A Preclinical Model for Colitis and Colitis-Associated Cancer – A Prerequisite for Drug Development

A thesis submitted by

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Abstract

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) that is located in the large intestine. Patients with UC have a shorter life expectancy, and an increased incidence of developing colorectal cancer (CRC). CRC is one of the major contributors to the high mortality rates of cancer related deaths. Along with the increased risk of developing CRC, UC patients are also at a 20% increased risk of developing colitis-associate cancer (CAC). While CRC has been well studied, CAC progression has not been well characterized, warranting new studies to better understand this disease. The Wnt signaling pathway has been identified as a key factor in the development of CRC. The transcriptional repressor gene, HBP1, has been shown to downregulate Wnt signaling. In this study we use a HBP1 knockout mouse model to investigate HBP1's role in UC and CAC. There are currently no biomarkers to predict UC or CAC. Our *in vivo* and *in vitro* studies suggest that the transcriptional repressor, HBP1, plays a role in the susceptibility and development of UC and CAC. These studies hint at HBP1 as a possible biomarker for UC and CAC, while also offering a potential animal model that recapitulates the two disorders. There is also a gap in knowledge when distinguishing the UC to CAC progression, in which this animal model could provide further insight on the molecular mechanisms contributing to the UC to CAC progression. Lastly, these studies suggest that a pharmacological compound that suppresses Wnt signaling could potentially be used to reduce the effects of UC and CAC and their development.

Acknowledgments

I would like to acknowledge everyone who has helped facilitate my experience throughout the duration of my masters education. The tremendous amount of support that I have received from faculty, family, and friends has been paramount to my time spent at Tufts University. There is only so much room in this section to allow me to acknowledge those who were involved throughout this process, so I apologize to those who were not mentioned. I am still appreciative and extremely blessed to have the support of all of those who were not mentioned.

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Beaugerie, L., & Itzkowitz, S. H. (2015). Cancers complicating inflammatory bowel disease. *N Engl J Med, 372*(15), 1441-1452. doi:10.1056/NEJMra1403718

List of Abbreviations

APC: Adenomatous polyposis coli
AOM: Azoxymethane
CAC: Colitis-associated Cancer
CD: Crohn's Disease
cDNA: Complementary Deoxyribonucleic Acid
CRC: Colorectal Cancer
CTT: Cotton Top-Tamarin
DSS: Dextran Sulfate Sodium
DVL: Dishevelled
EDTA: Ethylenediaminetetraacetic Acid
GSK3β: Glycogen synthase kinase 3β
HBP1: HMG Box-containing Protein 1
H&E: Hematoxylin & Eosin
IBD: Inflammatory bowel disease
GSK3β: Glycogen synthase kinase 3β
LGR5: Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
LRP5: Low-density lipoprotein receptor-related protein 5
LRP6: Low-density lipoprotein receptor-related protein 6
mRNA: Messenger Ribonucleic Acid
OCT: Optimal Cutting Temperature
PBS: Phosphate Buffer Saline
PORCN: Porcupine
PVFD: Polyvinylidene Difluoride
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TCF/LEF: T-cell factor/lymphoid enhancer-binding factor
UC: Ulcerative Colitis
WT: Wildtype

Chapter 1: Introduction

1.1. Inflammatory Bowel Disease

1.1.1. Fundamentals of Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is characterized as chronic inflammation of the small and large intestine (Molodecky et al., 2012). Patients who experience IBD will be diagnosed with ulcerative colitis (UC) or Crohn's disease (CD). While CD and UC have similar symptoms, they differ from one another by the area in which they located. CD can arise in any part of the gastrointestinal tract, while UC is located only in the colon. There is an estimated 0.4% of Europeans and North Americans that are currently diagnosed with IBD. Patients with IBD have a shorter life expectancy and an overall lower quality of life (Herrinton et al., 2012).

1.1.2. Inflammatory Bowel Disease Complications

Along with the symptoms from constant inflammation of the gastrointestinal tract, patents with IBD have an increased risk of developing colorectal cancer (CRC) (Herrinton et al., 2012). CRC ranks as one of the major causes of cancer related mortality (Tenesa & Dunlop, 2009). The increased risk of developing CRC within the UC patient population is dependent on the severity and duration of the UC, with the risk of CRC increasing every ten years. In a 30-year study of the disease progression, the risk for CRC increases from 2% to 18% in UC patients (Jess et al., 2012). Along with the increased risk of CRC, UC patients also have a 20% increased susceptibility to developing colitis-associated cancer (CAC) (Rubin, Shaker, & Levin, 2012). The current surveillance for CRC and CAC in UC patients is to have routine colonoscopies every 1-2 years once the patient has been diagnosed with UC for 8-10 years (R. Chen et al., 2005). The current

procedure to ensure an early detection of CRC and CAC in UC patients is extremely exhausting, both mentally and physically, which warrants investigations on UC and its progression into CRC and CAC to allow for new methods that are less invasive for the patients.

1.2. Colorectal Cancer & Colitis-Associated Cancer

CRC has been well characterized in terms of the genetic changes that occur during tumorigenesis (Vogelstein et al., 2013). The first major alteration identified in tumorigenesis is the initiation of an earl adenoma by the loss of function to Adenomatous polyposis coli (APC). This loss of function to the APC gene is crucial to the development of CRC, which allows a cascade of alterations to transpire. The next stage of tumorigenesis is initiated by a mutation to the KRAS gene, which transitions the epithelial into late stage adenoma. A p53 mutation that is promotes the late stage adenoma into a carcinoma is the last phase of tumor development. CAC is similar to CRC in terms of mutations, but the order in which the mutations occur is different between the two diseases (Figure 1.1.). Also, the polyps that develop during CRC are not found in CAC. Instead, an area of inflamed epithelial that is exhibiting abnormal cellular growth is the product of CAC, which is defined as dysplasia (Beaugerie & Itzkowitz, 2015).

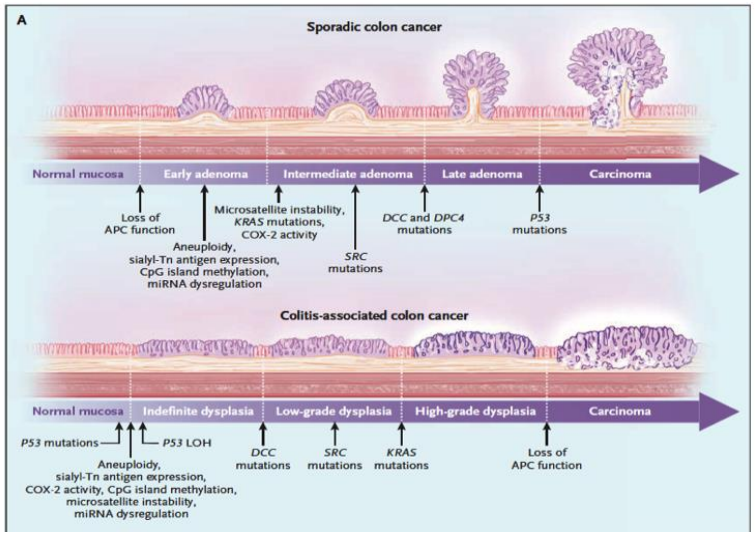


Figure 1.1. Pathogenesis of Colorectal Cancers. CRC and CAC has similar mutations, but the order in which these mutations occur is the main difference between the two diseases. Another difference is in the formation of the carcinomas, with the difference being the formation of polyp in CRC and dysplasia in CAC. Diagram reprinted with permission from [Beaugerie, L., & Itzkowitz, S. H. (2015). Cancers complicating inflammatory bowel disease. *N Engl J Med, 372*(15), 1441-1452. doi:10.1056/NEJMra1403718]

1.3 Preclinical Models of Ulcerative Colitis and Colorectal Cancers

1.3.1. Apc Mouse Model

The first step to a majority of CAC's is caused by mutations to the Apc gene. Stated earlier, the APC gene mutation is the initiator of the cascade of mutations that lead to colonic carcinomas (Beaugerie & Itzkowitz, 2015). There are many Apc mouse models that develop tumors in the gastrointestinal tract, but there are two that produce an extremely high number of tumors. The Apc^{Min} mouse was generated on a C57BL/6J background resulting in the development of over 100 intestinal tumors in each mouse. Another model that produced intestinal tumors is the $Apc^{\Delta 716}$ mouse. Homozygosity for either of these mouse models results in embryonic lethality, which some believes hinders the validity of exactly how Apc is playing a role in tumors in mice. Also, a majority of

the tumors are found in the small intestine, suggesting that these models are unable to characterize CRC properly (Fodde & Smits, 2001).

1.3.2. IL-10 Mouse Model

The IL-10 gene has been well established as an anti-inflammatory regulator by lowering the number of immune cells to the site of interest. IL-10 plays a major role in inflammation, and because of IL-10's suppressive characteristics of immune cells, it was believed to be a promising target gene to investigate UC. This lead to the development of the IL-$10^{-/-}$ mouse (Rennick & Fort, 2000). The IL-$10^{-/-}$ mice naturally developed UC and CAC, suggesting that this model recapitulates the human progression of UC and CAC. Although these findings were novel, a majority of IL-$10^{-/-}$ results focus on the microbiota and the importance of homeostasis within the intestines (Keubler, Buettner, Hager, & Bleich, 2015).

1.3.3. Other Pre-Clinical Models

Another model that was developed to investigate UC and CAC is the Gαi2 KO mouse. This mouse model targeted G proteins, specifically the GαI subunit. These KO mice naturally developed UC and adenocarcinomas in the colon (Kanneganti, Mino-Kenudson, & Mizoguchi, 2011). This model gave rise to the anti-α4 therapeutic which reduced inflammation in the intestine in various pre-clinical and clinical trials. Studies later identified that prolonged use of the anti-α4 antibody will actually increase the severity of UC, along with many other harmful side effects.

The best pre-clinical model for UC and CAC was the primate, Cotton Top-Tamarin (CTT). These primates developed UC and CAC while living in their natural habitat, which was remarkably similar to the human development of these diseases

(Kanneganti et al., 2011). Utilizing the CTT primates, an anti-TNFα antibody treatment was administered, which successfully demonstrated that TNFα is promising therapeutic target for IBD. Unfortunately, the CTT primate population has been designated as an endangered species, no longer allowing for the use of CTT primates as a pre-clinical model.

1.4 WNT Signaling

1.4.1. Fundamentals of Wnt Signaling

The Wnt signaling pathway is present in many organs in the body, by contributing to proper development and bodily homeostasis. The Wnt signaling pathway begins a cascade of events through the activation of β-catenin. Wnt ligands are secreted and bind to extracellular receptors that control different intracellular mechanisms. When Wnt is not present, a destruction complex involving adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) and AXIN, becomes activated and phosphorylates β-catenin. When extracellular Wnt is present, it binds to the frizzled receptor and the co-receptors low-density lipoprotein receptor-related protein 5/6(LRP5, LRP6). These receptors initiate the Dishevelled proteins (DVL), which deactivates the destruction complex allowing for β-catenin to enter the nucleus. When β-catenin enters the nucleus it binds with the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) yielding gene expression (Kahn, 2014). There are many components that contribute to the Wnt signaling pathway. There are many different Wnt ligands as well as multiple receptors and co-receptors involved. The complexity of the Wnt pathway makes it difficult to develop safe and effective therapeutics, but it still does serve as a promising target for many diseases.

1.4.2. Wnt Signaling in Disease

Because Wnt signaling is crucial in such a vast majority of the body, it is inevitable that the Wnt signaling pathway also plays a role in the development and onset of many diseases. Various cancers have identified the Wnt signaling pathway as a prominent factor, including CRC (Grady & Pritchard, 2014). Wnt signaling has also been identified as an important component within stem cells. Stem cells play a major role in tissue renewal and regeneration, contributing many types of cancer (Nusse & Clevers, 2017).

1.4.3. HBP1 Inhibit WNT Signaling

The HMG box-containing protein 1 (HBP1) gene has been identified as a transcriptional repressor that regulates the Wnt signaling pathway. It has been shown that the Wnt pathway is repressed by HBP1 because it inhibits TCF/LEF binding, as well as a repress Cyclin D1(Sampson et al., 2001). This previous data suggest that investigating the mechanisms associated with HBP1 can provide useful insight to several Wnt related illnesses, specifically illnesses related to increase Wnt signaling. The Yee lab's previously developed HBP1$^{-/-}$ mouse model can provide a more defined characterization of the Wnt signaling pathway and its role in the pathogenesis of UC and CAC.

1.5. Study Aims

1.5.1. Gap in Knowledge

While Wnt signaling has been well characterized in CRC, its role has not been well defined in the onset of CAC from UC. Because CAC is understudied, there is an opportunity for investigators to better characterize Wnt signaling in the progression of

CAC from UC. There are also few animal models available that mirror the natural development of UC to CAC.

1.5.2. HBP1: A target gene for Ulcerative Colitis and Colitis-Associated Cancer

In this paper we demonstrate that the HBP1 gene plays a role in UC and CAC through the WNT signaling pathway, while also demonstrating that HBP1$^{-/-}$ mice elicit an increased immune response. With hopes to fill the void in the lack pre-clinical models that recapitulate the disease progression of UC and CAC. This papers data suggest that our HBP1$^{-/-}$ mouse model can provide a more sufficient pre-clinical model to investigate UC and CAC and possible therapeutics.

Chapter 2: Methods

2.1. Animal Studies

2.1.1. HBP1 Animal Housing

All animals were housed in the Tufts University Animal Facilities with normal chow and water in a 12 hour light and dark interval. All experiments and protocols were approved by the Tufts Institutional Animal Care and Use Committee. All HBP1$^{-/-}$ and HBP1 WT mice that were used in these experiments were 3 months of age. The construction of the HBP1transgenic mouse has been described in previous studies(Shih et al., 2001).

2.1.2. Dextran Sodium Sulfate (DSS) Studies

HBP1$^{-/-}$ and WT mice underwent 2 rounds of DSS treatment. The mice were administered 1% Dextran Sulfate Sodium (DSS) drinking water for 1 week, and then put back on normal drinking water for 2 weeks. Body weights were recorded daily, and stool samples were taken throughout DSS exposure.

2.2. Intestinal Crypt Isolation

Colons were harvested from HBP1$^{-/-}$ and WT mice, washed with ice cold PBS, and lacerated. The colons were then placed in an ice could EDTA/PBS solution at a concentration of 1:50. The colons were incubated on an orbital shaker in a cold room for 1 hour. Next, the colons were transferred 50ml tubes containing ice cold PBS. Then, the crypts were removed from the colon by 3-5 minutes of vigorous shaking. The colon was removed from the 50ml tube, and the 50ml tube was centrifuged at 300g for 5 minutes to pellet the crypts. The supernatant was removed from the 50ml tube and the crypts were collected and put in a -80°C freezer.

2.3 Organoid Experiments

Intestinal crypts were isolated from HBP1$^{-/-}$ and WT mice and seeded into matrigel for crypt culture. Organoid formation was assessed at Day 5 of culturing. Organoids were then dissociated and sorted for live cells, then seeded into new wells. Secondary clongenicity was then assessed after 4 days.

2.4 Western Blot Analysis

Western blotting was performed using an SDS-PAGE electrophoresis system. 20μg of protein was suspended into sample buffer and loaded into a bio rad precast 4-20% gradient gel. The protein was electrophoresed in Tris running buffer and blotted to a PVDF membrane. The membrane was probed with primary antibodies against phosphorylated serine 9 GSK3β (pGSK3β^{S9}) total GSK3β, β-catenin, and α-tubulin (used as the protein loading control).

2.5 Quantitative real-time PCR

Total RNA was isolated from the intestinal crypts using the Qiagen RNA isolation protocol. Reverse transcription was performed on the isolated RNA in a thermos cycler, and cDNA was placed in 4°C fridge. mRNA expression levels of Leucine Rich Repeat Containing G Protein-Coupled Receptor 5 (LGR5) and Axin-2 were measured as the number of transcripts relative to those of 18S, and normalized to the mean value of the control.

2.6 Hematoxylin and Eosin Stain (H&E)

HBP1$^{-/-}$ and WT colons were embedded in optimal cutting temperature (OTC) before sectioning, and sectioned at Tufts University's Sackler Graduate School of Biomedical Sciences. The slides were rehydrated in distilled water, and placed in CAT

hematoxylin for 1 minute. Next, the slides were washed with 4-5 changes of tap water. The slides were then placed in 1X PBS for 1 minute. Next, the slides were then washed with 3 changes of distilled water. Lastly, the slide was placed in Eosin for 1 minute, and dehydrated by undergoing 3 changes of 95% ethanol and 2 changes of 100% ethanol.

Chapter 3: Results

3.1. Preliminary Results

3.1.1. Decreased Lifespan of HBP1$^{-/-}$ Mice

There is evidence that suggest that single genes can have large effects on the lifespan of an organism (Van Zant & de Haan, 2004). A traditional life span study using HBP1$^{-/-}$ and WT mice was conducted to see if the HBP1 gene had an effect on survival. As seen in Fig. 3.1, the HBP1$^{-/-}$ mice had a 65-day shorter life span than WT mice.

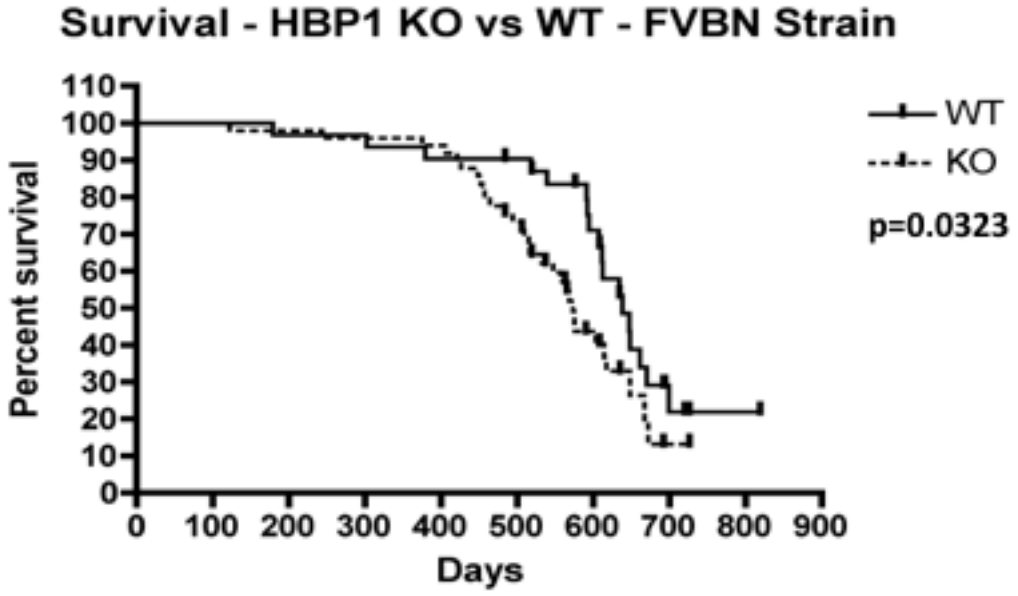


Fig. 3.1. Kaplan-Meir Survival of WT and HBP1$^{-/-}$ Mice. 31 WT and 49 HBP1$^{-/-}$ mice were allowed to naturally live, with the age of death recorded for each mouse.

3.1.2. Deletion of the HBP1 gene causes Ulcerative Colitis and Colitis-Associated Cancer

To evaluate the cause of death of HBP1$^{-/-}$ mice, 9 WT and 13 HBP1$^{-/-}$ mice were randomly selected to have a pathological analysis performed (collaboration with Dr. Roderick Bronson, Harvard Medical School). The pathological analysis revealed that 6 of the 13 HBP1$^{-/-}$ mice identified UC and/or CAC as the cause of death. There were no signs of UC or CAC present in the WT mice (Table 3.1). The colons displaying UC from the

HBP1$^{-/-}$ mice mirrored the human characteristics with the infiltration of lymphocytes, and the onset of CAC from severe UC (Figure 3.2).

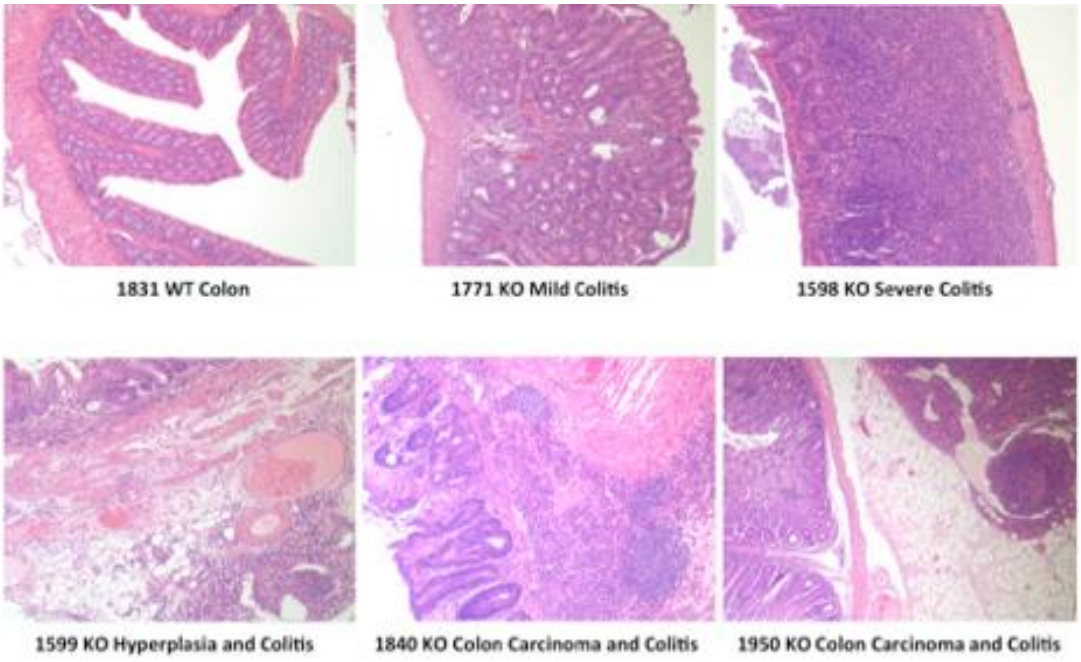


Figure 3.2 Colitis and Colitis-Associated Cancer in HBP1$^{-/-}$ Mice. H&E staining of the colons from WT and HBP1$^{-/-}$ mice at 10X Magnification. The different stages of colitis are presented in HBP1$^{-/-}$ mice (1771,1598) due to the infiltration of immune cells and the morphological changes to the epithelium. Hyperplasia and carcinomas are displayed in the HBP1$^{-/-}$ mice (1599,1840,1950) who exhibit sever colitis by the invasion of the colonic epithelium into the smooth muscle. The WT mouse has the morphology of a normal colon without the infiltration of immune cells.

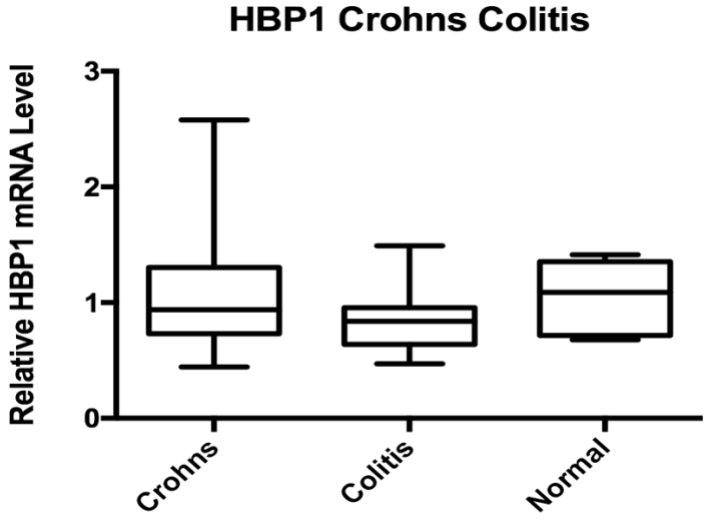
	HBP1 WT	HBP1$^{-/-}$	Total
No Colitis	9	7	15
Colitis	0	6	6
Total	9	13	22

Fisher's Exact Test p=0.046

Table 3.1 Incidence of Colitis in HBP1$^{-/-}$ mice. The significance test was determined by Fisher's Exact Test.

3.1.3. Decreased HBP1 Expression in Colitis Patients

Due to the results from HBP1$^{-/-}$ mice suggesting that the HBP1 gene is related to UC and CAC, a look at HBP1 gene expression was performed on patients with IBD. Human cDNA samples were obtained from normal, Crohn's, and UC patients. The cDNA was used to measure the mRNA levels of HBP1. The HBP1 mRNA expression was significantly lower in the UC group when compared to the normal group and the Crohn's respectively (Figure 3.3).



Unpaired T-test with Welch's Correction

Crohns vs Colitis	0.0128
Normal vs Colitis	0.0420
Normal vs Crohns	0.8462

Figure 3.3. HBP1 mRNA expression in Human Colitis Patients. Human tissue cDNA arrays from patients with UC and CD were analyzed by qPCR for HBP1 expression.

3.2 Increased Wnt Signaling in HBP1$^{-/-}$ Colons

3.2.1. Protein Expression of HBP1$^{-/-}$ Mice

The tissue of gastrointestinal tract is the most rapidly renewing tissue in the body, requiring constant tissue regeneration. There has been extensive research on the small intestinal epithelial providing a well characterized structure of the small intestine(Andersson-Rolf, Zilbauer, Koo, & Clevers, 2017). The Wnt pathway has been identified as an important regulator of intestinal homeostasis, by controlling the adult

stem cells within the intestine, which are labeled the crypt cells of the intestine (Doerks, Copley, Schultz, Ponting, & Bork, 2002; Kuhnert et al., 2004). Based on the previous work from others, we believe that the Wnt pathway must be playing a role in the onset of UC and CAC in the HBP1$^{-/-}$ mice. A western blot analysis was performed on the intestinal crypts of HBP1$^{-/-}$ and WT mice. When the two groups were compared, the HBP1$^{-/-}$ mice had increased β-catenin protein levels (Table 3.4 & 3.5).

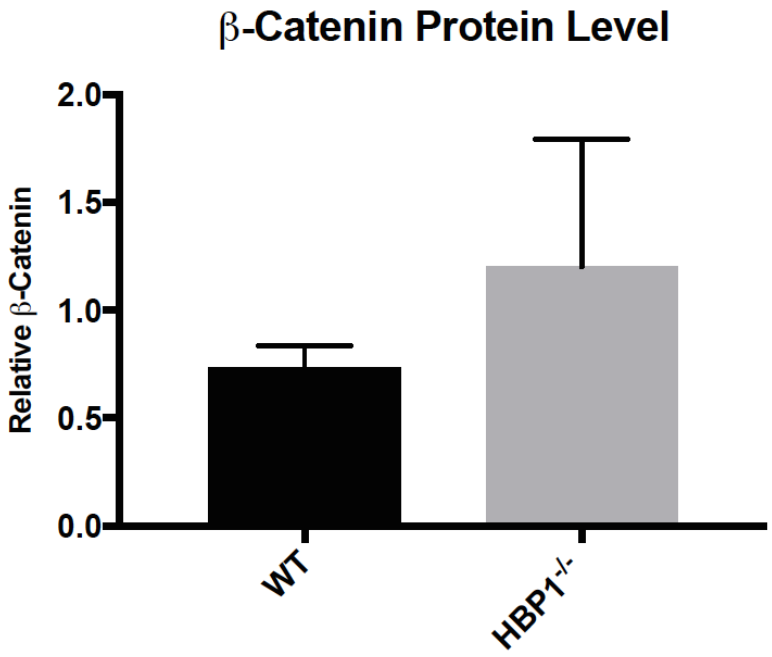


Fig. 3.4. β-catenin Protein Level. A quantitative expression of the protein level of β-catenin from intestinal crypts. β-catenin expression was normalized to actin.

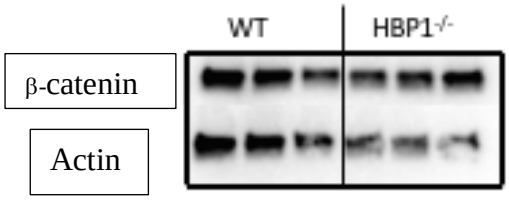


Fig. 3.5. Western Blot analysis of β-catenin. Protein was extracted from in the intestinal crypts located in the colon of WT and HBP1$^{-/-}$ mice, and a western blot was performed on the protein level of β-catenin.

3.2.2. Wnt target gene expression in HBP1$^{-/-}$ mice

As previously stated, AXIN is part of the destruction complex that phosphorylates β-catenin(Kahn, 2014). Because β-catenin is typically the target for a variety of therapeutics, many new therapeutics have begun targeted Axin2 in hopes to phosphorylate β-catenin. When Axin2 mRNA levels were evaluated through qPCR, Axin2 was increased in HBP1$^{-/-}$ mice when compared to the WT mice (Figure 3.6). LGR5 expression was also measured, and the mRNA expression of LGR5 was increased in the intestinal crypts in the HBP1$^{-/-}$ mice when compared to the WT (Figure 3.7).

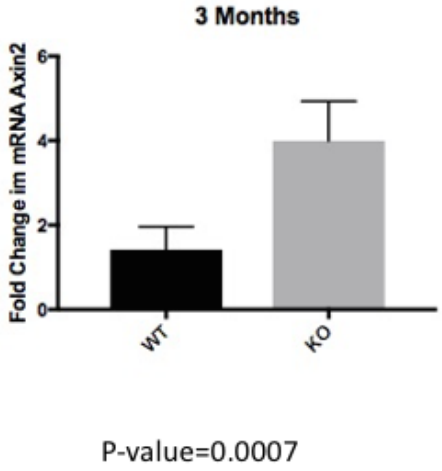
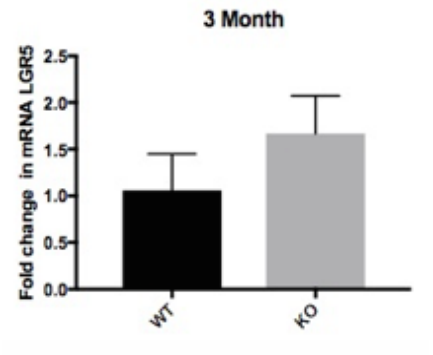


Fig. 3.6. mRNA Expression of Axin2. RNA was isolated from the colonic intestinal crypts of HBP1$^{-/-}$ and WT mice. qPCR was performed and the mRNA expression of Axin2 was measured. Expression levels were normalized to 18S control.



P-value=0.0417

Fig. 3.7. mRNA Expression of LGR5. RNA was isolated from the colonic intestinal crypts of HBP1$^{-/-}$ and WT mice. qPCR was performed and the mRNA expression of LGR5 was measured. Expression levels were normalized to 18s control.

3.3. Increased Tissue Renewal and Regeneration in HBP1$^{-/-}$

3.3.1 Organoid Formation is Increased in HBP1$^{-/-}$

Organoid formation experiments are instrumental in the field of translational science. This method provides insight into the driving forces behind intestinal renewal and homeostasis. Organoid formation experiments can also be used as a model for understanding intestinal complications like inflammation and cancer (Barker et al., 2010). Crypts were isolated from HBP1$^{-/-}$ and WT mice and plated in in matrigel to measure organoid proliferation. The HBP1$^{-/-}$ mice had a 2-fold increase of crypt-organoid formation when compared to WT mice (Figure 3.8). It was also observed that the organoids that were formed in the HBP1$^{-/-}$ mice were larger than the WT organoids (Figure 3.9).

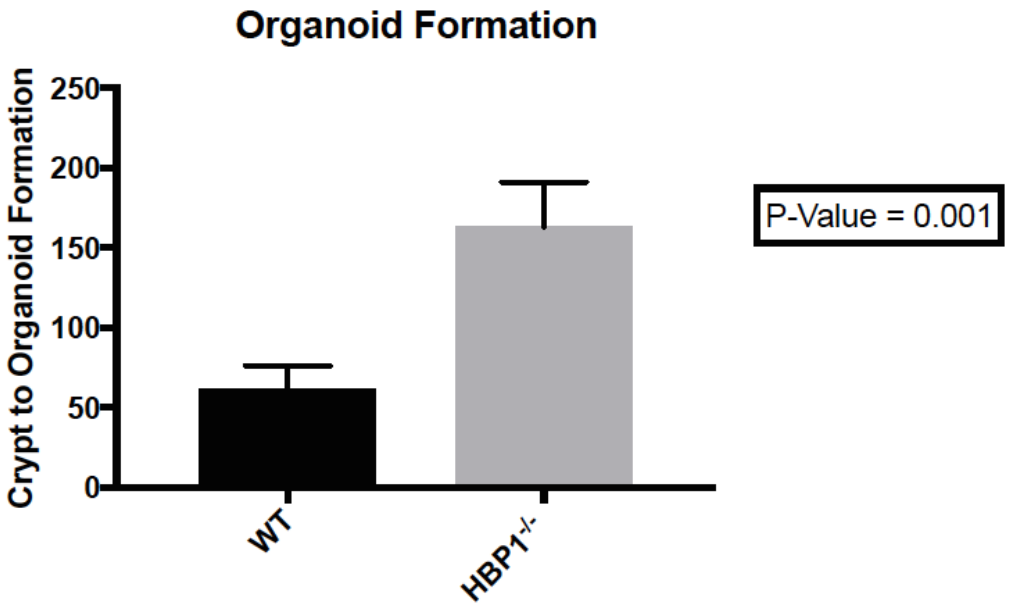


Figure 3.8. Organoid Formation Experiments. Intestinal crypts were isolated from WT and HBP1$^{-/-}$ mice and cultured for 4 days to record the amount of organoids formed. The number of organoids formed in the HBP1$^{-/-}$ mice was significantly higher than the WT. The crypts were seeded into 5 wells at 200-250 crypts per well. The statistical significance was calculated by a two tailed t-test.

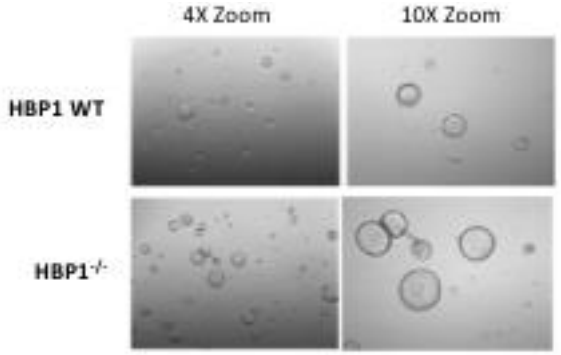


Figure 3.9. Increased Organoid Size. The size of organoids formed in the HBP1$^{-/-}$ mice was significantly higher than the WT. Intestinal crypts were isolated from WT and HBP1$^{-/-}$ mice and cultured for 4 days to record the amount of organoids formed. On the 4th day of culturing, images were taken of the formed organoids at 4X and 10X.

3.4. HBP1$^{-/-}$ Mice are More Susceptible to DSS-induced Colitis

3.4.1. DSS Experiments

HBP1$^{-/-}$ and WT mice were administered 1% DSS drinking water for 7 days, in which the mice would be monitored for UC symptoms. The HBP1$^{-/-}$ mice had an increased susceptibility to DSS-induced colitis when compared to the WT mice. HBP1$^{-/-}$ mice experience increased average weight loss after two rounds of DSS treatment (Figure 3.1). The HBP1$^{-/-}$ mice also had severe rectal bleeding, and a higher incidence of blood found in their fecal matter when compared to the WT mice (Figure 3.11). Also, relative to the WT, the HBP1$^{-/-}$ mice had decreased survival during the 1% DSS-induced colitis experiments (Figure 3.12). Finally, histological analysis of HBP1$^{-/-}$ mouse colon shows increased immune cell infiltration relative to the WT (3.13).

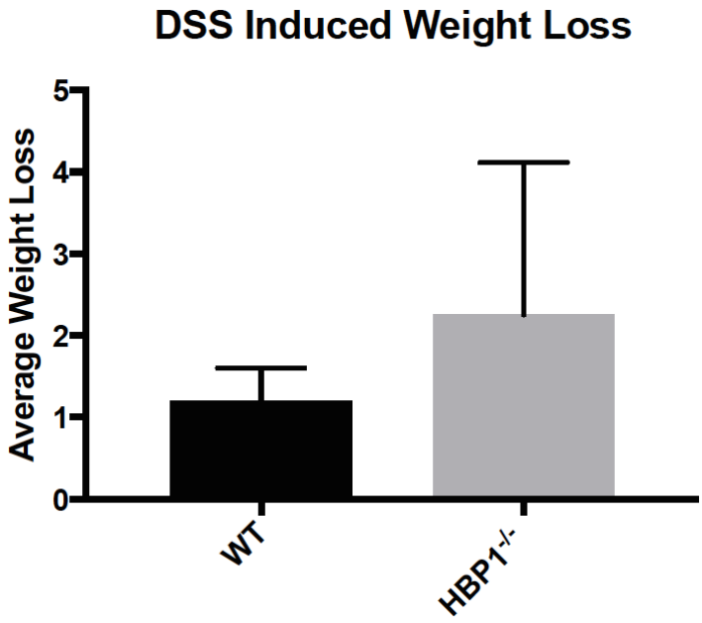


Figure 3.10. DSS induced Weight Loss. On average, the HBP1$^{-/-}$ mice experienced severe weight loss relative to the WT mice. 3 WT and 3 HBP1$^{-/-}$ mice, aged 3 months, underwent 2 rounds of 1% DSS treatment for 7 days, followed by 2 weeks of recovery with regular water. The average weight was of each mouse was recorded after each round.

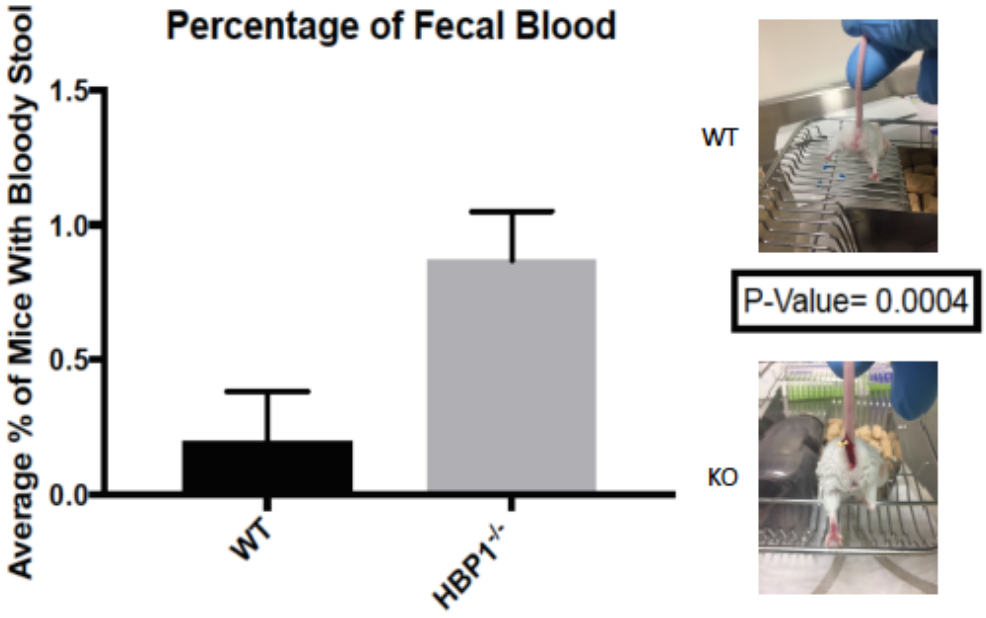


Figure 3.11. Increased of Fecal Blood. Relative to the WT, the HBP1$^{-/-}$ mice had an increased incidence of fecal blood during 1% DSS treatment. 3 WT and 3 HBP1$^{-/-}$ mice, aged 3 months, underwent DSS treatment and had their stool tested for blood throughout the treatment (sample taken: n=5). The statistical significance was calculated through a t-test in Prism 7. Images of a WT and HBP1$^{-/-}$ were taken on the last day of DSS treatment.

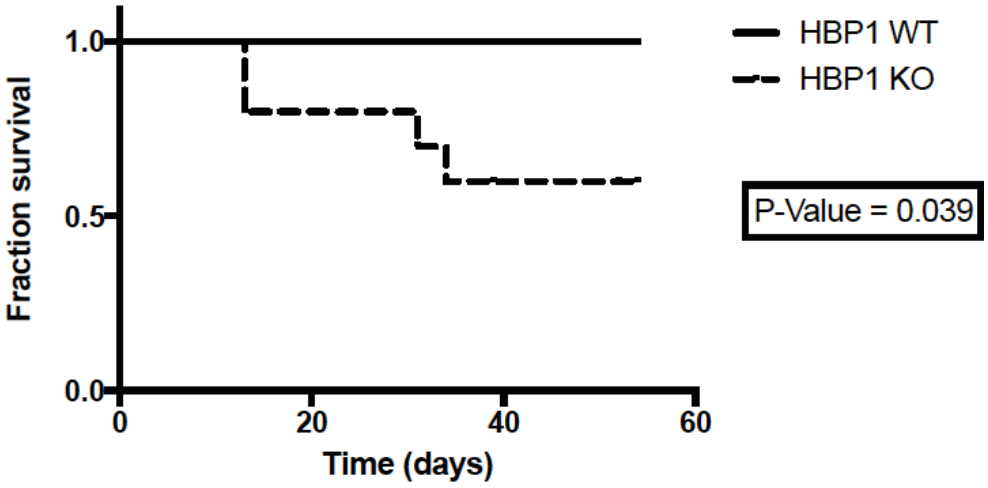


Figure 3.12. Decreased Survival Rate. Relative to the WT, the HBP1$^{-/-}$ mice experience decreased survival during the 1% DSS treatment. 9 WT and 10 HBP1$^{-/-}$ mice, aged 3 months, were put through 2 rounds of 1% DSS treatment, and the survival of these mice were recorded. A Kaplan-Meir survival was used in Prism 7 to calculate the fraction of survival and the p-value was calculated using the Gehan-Breslow-Wilcoxon test in Prism 7.

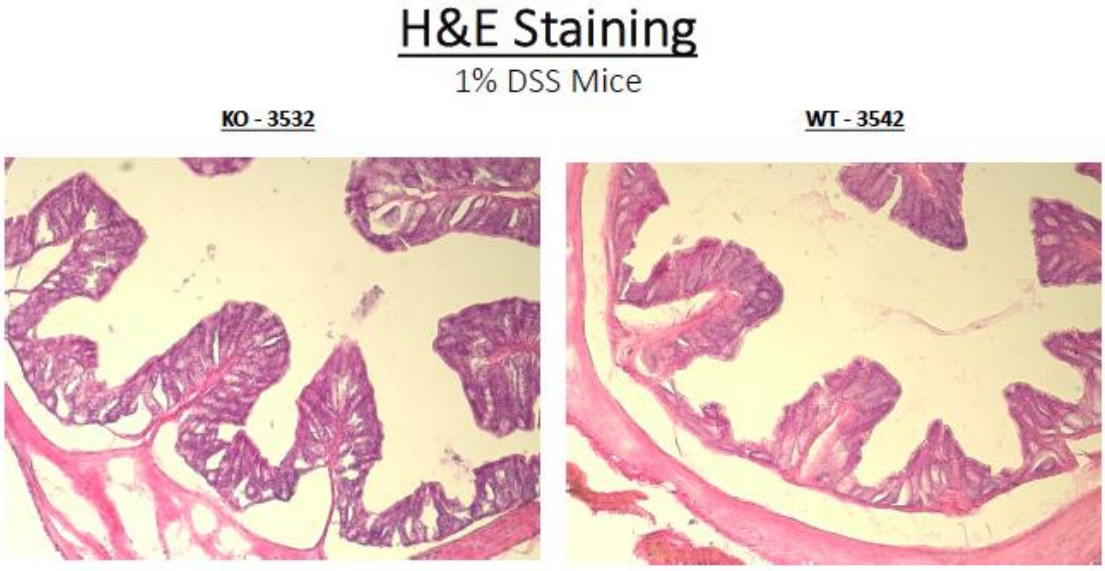


Figure 3.13. Immune Cell Infiltration. The HBP1$^{-/-}$ mice presented an increased immune cell infiltration when compared to the WT. A routine H&E staining was performed on the mice administered 1% DSS. The colons were harvested after the 2 rounds of DSS and embedded in OCT before being sectioned.

3.4.2. Gender Differences in DSS-induced Colitis

Another interesting finding during the DSS experiments was the difference of severity and survival rate between genders. The males in the DSS experiments experience a lower survival rate than females (Figure 3.14). While not significant, it is important to note that the majority of deaths during the DSS experiments were amongst males.

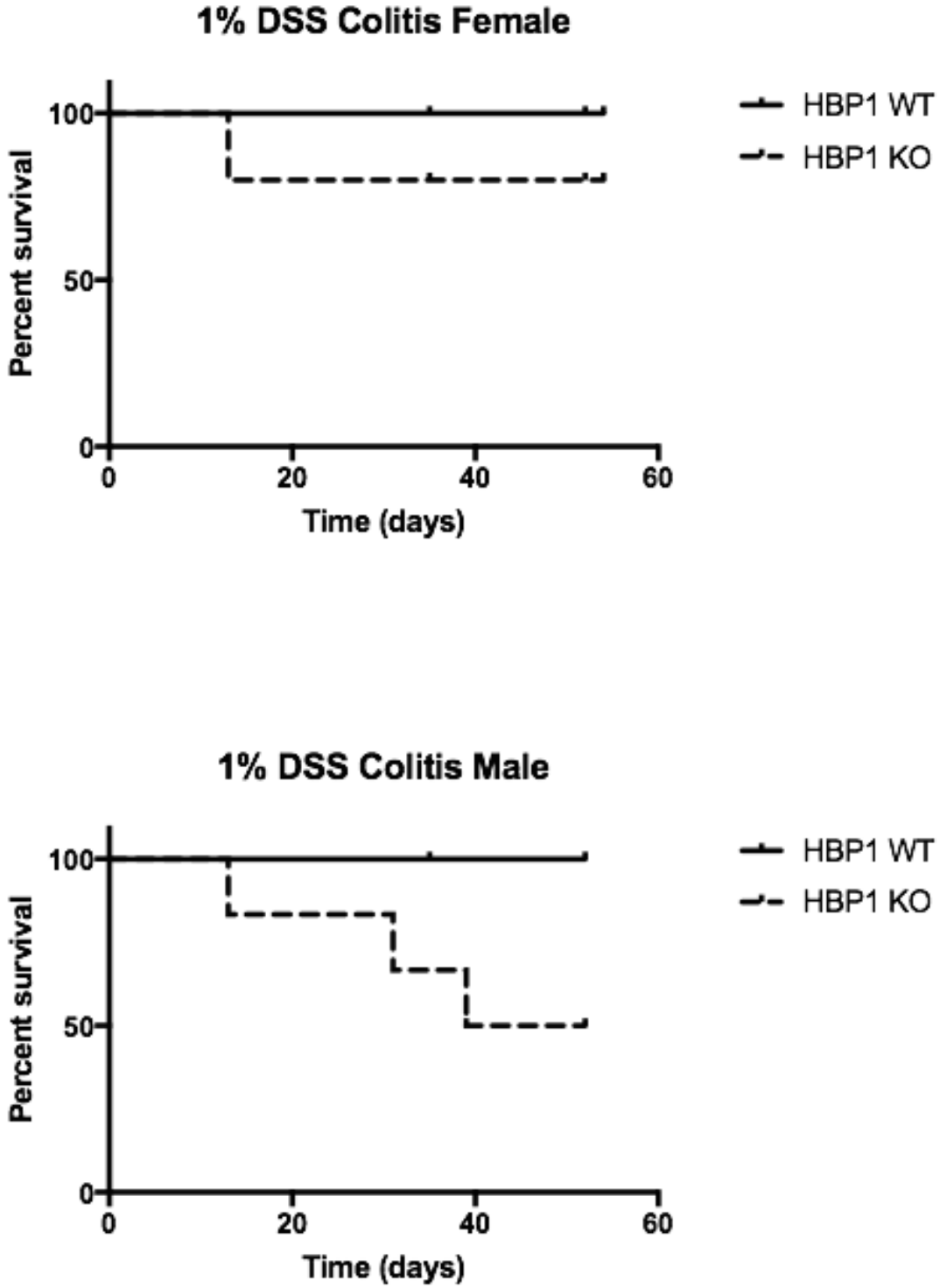


Figure 3.14. Differences amongst Genders in DSS-induced colitis. These graphs represent the survival rate of mice in the DSS-induced colitis experiments. The two different graphs represent the different survival rate between male and female mice.

Chapter 4: Discussion

4.1. Increased Wnt Signaling in HBP1$^{-/-}$ Colons

The data suggest an upregulation in the Wnt signaling pathway in HBP1$^{-/-}$ mice with observations of increased β-catenin protein levels, and increased Axin2 mRNA expression from the intestinal crypts from the colon. High β-catenin levels are correlated with high levels of Wnt signaling, while Axin2 is also important because it has been linked to the initiation of CRC (Kahn, 2014; Wu et al., 2012). These results strengthen the hypothesis that HBP1 deletion could contribute to UC and CAC by increasing Wnt signaling and tumor initiation. This data gives a glance at the molecular components of UC and CAC progression, suggesting the Wnt pathway as a possible therapeutic target.

The mice used in these experiments are 3 months of age, which does not give a true representation of mice that experience UC and CAC. To further investigate the role of Wnt signaling in UC and CAC, it would be beneficial to better understand the progression of the disease. Repeating the western blot, qRT-PCR and histology experiments in HBP1$^{-/-}$ mice at different ages may open a window into actual disease progression. Because the UC and CAC increase with age, it would be interesting to see a time-line during HBP1$^{-/-}$ mouse lifespan. This would allow for the a better characterization of the molecular mechanisms associated with Wnt signaling that are believed to be contributing to the progression and onset of UC and CAC.

4.2. HBP1$^{-/-}$ Mice Exhibit Increased Tissue Renewal

The organoid formation assay suggests that mice lacking the HBP1 gene have increased tissue renewal and regeneration in the colon relative to WT mice. A prominent component of tissue regeneration in the colon is due to Wnt signaling, in which these

results suggest that Wnt signaling may be increased in HBP1$^{-/-}$ crypts (Barker et al., 2010). Again, the age of the mice experimented on in this study are not representative to the tissue renewal at later stages of the HBP1$^{-/-}$ life span. So, it would be useful to run these experiments on crypts that have been isolated from older mice.

4.3. HBP1$^{-/-}$ Mice are More Susceptible to DSS-induced Colitis

These results allow us to have a pre-clinical explanation of the role of HBP1 in UC and CAC. The results suggest that the deletion of HBP1 has an effect on the susceptibility of DSS-induced colitis by causing more severe signs of UC, as well as eliciting a stronger immune response comparing HBP1$^{-/-}$ to WT from the H&E staining. This leads us to believe that HBP1 plays a role in the onset of UC that will later develop CAC. The interesting results regarding UC susceptibility in the HBP1 mouse model suggests its possible use as a pre-clinical model for the study of UC to CAC progression, and a possible biomarker for UC and CAC. While DSS-induced colitis is a good model to demonstrate acute colitis, it does not give an accurate representation of cancer progression. Azoxymethane (AOM) is a chemical compound that is used to induce cancer in mice, and can be beneficial in these experiments. To better investigate the progression of CAC from UC, it would be beneficial to see if AOM induced cancer is more severe/increased in HBP1$^{-/-}$ mice.

4.4. HBP1$^{-/-}$ Mouse Model Limitations

These studies demonstrated that the deletion of the HBP1 gene contributes to the UC and CAC. While these findings are extremely significant and useful, there are questions surrounding the limitations of the HBP1 mouse model. The mouse model used in these experiments exhibits a global knockout of the HBP1 gene, which does not allow for a

concise characterization of the HBP1 gene deletion in the colon or immune cells. As stated earlier, LGR5 is an integral component of the intestinal stem cell proliferation in the intestine (Barker et al., 2010). Therefore, a LGR5-Cre/HBP1 flox/flox mouse model can be used to better describe the molecular mechanisms involved in UC and CAC in a colon epithelia-specific setting.

Another important component of UC and CAC is the immune response that sequesters proinflammatory cells to the site of UC. It has been established that proinflammatory cytokines are increased in colitis patients, specifically IL-17 (Xhu et al., 2018). Because of IL-17's contribution to UC, it would be useful to produce an IL17-Cre/HBP1 flox/flox mouse model. There are other proinflammatory cytokines of the T-cell lineage that contribute to UC, which could also provide useful models to investigate the HBP1 gene's role in UC and CAC.

4.5. Pharmacological Implications

The data in this study suggest that the Wnt signaling pathway can provide therapeutic targets for UC patients, as well as propose HBP1 as a possible biomarker for the UC and CAC population. It would be interesting to see if inhibiting Wnt signaling in the $HBP1^{-/-}$ mice would improve the outcome or decrease the number of mice who develop colitis. These experiments would need to be well thought out and designed properly to fully test the therapeutic results on the $HBP1^{-/-}$ mice. For example, to test therapies for patients who have already been diagnosed, the $HBP1^{-/-}$ and WT mice should be administered the therapeutic after symptoms have been observed in DSS treated mice. Or, if the onset of UC was well defined from the previously suggested studies, the therapeutic could be administered to free living mice at the time of the known early onset

of UC. This would be useful to see if inhibiting Wnt signaling would prevent/lessen UC development. These studies would give insight into not only possible Wnt signaling inhibiting therapies, but also provide insight into how applicable HBP1$^{-/-}$ mice will be as pre-clinical model.

There are currently no approved pharmacologic therapies that directly target the Wnt pathway, and the available therapies that do alter the Wnt pathway have mild alterations causing for a weak inhibition of Wnt signaling. A major setback for therapies that are targeting the Wnt pathway are the off target effects. Wnt signaling is crucial in many tissues throughout the body, which makes altering Wnt signaling dangerous if it is not directed at the specific site of interest. Many studies have indicated that Wnt signaling agonist and antagonist end up causing severe side effects, with the intestine being a common site of the adverse side effects(Kahn, 2014).

While there are many drugs in the preclinical phase, there are presently phase I clinical studies that are yielding promising results. Specifically, the porcupine (PORCN) inhibitor LGK974, also known as WNT974. PORCN has been identified as a requirement for Wnt signaling in regards to tissue regeneration and cancer (B. Chen et al., 2009). LGK974 is currently in phase I trials at Novartis, and it has shown promising in vitro and in vivo results indicating a strong Wnt signaling inhibition (Liu et al., 2013). Liu et al. wonderfully presented LGK974's Wnt inhibition properties, its pharmacokinetics and pharmacodynamics, and its efficacy in human head and neck squamous cell carcinoma model. This PORCN inhibitor surprisingly presented little to no side effects in multiple Wnt dependent tissues, specifically the intestine. It would be interesting to see the results of LGK974 in our HBP1$^{-/-}$ mouse to see if its Wnt inhibiting properties truly stands the

test. Also, if the trials were successful in reversing the onset/severity of colitis in the HBP1$^{-/-}$ model, it would further strengthen our HBP1 mouse model's ability to be a suitable preclinical model to study other possible Wnt inhibiting therapies.

Chapter 5: Conclusion

The deletion of the HBP1 gene in this mouse model suggests that HBP1 plays a role in the development of UC with signs of CAC progression. These finding suggest that HBP1 could be a possible stratification biomarker, while also providing possible therapeutic targets within the Wnt signaling pathway. The $HBP1^{-/-}$ mouse model also has promising results that make it eligible for a possible pre-clinical animal model to investigate the pathophysiology of UC and CAC progression.

Bibliography

Andersson-Rolf, A., Zilbauer, M., Koo, B. K., & Clevers, H. (2017). Stem Cells in Repair of Gastrointestinal Epithelia. *Physiology (Bethesda), 32*(4), 278-289. doi:10.1152/physiol.00005.2017

Barker, N., Huch, M., Kujala, P., van de Wetering, M., Snippert, H. J., van Es, J. H., . . . Clevers, H. (2010). Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell, 6*(1), 25-36. doi:10.1016/j.stem.2009.11.013

Beaugerie, L., & Itzkowitz, S. H. (2015). Cancers complicating inflammatory bowel disease. *N Engl J Med, 372*(15), 1441-1452. doi:10.1056/NEJMra1403718

Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C. W., . . . Lum, L. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol, 5*(2), 100-107. doi:10.1038/nchembio.137

Chen, R., Rabinovitch, P. S., Crispin, D. A., Emond, M. J., Bronner, M. P., & Brentnall, T. A. (2005). The initiation of colon cancer in a chronic inflammatory setting. *Carcinogenesis, 26*(9), 1513-1519. doi:10.1093/carcin/bgi106

Doerks, T., Copley, R. R., Schultz, J., Ponting, C. P., & Bork, P. (2002). Systematic identification of novel protein domain families associated with nuclear functions. *Genome Res, 12*(1), 47-56. doi:10.1101/

Fodde, R., & Smits, R. (2001). Disease model: familial adenomatous. *Cell Press, 7*(8), 5.

Grady, W. M., & Pritchard, C. C. (2014). Molecular alterations and biomarkers in colorectal cancer. *Toxicol Pathol, 42*(1), 124-139. doi:10.1177/0192623313505155

Herrinton, L. J., Liu, L., Levin, T. R., Allison, J. E., Lewis, J. D., & Velayos, F. (2012). Incidence and mortality of colorectal adenocarcinoma in persons with inflammatory bowel disease from 1998 to 2010. *Gastroenterology, 143*(2), 382-389. doi:10.1053/j.gastro.2012.04.054

Jess, T., Simonsen, J., Jorgensen, K. T., Pedersen, B. V., Nielsen, N. M., & Frisch, M. (2012). Decreasing risk of colorectal cancer in patients with inflammatory bowel disease over 30 years. *Gastroenterology, 143*(2), 375-381 e371; quiz e313-374. doi:10.1053/j.gastro.2012.04.016

Kahn, M. (2014). Can we safely target the WNT pathway? *Nat Rev Drug Discov, 13*(7), 513-532. doi:10.1038/nrd4233

Kanneganti, M., Mino-Kenudson, M., & Mizoguchi, E. (2011). Animal Models of Colitis-Associated Carcinogenesis. *Journal of Biomedecine and Biotechnology, 2011*(Article ID 342637), 23. doi:0.1155/2011/342637

Keubler, L. M., Buettner, M., Hager, C., & Bleich, A. (2015). A Multihit Model: Colitis Lessons from the Interleukin-10-deficient Mouse. *Inflamm Bowel Dis, 21*(8), 1967-1975. doi:10.1097/MIB.0000000000000468

Kuhnert, F., Davis, C. R., Wang, H. T., Chu, P., Lee, M., Yuan, J., . . . Kuo, C. J. (2004). Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci U S A, 101*(1), 266-271. doi:10.1073/pnas.2536800100

Liu, J., Pan, S., Hsieh, M. H., Ng, N., Sun, F., Wang, T., . . . Harris, J. L. (2013). Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. *Proc Natl Acad Sci U S A, 110*(50), 20224-20229. doi:10.1073/pnas.1314239110

Molodecky, N. A., Soon, I. S., Rabi, D. M., Ghali, W. A., Ferris, M., Chernoff, G., . . . Kaplan, G. G. (2012). Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology, 142*(1), 9. doi:10.1053/j.gastro.2011.10.001

Nusse, R., & Clevers, H. (2017). Wnt/beta-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell, 169*(6), 985-999. doi:10.1016/j.cell.2017.05.016

Rennick, D. M., & Fort, M. M. (2000). IL-10-defecient (IL-10-/-) mice and intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol, 278*(6), 4.

Rubin, D. C., Shaker, A., & Levin, M. S. (2012). Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer. *Front Immunol, 3*, 107. doi:10.3389/fimmu.2012.00107

Sampson, E. M., Haque, Z. K., Ku, M.-C., Tevosian, S. G., Albanese, C., Pestell, R., . . . Yee, A. S. (2001). Negative regulation of the Wnt-B-catenin pathway by the transcriptional repressor HBP1. *The EMBO Journal, 20*(16), 12.

Shih, H. H., Xiu, M., Berasi, S. P., Sampson, E. M., Leiter, A., Paulson, K. E., & Yee, A. S. (2001). HMG Box Transcriptional Repressor HBP1 Maintains a Proliferation Barrier in Differentiated Liver Tissue. *Molecular and Cellular Biology, 21*(17), 5723-5732. doi:10.1128/mcb.21.17.5723-5732.2001

Tenesa, A., & Dunlop, M. G. (2009). New insights into the aetiology of colorectal cancer from genome-wide association studies. *Natur Reviews, 10*.

Van Zant, G., & de Haan, G. (2004). Genetic control of lifespan: studies from animal models. *Expert Reviews in Molecular Medicine, 1*(20). doi:10.1017/s1462399499001441

Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A., Jr., & Kinzler, K. W. (2013). Cancer genome landscapes. *Science, 339*(6127), 1546-1558. doi:10.1126/science.1235122

Wu, Z. Q., Brabletz, T., Fearon, E., Willis, A. L., Hu, C. Y., Li, X. Y., & Weiss, S. J. (2012). Canonical Wnt suppressor, Axin2, promotes colon carcinoma oncogenic activity. *Proc Natl Acad Sci U S A, 109*(28), 11312-11317. doi:10.1073/pnas.1203015109

Xhu, Q., Zheng, P., Chen, X., Zhou, F., He, Q., & Yang, Y. (2018). Andrographolide presents therapeutic effect on ulcerative colitis through the inhibition of IL-23/IL-17 axis. *Am J Transl Red., 10*(2), 8.