *mcp1a*(-/-) *Lgr5*-EGFP-IRES-CreERT2(+/-) F3 mice. The homozygous hCYP1A1/2 transgene, homozygous mouse *Cyp1a1/2* null allele, and heterozygous *Lgr5*-EGFP-IRES-CreERT2 allele in the mice were confirmed by PCR genotyping using vender's protocols.

The hCYP1A/*Lgr5*-EGFP mice were treated with PhIP and DSS as described in the hCYP1A mice study. At days 1, 3, 7, 11, 14, and 21 and week 10, colons from PhIP/DSS-treated mice were carefully excised, cleansed with phosphate-buffered saline, and fixed in 4% formaldehyde solution overnight. Next, fixed colons were Swiss rolled, frozen in OCT, cut into 8 μ m thick cryostat sections, and frozen in -80°C. For the initial fluorescence analysis, cryosections were fixed with methanol/acetone (50:50) solution, washed in three changes of phosphate-buffered saline, and directly mounted with ProLong Gold Antifade Mountant with

DAPI (Invitrogen, Grand Island, New York). For immunofluorescence analysis, cryosections were incubated with rat anti-mouse CD326 (Ep-CAM) (1:250) (BioLegend, San Diego, California) and rabbit anti-mouse β -catenin (1:250) (Santa Cruz Biotechnology, Santa Cruz, California) primary antibodies overnight at 4°C. Next day, sections were then incubated with Alexa Fluor 568 Goat anti-rat or anti-rabbit antibody (Invitrogen, Grand Island, New York) and mounted with ProLong Gold Antifade Mountant with DAPI. Evaluation and fluorescence imaging was done using Olympus BX41 Fluorescence Microscope (Olympus America Inc., Center Valley, Pennsylvania).

Statistical analysis. Results were analyzed using the GraphPad Prism software (GraphPad, California). Student's t-test was used to determine whether there were any differences between 2 groups. One-way ANOVA followed by Dunnett's post hoc test was used to determine differences between multiple groups. All reported P-values were 2-sided. Differences were considered statistically significant when $P < .05$.

RESULTS

Effects of PhIP and DSS Treatments on hCYP1A Mice

To induce colon carcinogenesis, hCYP1A mice were given two doses of PhIP (100 mg/kg), followed by DSS (1.5%) in drinking water for 4 days (Figure 1A). This treatment plan was adapted to improve animal health and optimize tumorigenesis from the previous study with one high dose (200 mg/kg) of PhIP and 7 days of DSS exposure (Cheung et al., 2011). The body weight was closely monitored and showed approximately 5% decrease after PhIP administrations and approximately 10% decrease after DSS exposure (Figure 1B). No death occurred as a result of the PhIP or DSS treatment, and mice eventually recovered to normal body weight. Occult bloods in stools and/or mild diarrhea were observed after 3 days of DSS exposure. After 4 days of DSS treatment, mice manifested bloody stools or diarrhea, which gradually disappeared after DSS was withdrawn. After cessation of DSS exposure, mice were sacrificed at day 1 (D1), 3 (D3), 7 (D7), 11 (D11), 14 (D14), and 21 (D21) as well as week 10 (W10). Macroscopic evaluation of the mouse colons at the early time-points revealed a significant reduction in colon length (Figure 1C). The reduction was most significant at D3 and D7 and persisted to W10 (Figure 1D). Visible colonic tumors were found as early as D21 and increased in size and number by W10. Interestingly, mice that did not manifest blood in their stools after PhIP/DSS treatments had lower incidence of colon tumors at W10 (Supplementary Figure S1A). Likewise, mice that were administered lower doses of PhIP (ie, 50 and 25 mg/kg) or have DSS exposure delayed by 1 week developed significantly fewer tumors (Supplementary Figure S1B). Mice treated with DSS alone exhibited similar loss of body weight, colon shortening, and bloody stools or diarrhea, but no incidence of colon tumor at W10 (Supplementary Figure S1B).

Histopathologic Progression and Molecular Changes in PhIP/DSS-Induced Colon Carcinogenesis in hCYP1A Mice

Histological evaluation of the vehicle-treated mouse colon showed normal mucosa with intact epithelium, well-defined crypts, and sparse resident immune cells in the lamina propria (Figure 2A). Evaluation of PhIP/DSS-treated mice, however, revealed loss of surface epithelia and crypts with severe ulcerations and infiltrations of mononuclear cells in the mucosa at D1. At D3, extensive mucosal ulcerations were observed with

A

B

C

D

FIG. 2. Histopathologic progression in PhIP/DSS-induced colon carcinogenesis. **A**, Representative micrographs of VC colon show normal colonic mucosa. PhIP/DSS-treated mice colons show: severe degeneration of the mucosa with ulcerations and infiltration of mononuclear cells at D1; extensive mucosal ulceration with reactive hyperplastic crypts and expansion of inflammatory cells into the submucosa at D3; severe mixed inflammation and focal low-grade dysplastic crypts with mild cytologic atypia (ie, pleomorphic cells, hyperchromatic nuclei, and nuclear crowding/overlapping and pencil-like nuclei) at D7; high-grade dysplastic lesions with proliferative crypts in mild architectural disarray, severe cytologic atypia (ie, pleomorphic cells, loss of cell polarity, cribriform/complex architecture, and irregular and hyperchromatic nuclei) and severe inflammation at D11; high-grade dysplastic lesions with back-to-back disorganized crypts, severe cytologic atypia and severe inflammation at D14; adenomas with highly dysplastic crypts in cribriform architecture and focal cell necrosis at D21; and adenocarcinomas with neoplastic epithelia, highly disorganized crypts, severe cytologic atypia, and crypt abscesses at W10. **B**, micrographs of an adenomatous crypt infiltrating into the submucosa. Scale bar represents 200 μ m. **C**, PhIP/DSS-treated mice showed significant levels of colon ulceration at D1, D3, D7, and D11 that resolved gradually. Box-and-whisker plot presented in mean with max/min ($n = 4$). Statistical analysis was done using ANOVA-Dunnett (** $P < .001$, ** $P < .01$, * $P < .05$). **D**, PhIP/DSS induced high-grade dysplastic lesions in 1 out of 4 mice at D7 ($n = 1$) to 3 out of 4 mice at D11 ($n = 3$), and D14 ($n = 3$), and to 4 out of 4 mice at D21 ($n = 8$) and W10 ($n = 13$), with increasing numbers and sizes. NO, none observed.

appearance of reactive hyperplastic crypts and expansion of inflammatory cells into the submucosa. At D7, severe mixed inflammation and focal low-grade dysplastic crypts with mild cytologic atypia (ie, pleomorphic cells, hyperchromatic nuclei, nuclear crowding/overlapping and pencil-like nuclei) were found in the areas of ulceration. At D11 and D14, high-grade dysplastic lesions with proliferative crypts in increasing architectural disarray and severe cytologic atypia (ie, pleomorphic cells, loss of cell polarity, cribriform/complex architecture and irregular and hyperchromatic nuclei) were found with widespread inflammation. At D21, tubular adenomas with highly dysplastic crypts in cribriform architecture were observed with focal necrosis. At W10, adenocarcinomas with neoplastic epithelia, highly disorganized crypts, severe cytologic atypia, and crypt abscesses were identified, and 1 (out of 13) adenocarcinoma showed evidence of invasion into the submucosa (Figure 2B). Mice treated with DSS alone also exhibited similar histopathology in the colon at D1, D3, and D7, but only low-grade dysplastic crypts was observed at D11, D14, and D21 (data not shown). By W10, mucosa of these mice retained an intact epithelium and crypts with no evidence of neoplasia, suggesting low-grade dysplastic crypts are regenerative epithelium from tissues injury caused by DSS treatment.

Further assessments of the colon mucosa of PhIP/DSS-treated mice revealed high levels of ulceration and rapid development of high-grade dysplastic lesions at the early time-points (Figure 2C and D). Compared to the vehicle control, significant ulcerations were found from D1 to D11 with peak levels at D3 and D7 (Figure 2C). In addition, high-grade dysplastic lesions were detected in 1 of 4 (25%) mice at D7, 3 of 4 (75%) mice at D11 and D14, and in 4 of 4 (100%) mice at D21 and W10 with increasing number and size (Figure 2D). In mice treated with DSS alone, similar levels of colon ulceration were also found from D1 to D11, but no high-grade dysplastic lesions were observed at any time-point (data not shown).

Immunohistochemical analysis was used to characterize the expressions of β -catenin (a key Wnt pathway protein) and Ki-67 (a proliferation marker) in the progression of PhIP/DSS colon carcinogenesis. In the colon mucosa of vehicle-treated mice, β -catenin was expressed at a basal level in the membrane staining in the colon epithelial cells (Figure 3A). In the PhIP/DSS-treated mice, β -catenin expression levels remained low in the cells of the degenerating crypts at D1, reactive hyperplastic crypts at D3, and low-grade dysplastic crypts at D7. However, β -catenin was overexpressed and nuclearized in numerous epithelial cells of the high-grade dysplastic lesions at D11 and D14, and in majority of the epithelial cells in the adenomas at D21 and adenocarcinomas at W10. Unlike β -catenin, Ki-67 was normally expressed in a few cells at the base of colon crypts, where the intestinal stem cells were located (Figure 3B). In the PhIP/DSS-treated mice, Ki-67 was lost in the degenerating crypts at D1, but was highly expressed in the epithelial cells of the reactive hyperplastic crypts at D3 and low-grade dysplastic crypts at D7. The Ki-67 was also overexpressed in the cells of the high-grade dysplastic lesions at D11 and D14, adenomas at D21, and adenocarcinomas at W10. Altogether, these results suggest PhIP/DSS-induced colon carcinogenesis may have arisen from the proliferation of high-grade dysplastic cells with aberrant β -catenin.

Dominant Active *Ctnnb1*/ β -Catenin Mutations in the High-Grade Dysplastic Lesions

Previous study in our lab showed a majority of PhIP/DSS-induced colon tumors carried dominant active mutations of the *Ctnnb1*/ β -catenin gene, chiefly in codon 32 and 34 of exon 3 (Wang et al.,

2015). To determine whether these mutations can be detected in earlier preneoplastic colon, laser capture microdissection and targeted sequencing were employed. Epithelial tissues from high-grade dysplastic lesions, low-grade dysplastic crypts, and adjacent normal epithelia were microdissected from the colon mucosa of PhIP/DSS-treated mice at D7, D11, D14, and D21 (Supplementary Figure S3). High-grade dysplastic lesions selected for microdissection were also positive for β -catenin staining (data not shown). After DNA extraction and PCR amplification, the *Ctnnb1*/ β -catenin exon 3 region was sequenced and revealed either codon 32 or 34 mutations in the high-grade dysplastic lesions (Table 1 and Supplementary Figure S3). Similar mutations were not found in the low-grade dysplastic crypts or adjacent normal epithelia at any early time-points. These results indicate that the high-grade dysplastic lesions carrying *Ctnnb1*/ β -catenin codon-32 or codon-34 mutation are the early preneoplastic manifestation of PhIP/DSS-induced colon tumors.

Role of *Lgr5*-EGFP-Expressing Stem Cells in PhIP/DSS-Induced Colon Carcinogenesis

Studies with *Lgr5*⁺ stem cells, a well-defined stem cells type in the intestinal crypts, have suggested these cells could be the cell-of-origin for intestinal cancer (Barker et al., 2007, 2009). To investigate whether *Lgr5*⁺ stem cells are involved in the PhIP/DSS-induced colon carcinogenesis, we generated *hCYP1A/Lgr5*-EGFP mice, which harbor human CYP1A2 and one EGFP-tagged *Lgr5* allele (Supplementary Figure S2A). Interestingly, the *hCYP1A/Lgr5*-EGFP mice were found to have a greatly variegated expression of *Lgr5*-EGFP in the colon, leading to infrequent appearance of *Lgr5*-EGFP-expressing cells in the colon crypts of vehicle-treated control mice (Figure 4A and Supplementary Figure S2B). In the PhIP/DSS-treated mice, residual *Lgr5*-EGFP-expressing cells were detected amidst the ulcerated mucosa at D1 and D3 time-points. At D7, D11, and D14, clusters of *Lgr5*-EGFP-expressing cells were observed in the mucosa or within some high-grade dysplastic lesions. By D21 and W10, *Lgr5*-EGFP-expressing cells were found to constitute a large part of certain adenomas and adenocarcinomas, respectively. However, the majority of high-grade dysplastic lesions, adenomas, and adenocarcinomas in the later time-points were found to contain very few *Lgr5*-EGFP-expressing cells.

Colon epithelium is a rapid turnover tissue and cryptic epithelial cells are replaced by new ones in 4–7 days (Barker et al., 2007). Such an active regeneration also effectively repairs injured epithelium. Using EpCAM, a transmembrane glycoprotein and epithelial cells marker, we found that the entire colon epithelium sloughs off after treatment with DSS (Figure 4B). However, EpCAM staining also revealed a few residue epithelial cells embedded in the damaged area filled with inflammatory and stromal cells (Figure 4B, D1, and D3). In approximately 2 weeks after withdrawing DSS (D14), the epithelium was almost completely regenerated. The regeneration source is expected to be the residue epithelial cells left in the damaged area. Furthermore, immunofluorescence stainings with β -catenin showed many of the *Lgr5*-EGFP-expressing cells in the high-grade dysplastic lesions, adenomas and adenocarcinomas were co-stained with high levels of β -catenin (Figure 4C). These results demonstrate that *Lgr5*⁺ colon stem cells are the residue EpCAM-staining epithelial cells after DSS-induced colon damage and are the source of the regenerated colon epithelium after withdrawing DSS treatment. Under this condition, PhIP/DSS-induced tumors are likely to be derived from the residue *Lgr5*⁺ colon stem cells carrying dominant active β -catenin mutations induced by PhIP.

FIG. 3. β -catenin nuclear accumulation and Ki-67 overexpression in PhIP/DSS-induced colon carcinogenesis. **A**, Representative micrographs of β -catenin immunostaining in colon showing: basal membrane staining of epithelial cells in vehicle-treated control mice; weak stainings of epithelial cells in PhIP/DSS-treated mice at D1, D3, and D7; strong nuclear staining of some epithelial cells in high-grade dysplastic lesion at D11 and D14; and pervasive nuclear staining of most epithelial cells in polypoid adenoma at D21 and adenocarcinoma at week 10. **B**, Representative micrographs of Ki-67 immunostaining in colon showing: few positive-stained cells in the base of colon crypts of vehicle-treated control mice; loss of positive-stained cells in the degenerating crypts of PhIP/DSS-treated mice at D1; overexpression of positive-stained cells in reactive hyperplastic crypts at D3, low-grade dysplastic crypts at D7, high-grade dysplastic lesions at D11 and D14, adenoma with high-grade dysplasia at D21, and adenocarcinomas at week 10. Scale bars represent 200 μ m.

TABLE 1. Mutations in Codons 32 and 34 of *Ctnnb1*/ β -catenin Exon 3 in Colon Tissues of PhIP/DSS-Treated Mice

Sample ID		Adjacent Normal Epithelia		Low-grade Dysplastic Lesion		High-grade Dysplastic Lesion	
		Codon 32	Codon 34	Codon 32	Codon 34	Codon 32	Codon 34
D7	mouse 1	wt (ATC)	wt (TTC)	wt (ATC)	wt (TTC)	mutant (ATA)	wt (TTC)
D11	mouse 1	wt (ATC)	wt (TTC)	wt (ATC)	wt (TTC)	wt (ATC)	mutant (TAC)
	mouse 2	wt (ATC)	wt (TTC)	wt (ATC)	wt (TTC)	mutant (ATA)	wt (TTC)
	mouse 3	wt (ATC)	wt (TTC)	wt (ATC)	wt (TTC)	mutant (ATA)	wt (TTC)
D14	mouse 1	wt (ATC)	wt (TTC)	wt (ATC)	wt (TTC)	wt (ATC)	mutant (TAC)
	mouse 2	wt (ATC)	wt (TTC)	wt (ATC)	wt (TTC)	mutant (ATA)	wt (TTC)
D21	mouse 1	wt (ATC)	wt (TTC)	wt (ATC)	wt (TTC)	mutant (ATA)	wt (TTC)
	mouse 2	wt (ATC)	wt (TTC)	wt (ATC)	wt (TTC)	wt (ATC)	mutant (TAC)

wt, wildtype.

FIG. 4. Characterization of Lgr5-EGFP-expressing colon stem cells in PhIP/DSS-induced colon carcinogenesis in hCYP1A/Lgr5-EGFP mice. **A**, Representative fluorescence micrographs of colons showing variegated appearance of Lgr5-EGFP-expressing cells (Green) in the crypts of vehicle-treated control mice; residual Lgr5-EGFP-expressing cells amidst the ulcerated mucosa at D1 and D3; clusters of Lgr5-EGFP-expressing cells in the mucosa or within high-grade dysplastic lesions at D7, D11 and D14; and a large part of certain adenomas and adenocarcinomas at D21 and W10, respectively. **B**, Representative immunofluorescence micrographs of hCYP1A/Lgr5-EGFP mice colons co-stained with epithelial cell marker EpCAM (Red) showing EpCAM-positive residual Lgr5-EGFP-expressing cells (Green) in the ulcerated mucosa at D1 and D3, high-grade dysplastic lesions at D7, D11, and D14, adenomas with high-grade dysplasia at D21, and adenocarcinomas at W10. **C**, Representative immunofluorescence micrographs of hCYP1A/Lgr5-EGFP mice colons co-stained with β -catenin (Red) showing high levels of β -catenin expression in the Lgr5-EGFP-expressing cells (Green) within high-grade dysplastic lesions at D7, D11, and D14, adenomas with high-grade dysplasia at D21, and adenocarcinomas at W10. DAPI staining (blue). Scale bars represent 200 μ m.

DISCUSSION

In this study, we investigated the early stages of a colon carcinogenesis model induced by a dietary carcinogen, PhIP, and promoted by DSS-induced colitis in CYP1A-humanized mice. Previous studies in our lab utilized one high-dose (200 mg/kg) of PhIP and 7 days of DSS exposure, but this combination resulted in death in some of the mice (Cheung et al., 2011). To reduce toxicity, our current study adapted a new treatment procedure using two lower doses (100 mg/kg) of PhIP at 3 days apart and then followed by DSS exposure for only four days (Figure 1A). Mice under the new procedure still experienced abrupt body weight loss after the treatments, but deaths were completely avoided while tumor incidence was enhanced to 100% at week 10. Interestingly, mice that did not manifest blood in their stools after the PhIP/DSS treatments were much less likely to develop colon tumors, suggesting that intestinal damage as reflected in bloody stools was vital for the colon carcinogenesis of this model. The observations that mice did not develop tumors when treated with DSS alone, or had significantly fewer tumors when administered lower doses of PhIP or delayed exposure to DSS, highlighted the importance of PhIP-initiated *Cttnb1*/ β -catenin mutation and DSS-induced colitis in the model.

In the hCYP1A mice, PhIP/DSS treatments caused rapid destruction of colon mucosa with severe inflammation, followed by the appearance of reactive hyperplastic and low-grade dysplastic crypts, and then manifestation of high-grade dysplastic lesions, adenomas, and finally adenocarcinomas (Figure 2A). These histopathologic features are very similar to those found in the murine AOM/DSS-induced models (Rosenberg et al., 2009; Tanaka, 2009) and resemble human colon carcinogenesis, which is believed to progress from indefinite dysplasia to low-grade dysplasia to high-grade dysplasia to carcinoma (Boivin et al., 2003; De Lerma Barbaro et al., 2014; Itzkowitz et al., 2004). However, only one out of thirteen (7.7%) PhIP/DSS-induced adenocarcinomas showed evidence of submucosal invasion at week 10. This is comparable to the AOM/DSS model, where only 2 of 25 (8%) adenocarcinomas showed submucosal invasion at the equivalent time-point (Suzuki et al., 2004). It is possible that week 10 is still in early period of cancer development process and further genetic alterations are necessary to advance the colon carcinogenesis to exhibit invasiveness.

Aberrant regulation of the Wnt/Apc/ β -catenin pathway is critical for the onset and progression of CRC, with over 90% of sporadic colon cancer cases harboring mutations in Apc or β -catenin (Polakis, 2007; Fearon, 2011). In our study, active mutations of *Cttnb1*/ β -catenin gene and nuclear accumulation of the β -catenin protein were observed in the high-grade dysplastic lesions at the early time-points. With high levels of Ki-67 (proliferative marker) expression, these preneoplastic lesions are strongly implicated to be early precursors of the colon tumors and consistent with a recent study in Apc ($\Delta 14/+$) mice (Nakanishi et al., 2015). However, further studies are necessary to determine whether other early driver events are also involved in the PhIP/DSS-induced colon carcinogenesis.

The hCYP1A/Lgr5-EGFP mice were generated in our lab to study the role of Lgr5+ intestinal stem cells in PhIP/DSS-induced colon carcinogenesis. In PhIP/DSS-treated mice, Lgr5-EGFP-expressing cells were observed amidst the ulcerated mucosa, within some high-grade dysplastic lesions, and constituted a large part of certain adenocarcinomas (Figure 4A). It is interesting to find that some colon stem cells are resistant to the PhIP/DSS-induced damage and remain in the ulcerated mucosa. In addition, it appears that a few of these scattered

Lgr5-EGFP-expressing stem cells are promoted to form cell clusters, which progress to high-grade dysplastic lesions and adenocarcinomas. These findings are in contrast to recent studies that showed Lgr5-EGFP expression is restricted to a small population of scattered cells in colon tumors induced by AOM/DSS (Barker et al., 2009; Hirsch et al., 2014). Although exact reason for the discrepancy is currently unclear and need to be further studied, we speculate that the greatly variegated expression of Lgr5-EGFP in the colon crypts may play a fundamental role. On the other hand, we demonstrate that under the conditions used in our experiment, Lgr5+ colon stem cells are the only residue EpCAM-labeled epithelial cells after DSS induced damage, and they are the cells that regenerate colon epithelium. The step-wise progression of Lgr5-EGFP-expressing cells at the early time points, accompanied with active Wnt signaling via elevated nuclear β -catenin, strongly suggests that PhIP-induced mutant Lgr5+ colon stem cells are the cell-of-origin in the PhIP/DSS-induced colon carcinogenesis. However, we cannot exclude other types of cells in the colon as the initiating cells for colon carcinogenesis because many of the high-grade dysplastic lesions, adenomas and adenocarcinomas do not contain Lgr5-EGFP-expressing cells. These non-Lgr5-EGFP-expressing lesions and neoplasia may have arisen from mutated transit-amplifying cells or even surface epithelial cells (Shih et al., 2001). It is also possible that the EGFP-tagged Lgr5 allele was randomly silenced during the expansion of the mutated Lgr5-EGFP-expressing cells (Koo et al., 2014). Future study employing lineage tracing to track the Lgr5+ stem cells during PhIP/DSS-induced colon carcinogenesis is necessary to further define the Lgr5+ stem cells as the origin of PhIP/DSS-induced colon tumors.

In summary, this study demonstrates that PhIP/DSS-induced colon carcinogenesis in the hCYP1A mice is a stepwise process from reactive changes and low-grade dysplastic crypts to high-grade dysplastic lesions to finally adenocarcinomas. The rapid induction of colon tumors is likely initiated by mutations of the *Cttnb1*/ β -catenin gene in certain colon epithelial cells that undergo proliferation when promoted by DSS-induced colitis. Our study also demonstrates a possible role of Lgr5+ colon stem cells in the dietary carcinogen-induced, inflammation-promoted colon carcinogenesis model. Altogether, the results provide a well-characterized, physiological relevant mice model for studying the dietary etiology and prevention of human CRC.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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