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Effects of microbiota modulations on profiles of intestinal stem cell markers

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Abstract

The intestinal epithelium consists of several cell types such as absorptive enterocytes, enterochromaffin cells, goblet cells and Paneth cells, which are influenced by different luminal factors that can lead to apoptosis of these cells. To maintain the function of the intestine over a whole life span, a system of replacement for these cells is necessary. Since the 1970s it is known that intestinal stem cells, which are located at the base of the gastrointestinal crypts, perform this task by forming progenitor cells and terminal differentiated cells of the intestinal epithelium. A dysfunction of stem cells may be an important factor for carcinogenesis in the gastrointestinal tract. It is also known that inflammatory bowel disease or metabolic syndrome are associated with alterations in stem cell function and a higher risk of gastrointestinal tumors.

Recent data suggest that changes in diet or microbial metabolites can influence epithelial self-renewal and intestinal stem cell function. There is a high correlation between high fat diet, stem cell dysfunction and tumorigenesis in the gastrointestinal tract. However, the effects of the microbiome on intestinal stem cell function have not yet been investigated in detail *in vivo*. To explore the biology of intestinal stem cells stem-cell-specific proteins like Leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5), Olfactomedin 4 (Olfm4), Achaete-scute family bHLH transcription factor 2 (Ascl2), Ring finger protein 43 (Rnf43) or SRY-box transcription factor 9 (Sox9) have been established as surrogate markers for these cells.

I examined how pharmacologically induced changes of the microbiome by pro- or antibiotics influenced the expression of stem cell markers in the intestines of laboratory mice. C57BL/6 inbred mice were treated with probiotics (a mixture of *Lactobacilli* and *Bifidobacteria*), the non-absorbable antibiotic rifaximin or a broad-spectrum mixture of antibiotic drugs (ampicillin, neomycin sulfate, vancomycin, metronidazole; AMT) for two weeks. As a main result, AMT induced a significant downregulation of Lgr5, Rnf43 and Ascl2 in large bowel of mice. Next, I generated intestinal organoids and treated them with microbial metabolites or pathogen-associated molecular patterns (PAMPs) to investigate putative microbiota-host interaction pathways. LPS, Reg3b and butyrate significantly modulated the expression of stem cell markers.

In a clinical pilot study performed at the Department of Internal Medicine II, Klinikum rechts der Isar, and Vorsorgezentrum für Innere Medizin und Gastroenterologie, Dr. med. Albert Eimiller, I investigated the association of the altered intestinal microbiome with the expression levels of intestinal stem cell markers in a cohort of patients with expected compositional microbiome differences: healthy controls (n = 10), study subjects with inflammatory bowel disease (n = 9), or with metabolic syndrome (n = 7). I found a significant upregulation of Olfm4 in patients with ulcerative colitis. The microbial analysis showed a clear decrease of microbial variety with a shift

towards more abundant Firmicutes (phylum) and changing profiles of bacterial families in the groups of patients with metabolic syndrome and UC compared to the fecal microbiomes of healthy individuals.

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2. Abbreviations

(d)dH_2O	(Double) distilled water
(m)M	(Milli)molar
AMT	Antimicrobial treatment
Ascl2	Achaete-scute family bHLH transcription factor 2
CBC	Lgr5+ crypt base columnar cell
cDNA	Complementary deoxyribonucleic acid
CTR	Control
d	Day(s)
DGVS	Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselerkrankungen
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotides
FZD	Frizzled receptor
g	Gram(s)
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
h	Hour(s)
HCL	Hydrochloric acid
HE-staining	Hematoxylin and Eosin staining
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IVC	Individually ventilated cage
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
Lgr5	Leucine-rich-repeat-containing G-protein-coupled receptor 5
LPS	Lipopolysaccharides
MIH	Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Munich, Germany
min	Minute(s)
MS	Metabolic syndrome

Myd88	Myeloid differentiation primary response 88
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
ng	Nanogram
Olfm4	Olfactomedin 4
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction
Reg3b	Regenerating islet-derived 3 beta
RNA	Ribonucleic acid
Rnf43	Ring finger protein 43
rpm	Revolutions per minute
sec	Second(s)
Sox9	SRY-box transcription factor 9
SPF	specific-pathogen-free
TLR	Toll-like receptor
UC	Ulcerative colitis
UK	United Kingdom
USA	United States of America
y	Year(s)

3. Introduction

3.1. Epithelial organization of colonic crypts

The human gut and its physiology are representing a fascinating network that can affect our physical wellbeing in various aspects. A broad spectrum of diseases is known to imbalance this finely engineered system. The causes of disease vary from infectious, autoimmune, allergic, inflammatory origin to malignant transformation. With a surface area of more than 30 m^2 (Helander & Fändriks, 2014) the intestinal mucosa faces various challenges. The intestinal epithelium is composed of millions of crypt-villus units (Lieberkühn, 1745) consisting of a finger-like protrusion of the mucosa in the lumen, the villi and several surrounding invaginations of the mucosa, the crypts. In the large intestine villi are missing, only crypts are folded by the epithelium.

The colonic mucosa is composed of functional units termed the crypts of Lieberkühn that are histologically organized in distinct layers. A single-layered columnar epithelium harbors the terminally differentiated cells that are located at the top third of the crypt and are permanently shed into the intestinal lumen after 3 – 5 d (Kaiko et al., 2016). The colon generates three epithelial cell types: the absorptive enterocytes, the mucus-secreting goblet cells and the hormone-producing enteroendocrine cells. The cellular composition of epithelium varies due to its more secretory or absorptive functions along the bowel and depends on the presence or absence of Notch signaling while the first fate decision. Absent Notch signals promote differentiation to secretory cells (Paneth cells, goblet cells, enteroendocrine cells or Tuft cells), whereas active Notch signaling is linked to the absorptive lineage (enterocyte or M cells) (Gehart & Clevers, 2019). The lower two thirds of the crypt correspond to the transit-amplifying zone (TA) and the stem cell-harboring base of the crypt (Radtke & Clevers, 2005). The multipotent stem cells undergo self-renewal and create a population of transit-amplifying cells during asymmetric division. During maturation process these cells leave the base of the crypts and move upward the crypt thus replacing all epithelial cell types. Ensuring these features is possible due to the highly sophisticated architecture, the crypt-villus structure, and the ability to maintain epithelial homeostasis by self-renewal.

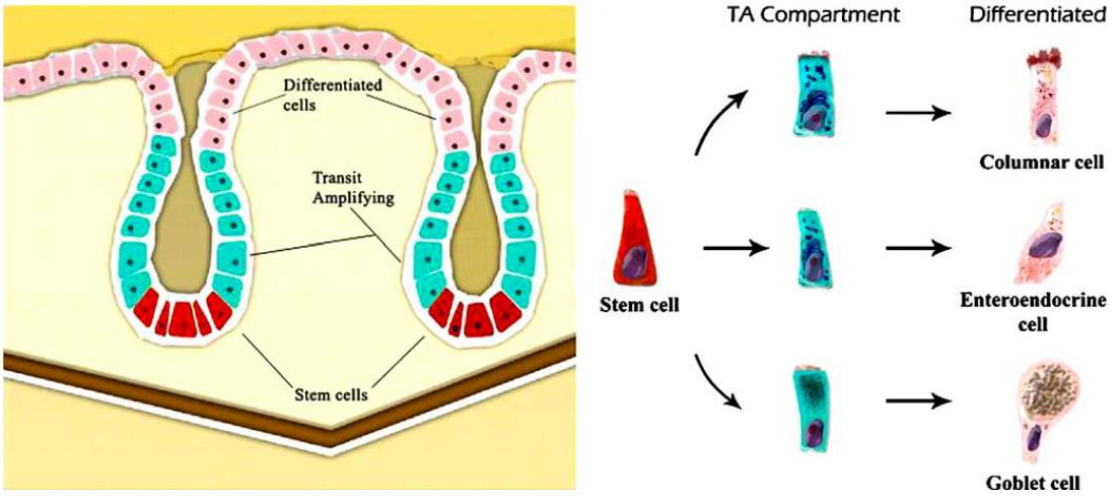


Figure 1: Colonic crypt organization and epithelial cell types

The lining of the colonic mucosa forms the crypts of Lieberkühn with intestinal stem cells (red) located at the crypts base. Due to asymmetric divisions the proliferating and differentiating daughter cells move upward the crypt by passing the transit-amplifying (TA) compartment (turquoise). The top third of the colonic crypt is composed of three terminally differentiated epithelial cells (pink): the absorptive columnar cells, the hormone-producing enteroendocrine cells and the mucus-secreting goblet cells. (source: Ricci-Vitiani et al., 2009)

Due to its architecture the large surface is primarily responsible for absorptive functions. Unique in its function the intestinal epithelium enables uptake of water and nutrients as well as metabolic exchange. Beside this, the gut epithelium acts as protective barrier against potential pathogens, toxins, extreme changes in pH value and mechanical damage. The physical barrier is enhanced by a narrow opening of the crypt (~ 6 µm) and mucus secretion from goblet cells, thereby providing a protective milieu for stem cells. In the large bowel the permanent fluid stream out of the crypt is necessary to effectively preserve the intestinal stem cells at the crypt's base from the pathogens, microbial metabolites, and digestive procedures in the gut lumen.

The specialized microenvironment at the crypt's base is called the intestinal stem cell niche and is composed either of Paneth cells or of Reg4+ deep crypt secretory cells, and the underlying mesenchyme. At the crypt's base of the small intestine each CBC cell needs to be in direct contact with at least one Paneth cell enabling stem cell activity. Paneth cells were shown to nurture CBC cells maintenance by providing Wnt ligands, EGF and Notch factors (Sato et al., 2011). They also act as "bodyguards" of the stem cell niche by producing several antimicrobial factors such as lysozyme, α-defensins and phospholipase A2 which are found highly concentrated in the crypt as well as in the intestinal lumen (Gassler, 2017). In the lumen the antimicrobial products blend into the goblet-cell secreted mucus and act as immunological barrier along the whole intestinal mucosa (Allaire et al., 2018). Additionally, Paneth cells produce glycolysis-derived lactate which serves as substrate for mitochondrial oxidative phosphorylation

in CBC cells enabling them to proliferate (Rodríguez-Colman et al., 2017). Whereas Paneth cells are missing in the colon, Reg4+ deep crypt secretory cells were found there fulfilling the same functions as Paneth cells (Sasaki et al., 2016). Beside the structural support, the underlying mesenchyme has an important role in regulating the stem cell activity, too. Represented by myofibroblasts, fibroblasts, pericytes, endothelial cells, neural cells, and smooth muscle cells (muscularis mucosae), the mesenchyme is located at the intestinal lamina propria and acts as a source of Wnt ligands, R-spondins, BMPs and BMB antagonists. It was shown that GLI1+ mesenchymal cells produce Wnt2B (Valenta et al., 2016) and CD34+GP38+aSMA- mesenchymal cells are secreting Wnt2B, Rspo1 and Gremlin 1 (Bmp inhibitor) (Stzepourginski et al., 2017).

Representing the intestinal stem cells (ISC) dividing crypt base columnar (CBC) cells were found at the bottom of the crypts of Lieberkühn in the small intestine and in the colon (Ricci-Vitiani et al., 2009). Active proliferating CBC cells were identified by the highly specific marker Lgr5 (Barker et al., 2007), a receptor for R-spondins that regulate Wnt signaling pathway (Glinka et al., 2011; Lau et al., 2011). Another stem cell marker is the e3 ligase RNF43, which is uniquely expressed in Lgr5+ CBC cells, and acts as a tumor suppressor by inducing endocytosis of Wnt receptors (Koo et al., 2012). Additionally, the transcription factor Ascl2, regulated through downstream Wnt signaling, was identified as ISC marker for modulating intestinal stem cell fate (van der Flier et al., 2009). Van der Flier et al. (2009) detected Olfm4 highly expressed in CBC cells, representing one more ISC marker. The gene Xenopus ONT1, part of the Olfm4 family, was shown to inhibit bone morphogenic protein (BMP) signaling, another pathway controlling the ISC's activity (Inomata et al., 2008). This indicates a long list of factors being involved in maintaining the necessary and protective environment that enables stem cells to provide tissue replenishment.

3.2. Wnt signaling pathway

The Wnt signal transduction cascade is one of several pathways which are all involved in developmental processes and are tightly connected to growth-related pathologies and cancer. Wnt signaling as a fundamental and ubiquitously active growth control pathway is a key player in shaping tissues during development (van Amerongen & Nusse, 2009) and correctly maintaining adult tissue architecture. In doing so, a variety in signal transduction steps mediated by receptors of different classes are necessary for a multitude of combinatorial Wnt signaling (Nusse & Clevers, 2017). Beyond the background of the high vertical evolutionary conservation of Wnt signals, it is assumed that modification of them is shared between all Wnt proteins. Beside high similarities in size and chemical constitution, all Wnts are equipped by the palmitoyl transferase termed Porcupine with a lipid (palmitoleic acid) (Rios-Esteves & Resh, 2013). The

lipid primarily serves to bind the Frizzled receptor (FZD) (Janda et al., 2012) as well as the hydrophobicity tethers the Wnt protein to cell membranes and may contribute to a limited Wnt spreading (Nusse & Clevers, 2017). Furthermore, the transmembrane protein Wntless/Evi (Wls) binds the lipidated protein transporting it outside the plasma membrane to become secreted. Outside of the cell the Wnts either can be deactivated by a lipid-removing enzyme called Notum (Kakugawa et al., 2015) or bound by Wnt receptors (e.g. FZD) exposed by target cells such as CBCs, located at the stem cell niche. Notoriously, Wnt signaling is the most important known pathway for regulating intestinal cells concerning their proliferative potential and fate. It was shown that Wnts are bound on FZDs of ISCs while accompanying the cell during the differentiation process and movement up the crypt-villus-axis. However, every cell division outside of the niche leads to the halving of receptor-bound-Wnt on stem cells surface. This generates a decreasing gradient of available Wnts from the base of the crypt towards the villus tip. As proliferative activity directly depends on the amount of available Wnt proteins, this represents an additional protective mechanism. Proliferation speed and the related movement of dividing cells shortens or lengthens the Wnt-gradient. Accordingly, increased cell division provokes a lack of Wnt ligands which acts as tumor suppressing mechanism. Otherwise, high levels of Wnts might support dedifferentiation of progenitor cells or symmetrical cell division of ISCs in the case of damaged or exhausted ISC population (Gehart & Clevers, 2019). Furthermore, R-spondin also influences the Wnt gradient by modulating the turnover of Wnt receptors. Farin et al. demonstrated that absent R-spondin results in less receptor-bound Wnt and therefore reduces the Wnt gradient (Farin et al., 2016).

Wnt ligands such as Wnt3, provided by Paneth cells (Sato et al., 2011), bind the FZD that forms a complex with LRP5 and LRP6. In the presence of R-spondin, activated Lgr5 binds Rnf43 and/or Znrf3 and enhances the number of FZDs and therefore the sensitivity for Wnt ligands. Free Rnf43 and Znrf3 are transmembrane E3 ubiquitin-ligases that control the turnover of available FZDs of the cell surface through ubiquitination.

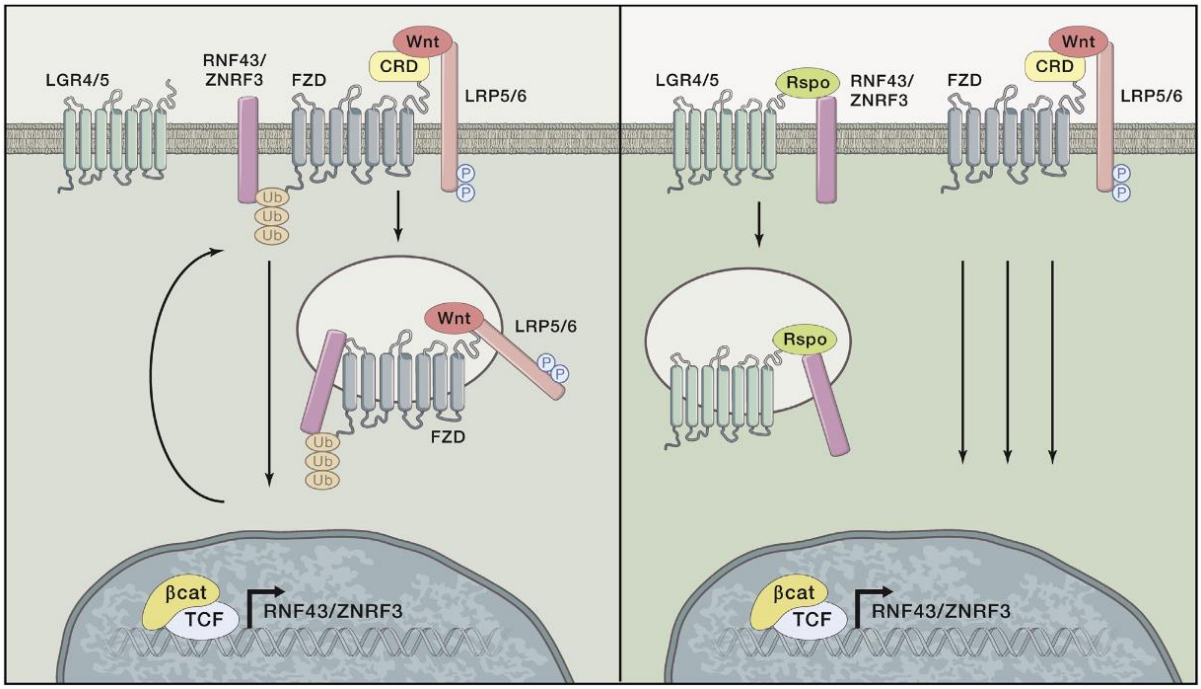


Figure 2: Receptors of Wnt signaling pathway

Frizzled (FZD) receptors which have 7-transmembrane (7TM) and an extracellular N-terminal cysteine-rich domain (CRD), are primary receptors for Wnt proteins. The co-receptors LRPs are transmembrane proteins that are phosphorylated by protein kinases such as GSK3 and CK1. R-spondins binding to members of the LGR5 family on the cell surface enhance Wnt signaling. The transmembrane E3 ubiquitin ligases ZNRF3 and RNF43 regulate Wnt signaling downstream by increasing the turn-over of FZD receptors. Interaction of R-spondin and ZNRF3 has been postulated to enhance Wnt signaling by decreasing the activity of ZNRF3 ligase activity and thereby more FZD receptors become available. (source: Nusse & Clevers, 2017).

The activated Frizzled-LPR5-LPR6-complex initiates the blocking of a destruction complex that is not able anymore to break down beta catenin. The accumulating beta catenin enters the nucleus and induces transcription of growth factors while binding to T cell factor (TCF) and replacing Groucho (Nusse & Clevers, 2017). Additionally, Rnf43 also localized at the nuclear envelope was detected to inhibit the Wnt pathway downstream of beta catenin by silencing the transcriptional activity of T cell factor 4 (TCF4) (Loregger et al., 2015). This Rnf43-mediated inhibition of the Wnt signaling cascade represents a promising strategy to regulate the pathway in the presence of activating mutations of APC or beta catenin which are known to modulate proliferation, polarity and stemness of intestinal epithelial cells, potentially towards tumorigenesis (Nusse & Clevers, 2017).

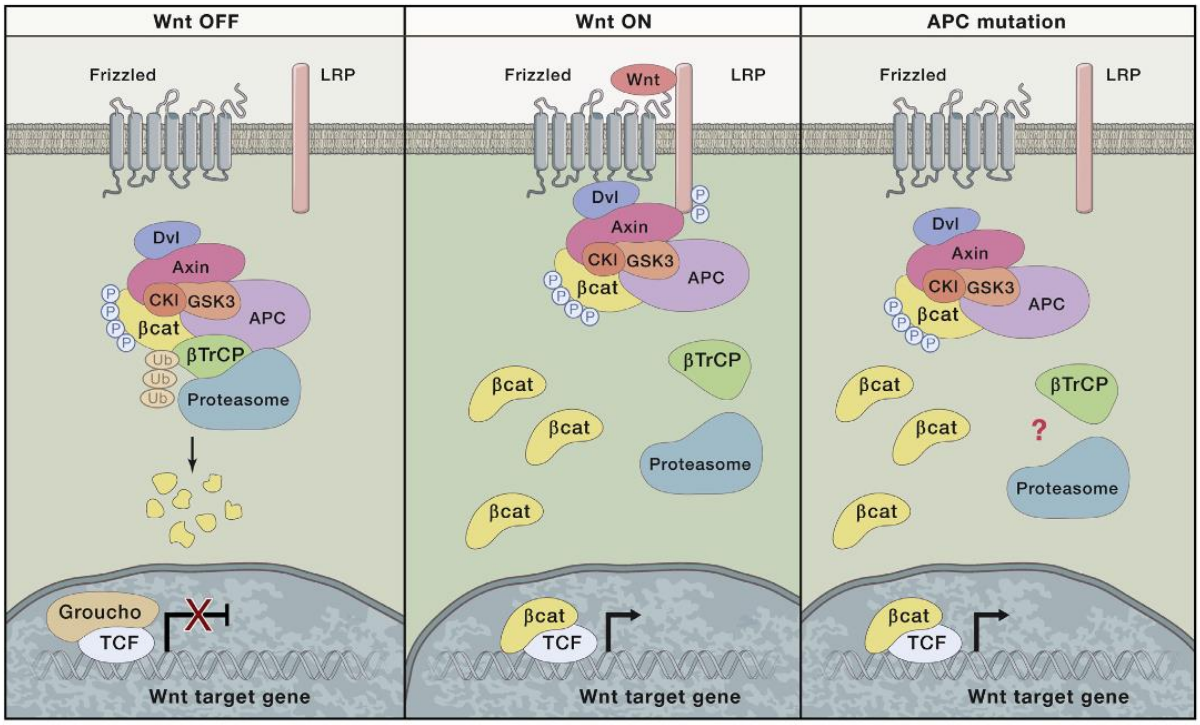


Figure 3: Wnt signal transduction in cells

Wnt OFF: *Without Wnt signals, β catenin is destructed by a complex composed of several proteins: Axin, APC, GSK3 and CK1, the phosphatase 2A (PP2A), and the E3 ubiquitin ligase β-TrCP. The degradation complex phosphorylates and ubiquitinates β catenin. Afterwards β catenin is degraded by the proteasome. Disheveled (Dvl) is another required molecule for activation of this cascade. Inside the nucleus, T cell factor (TCF) remains inactive as the consequence of binding to Groucho due to absent β catenin.*
Wnt ON: *Wnts binding to Wnt receptors leads to an association of Axin with phosphorylated LRP. The destruction complex stays inactive and β catenin accumulates. Next, β catenin enters the nucleus and induces transcription of target genes by binding TCF.*
APC mutation: *Mutated APC disturbs the destruction complex and consecutively activates transcription mediated by Wnt pathway. (source: Nusse & Clevers, 2017)*

3.3. Intestinal stem cell dysfunction and carcinogenesis

Interestingly, only a small spectrum of first-hit mutations are seen in sporadic CRC and over 90% of them are mutations activating Wnt signaling and more than 80% pertain to the Wnt antagonist Adenomatous Polyposis Coli (APC) (Atlas T.C.G.N., Muzny D., Bainbridge M., Chang K., Dinh H., Drummond J., Fowler G., Kovar C., Lewis L., Morgan M., et al., 2012). The human APC acts as tumor suppressor by ubiquitinating the transcription factor beta catenin with the help of a destruction complex. In the case of a dysfunctional tumor suppressor beta catenin accumulates, translocates to the nucleus, and activates the transcription of proto-oncogenes (Figure 3). Therefore, the mutated stem cells can uncontrolledly proliferate and form precursor lesions (adenomas). The development of CRC usually bases on the model of adenoma-carcinoma-sequence in most cases. Additional mutations of other genes such as TP53, Kras and BRAF and the development of chromosomal instability support the malignant transformation from adenomas to carcinomas. Hereditary CRC occurs in several genetic syndromes, whereby hereditary nonpolyposis colorectal cancer (HNPCC) represents the most common of these.

HNPCC arises from deficient Mismatch repair (MMR) proteins that lost their ability to detect and correct genetic errors and lead to a high number of poly-nucleotide tandem repeats instead.

Another signaling pathway that was investigated in the context of neoplastic growth is EGF pathway. Paneth cell-derived (Sato et al., 2011) EGF and transforming growth factor-α (TGFα) are activating the EGF receptor ERBB1, a tyrosine kinase that is highly expressed in CBC cells. Snippert et al. reported that overactive EGF signaling in Kras mutants induced more proliferative activity in ISC and higher dominance of mutated stem cells in the crypt (Snippert et al., 2014). At the same time EGF pathway is tightly controlled by the stem cell niche itself as ISC simultaneously express LRIG1, the inhibiting regulator of ERBB1. Wong et al. underlined the importance of EGF regulation when they observed distinct crypt expansion in LRIG1-knockout mice (Wong et al., 2012) which can facilitate tumor growth.

Other studies reported enormous ectopic crypt expansion and formation of polyps in mice when BMPRIA (also named BMPR-I (Shan Wang & Chen, 2018)), the type I receptor for BMPs in the intestinal epithelium, is deleted (He et al., 2004). Similar to the depletion of BMPRIA, the overexpression of Gremlin 1 and Noggin, two inhibitors of BMP signaling, have the same effect (Davis et al., 2015; Haramis et al., 2004). In this context, it is not surprising that hereditary juvenile polyposis bases on inhibiting mutations of the BMP pathway (Ma et al., 2018). BMP2 and BMP4 are BMP ligands, which belong to the TGFβ superfamily and are found in the intestine, are binding the type II receptors (BMPR-II) whereby inducing phosphorylation and subsequent dimerization of receptor regulated SMADs (rSMAD, SMAD1/5/8 for BMP signaling (Shan Wang & Chen, 2018)). In the next step, these are complexing with common mediator SMADs (co-SMAD) such as SMAD4 and enter the nucleus to regulate gene transcription. According to these findings BMPs act as negative regulators of epithelial proliferation in the intestine and drive cell differentiation. This powerful role of BMP pathway needs to be closely monitored to maintain the right balance of proliferation in the stem cell niche and cell differentiation above. For this reason, mesenchymal cells provide BMP inhibitors such as Gremlin 1, Gremlin 2, Chordin-like 1 or Noggin. As their source is mainly located under the crypts bottom, a natural gradient of highly inhibited BMP signaling arises at the bottom of the crypt and increases towards the villus tip (He et al., 2004; Kosinski et al., 2007; Stzepourginski et al., 2017).

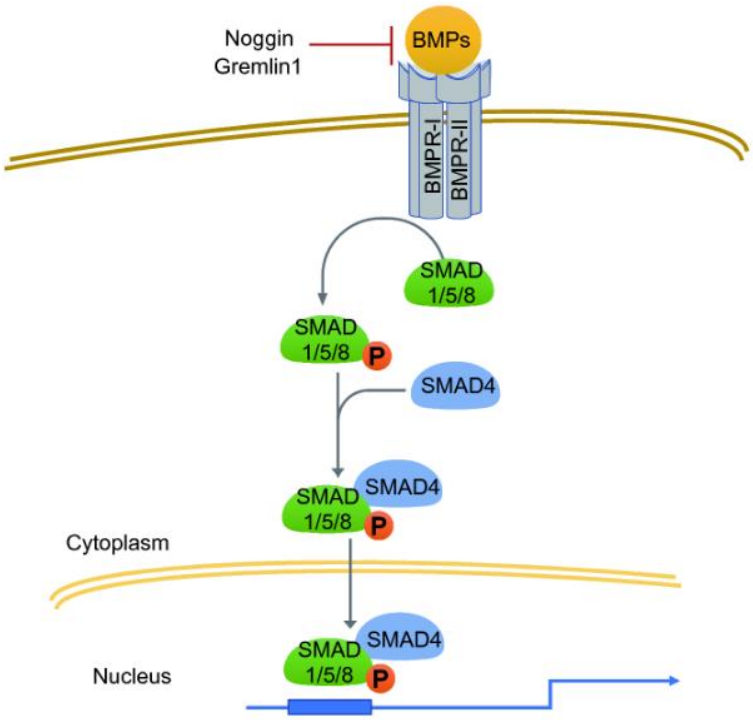


Figure 4: BMP signaling pathway

Ligands bind to the BMP receptors. Next, BMPR-II phosphorylates and activates BMPR-I which also phosphorylates SMAD1/5/8. The rSMAD molecules are complexing with SMAD4 and consecutively translocating to the nucleus. Accumulation of this complex regulates transcription of target genes. BMP inhibitors such as Noggin or Gremlin1 prevent BMP ligands from their binding to receptors. (source: Shan Wang & Chen, 2018)

Furthermore, Hippo signaling pathway was found to be another crucial modulator of intestinal crypt proliferation. The absence of YAP and TAZ, two downstream transcriptional regulators of the Hippo signaling, reduce regeneration capacity in the case of injury. Under normal conditions YAP and TAZ are strictly controlled by the inhibitory upstream kinases MST and LATS, which are turned off in the regenerative process (Gregorieff & Wrana, 2017). During regeneration, active YAP was reported to prevent excessive growth of ISCs and Paneth cells as well as the development of ectopic crypts and microadenomas by reducing the effects of Wnt signaling (Barry et al., 2013; Cai et al., 2010; Gregorieff et al., 2015). Beside this, YAP and TAZ were shown to suppress Wnt target genes and CBC cell markers e.g. Lgr5 or Axin2 (Barry et al., 2013). Hippo signaling also regulates EGF signaling by modulation of the EGFR ligand expression (Gregorieff et al., 2015). In line to these findings, human studies revealed that high activity of YAP and TAZ is tightly linked to CRC progression and overall poor prognosis (Wierzbicki & Rybarczyk, 2015).

One more pathway that promotes regulation of the ISC's fate is the Notch signaling pathway. It uniquely acts between adjacent cells through direct cell-to-cell-activation (Sancho et al., 2015) and is regulated by its own inhibition (Koch et al., 2013). It is well known that Notch pathway is

heavily involved in cell lineage specification of progenitor cells in the intestine (Noah & Shroyer, 2013). Recent studies also demonstrated that Notch signaling is crucial for the homeostasis of the intestinal epithelium as its inhibition caused reduced intestinal proliferation, decreased expression levels of Olfm4 and apoptosis of CBC cells (van Es et al., 2010; VanDussen et al., 2012).

This panoply of regulating pathways provides a broad spectrum of gene mutations and epigenetic variations that either can be inherited or acquired. This implies a longstanding multistep process that begins with the formation of a benign polyp and results in the transformation into a malignant carcinoma due to accumulating mutations of tumor suppressor or protooncogenic genes (Vogelstein et al., 1988). Additionally, local inflammatory processes and environmental factors can contribute to the polyp to cancer progression sequence. This variability in pathogenesis of CRC illustrates that distinct molecular alternations evoke various subtypes of CRC (Grady & Markowitz, 2015).

3.4. Multifactorial processes featuring the state of stemness

Since dysfunction of intestinal stem cells (ISC) is regarded to be an important trigger for gastrointestinal tumorigenesis (Barker et al., 2007) research particularly focuses on their stemness features. Strikingly, some findings indicate that stemness is a state that depends on location of cells in the crypt. Potten was the first who focused on another cell population with stemness features. The +4 cells are located between the stem cell zone and the progenitor zone, counting from the crypts bottom center in the fourth position along the crypt-villus-axis. Additionally, these cells can be identified by the expression of Bmi1 (Sangiorgi & Capecchi, 2008), Tert (Breault et al., 2008; Montgomery et al., 2011), Hopx (Takeda et al., 2011) and Lrig1 (Powell et al., 2012). Several studies reveal that +4 cells can restore the intestinal epithelium and CBC cells after injury (Breault et al., 2008; Montgomery et al., 2011; Sangiorgi & Capecchi, 2008; Takeda et al., 2011). Especially their resistance to radiation proves reserve stemness features (Tetteh et al., 2015). Therefore, +4 cells are debated as quiescent stem cells beside the active dividing Lgr5+ CBC cells. Recent studies reveal that plasticity in the intestinal epithelium can be maintained by dedifferentiation of progenitor cells originating from the secretory (Buczacki et al., 2013; Yan et al., 2017), as well as of the absorptive cell lineage (Tetteh et al., 2016). These findings were underlined by epigenetic investigations that discovered an open chromatin phase which is preserved throughout all levels of epithelial differentiation (Jadhav et al., 2016; Kaaij et al., 2013; Kim et al., 2014; Yu et al., 2015). Accordingly, the process of differentiation is completely set by the surrounding environment (Gehart & Clevers, 2019). This indicates a dynamic, multidirectional model of cell differentiation,

independent from one special stem cell population and completely different from the hematopoietic differentiation system (Gehart & Clevers, 2019).

Since ISC's activity underlies several regulating mechanisms, it was shown that their fate depends on their position in the crypt as well. The work of Ritsma et al. demonstrates that Lgr5+ ISC centrally located at the crypts base, directly adjacent to Paneth cells, rather succeed in becoming the dominant clone than those ISCs with less contact to Paneth cells or those further away from the crypt's center (Ritsma et al., 2014). Additionally, the Wnt signaling pathway with its poorly soluble ligands relies on short range signaling (Alexandre et al., 2014), highlighting the importance of proximity between the ISC and their regulators. This implicates a competitive behavior of dividing cells as niche space and available Paneth cell contacts are limited. Thus, the niche contains a high flow through of proliferating cells that compete for the role of the dominating clone in the stem cell zone. The daughter cells' competition for keeping their stem cell state and for the protective environment of the crypt, are aggravating circumstances for the survival of mutated stem cells. Owing to the strict surveillance mechanism on the proliferative activities in the niche, the pre-malignant cells need to acquire niche independency during cell division (Snippert et al., 2014; Vermeulen et al., 2013). As oncogenic mutations are tightly linked to prolonged, irregular cell cycles and more frequent apoptosis, mutated cells eventually are detected and pushed out from the crypts base. This mechanism can be regarded as natural guardian of a healthy and well-functioning source of intestinal epithelial cells.

Furthermore, recent research increasingly focuses on the microenvironment of ISCs that reveals a variety of other stem cell regulators such as cytokines, microbial metabolites, or dietary factors. In the case of intestinal injury of the epithelium Lindemans et al. discovered a mechanism how the immune system promotes regeneration of the epithelium by activation of the ISCs. They detected IL-22, produced by Group 3 innate lymphoid cells (ILC3s) increasing the proliferative activity of ISCs in organoids through activation of Jak/Stat signaling (Lindemans et al., 2015). Since gut commensals or also pathogens are known to release IL-22 by an immune dependent mechanism, this represents a crucial mechanism regulating ISCs. Another study performed by Beyaz et al. reports that high fat diet (HFD) in mice induces higher proliferation rates through Peroxisome Proliferator-Activated Receptor delta (PPARδ) activation in ISCs (Beyaz et al., 2016). PPARδ is also linked to pro-oncogenic effects on intestinal ISCs and progenitors (Alonso & Yilmaz, 2018; Beyaz & Yilmaz, 2016). Beside nutritional factors, the gut microbes and their metabolites were increasingly focused, by recent research.

3.5. The role of commensal bacteria in a healthy gut

The gut microbiome consists of estimated 100 trillion bacteria from several hundreds of different species (Rajilić-Stojanović & Vos, 2014) together weighting around 1,5 kg (Hill & Drasar, 1975),

and acts as a key player in health and disease of the human gut. Fulfilling a broad spectrum of beneficial functions by host-microbiota interactions the gut microbiome resembles an additional organ of the human body (O'Hara & Shanahan, 2006). The last two decades of research shed light on the lifetime-long symbiosis between commensal bacteria and the human gut. Representing a microbial fingerprint, the composition of the intestinal microbiome is unique in every individual and undergoes several transformations due to physiological aging, changing environments, exogenous factors, or disease. Considering the permanent flow of changes in the microbiome, exact understanding of the host-microbiota-interactions or the microbe-microbe-interactions remains a challenging chapter of science. The microbiome also reflects a fertile ground for new therapeutic approaches. Known in China since the 4th century (Zhang et al., 2012) and rediscovered in the 21st century, fecal microbiota transplantation (FMT) represents one therapeutic method of normalizing the microbial composition back to a healthy condition. The therapeutic benefit of FMT was demonstrated several times, amongst others by Li et al. in patients with recurrent *Clostridium difficile* infection (Li et al., 2016) or by Costello et al. in patients with active ulcerative colitis, who showed higher likelihood of remission 8 weeks after FMT (Costello et al., 2019). Commensal bacteria are necessary to maintain basic physiological functions. It was shown that commensals convert primary bile acids into secondary bile acids and are producers of vitamins of the B and K groups (Hill, 1997). The microbiota ferments indigestible plant-derived fibers to produce short-chain fatty acids (SCFAs) that either serve as energy source for enterocytes or are involved in several physiological processes. They expand the hosts genetic repertoire (Qin et al., 2010), support intestinal development (Sommer & Bäckhed, 2013) and provide protection from invading pathogens by conferring colonization resistance (Lawley & Walker, 2013). This implies direct (host-independent) and indirect (host dependent) mechanisms which can be disrupted by antibiotic treatment (Buffie & Pamer, 2013). The invading microbes compete for limited space and nutrients in the niche (Freter et al., 1983) or are confronted with growth inhibiting antimicrobial peptides (AMPs) such as bacteriocins produced by resident bacteria (Dobson et al., 2012). Indirectly, commensals induce the secretion of AMPs by the host such as the c-type lectins Reg3β and Reg3γ targeting gram-positive bacteria (Cash et al., 2006). Additionally, Wlodarska et al. observed that mucus production modulated by the microbiota is another mechanism of colonization resistance that affects susceptibility to pathogens (Wlodarska et al., 2011). Strikingly, commensal bacteria are tolerated by the hosts immune system which originally is programmed to defend potential pathogens with the help of mechanistic barriers as well as the innate and the adaptive immune system that are in turn educated by the host-microbiota interactions. Accordingly, the mucus layer and the one-layered intestinal epithelium represent the first and most important physical barriers to traversing pathogens from the intestinal lumen. The mucus predominantly contains Muc2, a highly glycosylated protein produced by goblet cells, that can be proteolytically degraded by some pathogens or commensal bacteria (Lidell et

al., 2006; van der Post et al., 2013). The mucin layer consists of a dense inner layer that rarely is penetrated by bacteria, and a more dispersed outer layer that occasionally serves for commensals habitat and metabolism (Johansson et al., 2011). Likewise, tight junctions between epithelial cells also contribute to a limited trans-epithelial permeability. Beside mechanistic separation the coexistence of host and commensals bases on intense crosstalk between the microbial flora and the innate immune system. Hence, several mechanisms are required to enable immune tolerance towards a permanently changing microbiome while ensuring immune defense against pathogens. Therefore, enterocytes secrete immunoglobulin A (IgA) antibodies (Hapfelmeier et al., 2010) and AMPs to support mucosal barrier function. Lately, intestinal AMPs, mainly derived by Paneth cells but also by pancreatic acini (Ahuja et al., 2017), were reported to play another pivotal role in shaping the microbiome (Ehmann et al., 2019). Another strategy of innate immunity are pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain-like receptors (NODs) that initially were observed sensing microbial ligands during infection. In addition, further investigations revealed activation of PRRs not only by pathogens but also by commensal bacteria in healthy colonization. It also supports the development of intestinal mucosa, intestinal immunity and defense of infections (Chu & Mazmanian, 2013). Most exact mechanisms that evoke distinct reactions after sensing of microbe-associated molecular patterns (MAMPs), either to tolerant or to pro-inflammatory immune responses, remain unclear. It is known that TLR-induced signaling contributes to host defense against infection, shapes the microbiome and preserves tissue integrity (Rakoff-Nahoum et al., 2004). Additionally, the patterns of membrane-bounded TLRs vary spatially, temporally (i.e. TLR5 gradually decreases in the SI during neonatal period) and between distinct cell types (i.e. TLR2, 4 and 5 expression is most dominant in colon), inducing a broad spectrum of TLR-dependent functions along the length of the intestine (Price et al., 2018). As key adapter molecule Myeloid differentiation primary response 88 (Myd88) senses inflammatory signals induced by TLRs, IL-1 and IL-18, and consecutively activates transcription factor NF-κB through IL-1R-associated kinase (IRAK) family kinases (Janeway & Medzhitov, 2002). In Myd88-deficient mouse models, altered microbial composition (Wen et al., 2008) and a significant increase in mucosal-associated bacteria compared to wild-type animals were observed (Chu & Mazmanian, 2013). Strikingly, deficiency for Myd88 in mice correlates with higher proliferative activity compared to wild type mice (Rakoff-Nahoum et al., 2004). Furthermore, Myd88 regulates the production of several AMPs such as Reg3γ, which limits the number of gram-positive bacteria at the mucosal surface and thereby reduces the activation of adaptive immunity through physical separation (Vaishnava et al., 2011). In addition, Wang et al. discovered Myd88 to be essential in mice for T cell differentiation and a homeostatic microbiome by regulating IgA levels and adapting the production of T helper 17 (Th17) cells by limiting the expansion of segmented filamentous bacteria (SFB) (Sen Wang et al., 2015). Myd88 was also reported to be required for self-renewal and proliferation of intestinal epithelial cells (IECs) by

demonstrating reduced Ki67-positive cells in the transit-amplifying compartment of globally Myd88-deficient mice (Holtorf et al., 2018). Additionally, Myd88 is a central protein connecting TLRs and potentially oncogenic signaling pathways (NFκB- and MAPK-pathway) and is linked to CRC initiation and progression (Holtorf et al., 2018). Moreover, lymphoid cells (ILCs) play a decisive role for host immunity and inflammation (Bostick et al., 2019) and are integrating microbial signals while developing phenotypic and functional plasticity (Gury-BenAri et al., 2016). Recent research increasingly focused on the influence of host-microbiota interactions on adaptive immune functions, which has been poorly understood so far. Several findings from the past years indicate an intense involvement of both, the adaptive immune system and the intestinal microbiome, in maintaining a healthy physiological condition. For example, a large set of secretory IgA antibodies responsive to commensal bacteria derived by B cells, enables immunoselection of bacterial epitope expression and thereby mediates gut homeostasis (Peterson et al., 2007). Furthermore, adhesion of intestinal microbiota to intestinal epithelium induces differentiation of Th17 (Atarashi et al., 2015) or microbiota-derived SCFAs are necessary for transition of antigen-activated CD8+ T cells into long-living memory cells (Bachem et al., 2019). Therefore, it is not surprising that dysregulations of microbiome-immunity interactions due to antibiotics, diets or genetic susceptibility are responsible for diseases such as inflammatory bowel diseases (IBDs) (Zheng et al., 2020). Considering the current state of knowledge, future research is confronted with the individualism of intestinal microbiome on the one hand and the individual immune system on the other hand, both highlighting the importance of a personalized approach of medicine.

3.6. Impact of microbial components and metabolites on intestinal mucosa

In the past two decades research focused on the role of the microbiome in ensuring hosts physiology through host-microbiota interactions. The underlying molecular mechanisms by which commensal bacteria interact with the IECs especially the ISCs, need to be elucidated. The effects of MAMPs, i.e. flagella, pili, and lipopolysaccharide (LPS) are mediated by PRRs in the intestinal epithelium which activate several signaling pathways such as NF-κB, mitogen-activated protein kinases (MAPK) or PPARγ (Siciliano & Mazzeo, 2012). Nigro et al. detected muramyl-dipeptide (MDP), a common peptidoglycan motif of nearly all bacteria, supporting survival of Lgr5+ ISCs in a Nod2 (NOD receptor) dependent way (Nigro et al., 2014). With the finding of a crypt-specific core microbiota (CSCM) which contains a stable array of certain bacterial species, further investigations focused on the interactions between most abundant bacterial components found in CSCM and the intestinal stem cell niche, the source of epithelial regeneration. This work revealed a dual role of LPS, which is observed at higher levels during inflammation (Pastor Rojo et al., 2007). On the one hand LPS reduced proliferation through

necroptosis of the transit-amplifying cells and ISCs, and on the other hand cell differentiation, especially of the goblet cell lineage, was augmented by LPS (Naito et al., 2017).

Beside the occurrence of natural components of bacteria, commensal bacteria feature several metabolic activities the products of which interact with the intestinal epithelium and adjacent microorganism due to their physical proximity. Currently, the bioactivity of short chain fatty acids (SCFAs) is widely discussed. The majority of SCFAs is produced through fermentation of indigestible carbohydrates originating from dietary fibers by commensal bacteria in the colon (Cummings, 1981). The most abundant SCFAs in the human gut are acetate and propionate, both mainly derived by *Bacteroidetes*, and butyrate, which is mainly produced by *Firmicutes* (Høverstad & Midtvedt, 1986; Macfarlane & Macfarlane, 2003). Likewise, butyrate serves as main energy source for colonic epithelial cells (Donohoe et al., 2011) and improves epithelial barrier function through several mechanisms (Burger-van Paassen et al., 2009; H. Liu et al., 2018; VanHook, 2015). Furthermore, Kaiko et al. observed that ISCs at the bottom of the crypts are preserved from exposure to high concentrations of butyrate through the butyrate consumption of IECs (Kaiko et al., 2016). This protective mechanism relieves the efficient butyrate-dependent inhibition of histone deacetylases (HDACs) seen in cancerous cells, where butyrate was found at 3-fold higher concentrations (Donohoe et al., 2012). Moreover, bile acids, trimethylamine N-oxide (TMAO) or protein fermentation products represent other microbial metabolites that affect host metabolism (Koh & Bäckhed, 2020). Further investigations of microbial components and metabolites, their impact on the intestinal microbiome and the host physiology, represent promising approaches for prevention or treatment of gastrointestinal diseases.

3.7. Does intestinal dysbiosis link gastrointestinal diseases to malignant transformation?

Several factors, e.g. the host's genetics (Gomez et al., 2017), environment, dietary habits (Graf et al., 2015) and lifestyle (David et al., 2014), are involved in shaping the microbiota which is of great importance concerning physical and mental health. Due to technical achievements in genome sequencing technologies and bioinformatics, scientists gained more and more insights into nature and functions of intestinal microorganisms such as interactions between microbiota with the host or other microbes. In consideration of all variables a healthy gut microbiome predominantly consists of the phyla *Firmicutes* and *Bacteroidetes*, followed by *Actinobacteria* and *Verrucomicrobia* (Jandhyala et al., 2015). Moreover, absence of the phylum *Proteobacteria* in combination with high abundance genera such as *Bacteroides, Prevotella* and *Ruminococcus* suggest a healthy gut microbiota (Hollister et al., 2014). Furthermore, the spatial composition of the intestinal microbiota shows differences between the lumen and the mucosal surface. While

Bacteroides, Bifidobacterium, Streptococcus, Enterobacteriacae, Enterococcus, Clostridium, Lactobacillus and *Ruminococcus* are more abundant in the lumen (can be identified in stool), only *Clostridium, Lactobacillus, Enterococcus and Akkermansia* are the predominant mucosa and mucus associated genera (Swidsinski et al., 2005). Disturbances of the delicate homeostasis between commensal bacteria and the host's immune system lead to microbiota dysbiosis and consecutive inflammatory state with long-term consequences in the intestinal tissue (Rastogi et al., 2020). Additionally, it was described that long-term antibiotic treatment correlates with a higher risk of developing colorectal adenomas associated with pharmacologically induced changes of the gut microbiota (Cao et al., 2018). Strikingly, recent research detected non-antibiotic drugs, including substances from all therapeutic classes such as chemically diverse antipsychotics, to extensively influence the composition of the gut microbiome (Maier et al., 2018). As an example, the antidiabetic substance Metformin, which is commonly used for treatment of type 2 diabetes, was demonstrated to significantly reduce the diversity of intestinal microbiota in healthy study subjects (Elbere et al., 2018). In several studies, exposure to antibiotics, especially in early-life period, was observed to cause a loss of species and strain diversity resulting in metabolic disturbances which impact adiposity, bone growth and normal development of the immune system (Cho et al., 2012; Cox et al., 2014). These effects are known to be accompanied by higher occurrence of antibiotic resistances in the remaining microbiota, which displays a fertile ground for invasion and spread of certain species or strains, thereby causing diseases such as *Clostridium difficile* infection. It was shown that altered compositions of intestinal microbiota correlate with certain human diseases such as inflammatory bowel diseases (IBDs) (Ferreira et al., 2014), metabolic diseases like obesity or diabetes (Karlsson et al., 2013), irritable bowel syndrome (Kennedy et al., 2014) or allergic diseases (Bisgaard et al., 2011). Interestingly, some of these diseases such as ulcerative colitis (UC) (Yashiro, 2014) and metabolic syndrome (MS) (Stürmer et al., 2006) were observed to predispose patients to CRC, too. Further investigations targeting the differences in composition and molecular impact of intestinal microbiota between individuals with or without CRC-predisposing diseases are needed. In addition, dysfunctional ISCs were reported in patients suffering IBD (Gersemann et al., 2011) as well as in CRC patients (Zeki et al., 2011) indicating a link between intestinal microbiota, function of ISCs, epithelial self-renewal and higher risk of intestinal tumorigenesis. Thus, mechanisms involved in ISC-regulation and disease-associated mutations of the same mechanisms are increasingly in the spotlight. For example, cancer-associated microbes are capable of stimulating Wnt/β-catenin signaling pathway either by binding E-cadherin on colonic epithelial cells (Rubinstein et al., 2013) or by injecting effector molecules such as CagA from *Helicobacter pylori* strains into host cells of the gastric mucosa (Abreu & Peek, 2014) or AvrA from *Salmonella* in colonic host cells (Lu et al., 2014). Chronic inflammation occurring in IBD, obesity or cancer evolves into a tumor-permissive milieu as soon as microbes can stimulate proinflammatory pathways due to mucosal barrier breach. The

immune system upregulates the concentration of inflammatory factors, e.g. reactive oxygen, nitrogen species, cytokines and chemokines that trigger tumor growth and spread (Garrett, 2015). Microbe-induced stimulation of PRRs releases cytokines such as IL-6, TNFα and IL-23 which consecutively activate NF-κB, a crucial regulator of cancer-associated inflammation (DiDonato et al., 2012). This mechanism mediating activation of NF-κB by highly abundant *Fusobacterium nucleatum* was frequently observed in human colorectal cancer tissue (Kostic et al., 2013). Additionally, bacteria promote cancer development in the host through DNA damage, that subsequently cause genome instability, resistance to cell death and increased proliferation signals (Garrett, 2015). These effects are mediated either directly by bacterial toxins, e.g. colibactin produced by *Escherichia coli* provoking DNA double-strand breaks and cell cycle arrest (Nougayrède et al., 2006) or indirectly by host-produced reactive oxygen and nitrogen species after exposure to bacterial toxins such as *Bacteroides fragilis* toxin (Goodwin et al., 2011). Considering the tremendous amounts of microorganisms inhabiting the human gut, the most molecular mechanisms that are responsible for a functioning ecosystem remain still elusive.

3.8. Aim of the project

Recent studies discuss the effect of microbial metabolites on the function of ISCs and epithelial self-renewal, whereas microbial biology, composition and impacts on the ISC *in vivo* have not been investigated in detail yet.

I. In a clinical pilot study, I explored how the colonic ISC signature from patients with CRC-disposing diseases (MS and UC) differs from the one in healthy individuals and hypothesized an altered microbiome in the probands with CRC-promoting illnesses.

II. In the experimental part I investigated the impact on ISC marker expression patterns by specific gene deficiency (Myd88) and dysbiotic or colitis-inducing treatment by using mouse models. Additionally, mice were screened for morphological changes after dysbiotic treatment.

4. Material and methods

4.1. Materials

4.1.1. Animals

8-10 weeks old male C57BL/6JOlaHsd and BALB/cOlaHsd mice (Harlan Winkelmann, Borchen, Germany, body weights from 22 to 28 g) were used. MyD88 knockout mice (C57BL/6JOlaHsd background, bred in-house, MIH) were kindly provided by Dr. Thorsten Buch. The animals were kept in IVCs under SPF-conditions containing three mice per cage with unlimited access to water and food. The mice were exposed to a 12 h light-dark cycle at room temperature of 22 ± 5 °C.

Thanks to Prof. Dr. Anne Krug colonic tissue from mice (C57BL/6JOlaHsd background, bred at Institute for Immunology, LMU) with dextran sodium sulfate (DSS) induced colitis was available.

4.1.2. Reagents for tissue fixation

Chloroform, Carl Roth GmbH + Co. KG, Germany

Formaldehyde, AppliChem GmbH, Germany

PBS, 137 mM NaCl; 2,7 mM KCL; 10 mM Na_2HPO_4; 1,8 mM KH_2PO_4 (pH 7,4)

4.1.3. Reagents for Hematoxylin-Eosin staining and Immunohistochemistry

Anti-Rabbit IgG (H+L), HRP Conjugate; Promega, USA

DPX mountant for histology, Sigma-Aldrich Chemie GmbH, Germany

Eosin (1%, watery), Morphisto GmbH, Germany

Ethanol absolute, Pharmacy of MRI of TU Munich, Germany

Hemalum solution acid according to Mayer, Carl Roth GmbH + Co. KG, Germany

Hydrochloric acid 37% (extra pure), Carl Roth GmbH + Co. KG, Germany

Hydrogen peroxide solution 30% in H_2O, Sigma-Aldrich Chemie GmbH, Germany

Ki-67 (D3B5) Rabbit mAb, Cell Signaling Technology Europe, B.V., Germany

Ki-67 Monoclonal Antibody (SolA15), PE, eBioscience™, Thermo Fischer Scientific, USA

Normal Goat Serum, Jackson ImmunoResearch Europe Ltd., UK

Roticlear, Carl Roth GmbH + Co. KG, Germany

Signal Stain® DAB Substrate Kit, Cell Signaling Technology Europe, B.V., Germany

SignalStain® Antibody Diluent, Cell Signaling Technology Europe, B.V., Germany

Sodium chloride, Carl Roth GmbH + Co. KG, Germany

Tris Pufferan® ≥99,9%, Carl Roth GmbH + Co. KG, Germany

Tri-Sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), Merck KGaA, Germany

Tween®20 Molecular biology grade, AppliChem GmbH, Germany

4.1.3.1. Primary antibody for immunohistochemistry

Target: Ki-67
Species: Rabbit (IgG)
Dilution: 1:400
Company: Cell Signaling Technology Europe, B.V.

} Used for murine tissue

Target: Ki-67
Species: Mouse
Dilution: 1:400
Company: eBiosciences, Thermo Fischer Scientific

} Used for human tissue

4.1.3.2. Secondary antibody for immunohistochemistry

Target: Anti-Rabbit (IgG)
Dilution: 1:200
Company: Promega

4.1.4. Reagents for quantitative real-time PCR and 16S ribosomal RNA sequencing

Bio-Rad C1000 Touch™ Thermal Cycler, Bio-Rad Laboratories GmbH, Germany

Bio-Rad CFX384™Real-Time System, Bio-Rad Laboratories GmbH, Germany

DNA-free™ DNA Removal Kit, Thermo Fischer Scientific, USA

dNTP, Promega, USA

FrameStar® 384-well PCR plate, 4titude Ltd, UK

GenElute™ Mammalian Total RNA, Sigma-Aldrich Chemie GmbH, Germany

HiSeq system, Illumina, USA

HUMAnN 2.0, The Huttenhower Lab, USA

iTaq™ Universal SYBR® Green One-Step Kit, Bio-Rad Laboratories, Inc., USA

MetaPHLAn 2.0, The Huttenhower Lab, USA

MicroAmp™ optical adhesive film, Applied Biosystem, USA

MiSeq system, Illumina, USA

NanoDrop 1000; Thermo Fischer Scientific, USA

Nuclease-free water, Promega, USA

OneStep-96 PCR Inhibitor Removal Kit, Zymo Research, USA

Quali-PCR Tubes, Kisker-Biotech GmbH & Co. KG

Random Primer, Promega, USA

Reverse Transcriptase 5x Buffer, Promega, USA

Reverse Transcriptase RNase, Promega, USA

TruSeq DNA library preparation kit, Illumina, USA

4.1.5. Primers for quantitative real-time PCR

mGAPDH	Forward GCCTTCTCCATGGTGGTGAA
	Reverse GCACAGTCAAGGCCGAGAAT
mRNF43	Forward GGGGCAAACTATGACGTGTG
	Reverse CTGCTGAAGAGGATCCGGTC
hGAPDH	Forward GAAGGTGAAGGTCGGAGT
	Reverse GAAGATGGTGATGGGATTTC
hLGR5	Forward TGATGACCATTGCCTACAC
	Reverse GTAAGGTTTATTAAAGAGGAGAAG
hTNFα	Forward CAGAGGGCCTGTACCTCATC
	Reverse GGAAGACCCCTCCCAGATAG
hIL-6	Forward GTAGCCGCCCCACACAGA

Reverse CCGTCGAGGATGTACCGAAT

4.1.6. Microscope and camera

AxiocamMRm, Zeiss, Germany

LEICA DMRBE, Leica, Germany

4.1.7. Software

Adobe Illustrator, Adobe Systems, USA

Axio vision imaging software 4.8, Zeiss, Germany

Bio-Rad CFX Manager software, Bio-Rad Laboratories, Inc., USA

GraphPad Prism 5 - 9, Graphpad Software, Inc., USA

KneadData, The Huttenhower Lab, USA

4.1.8. Reagents for experimental treatment

DSS, MP Biomedicals, USA

4.1.9. Antibiotic treatment

AMT: Metronidazole (1 g/l)
Ampicillin (1 g/l)
Vancomycin (0,5 g/l)
Neomycinsulfate (1 g/l)

RFX: Rifaximin (150 mg/l)

4.1.10. Probiotic treatment

OMNi-BiOTiC® 10 (= 5 g); Institute AllergoSan; Austria

Containing at least 5 billion of following bacteria:

Lactobacillus acidophilus W55
Lactobacillus acidophilus W37
Lactobacillus paracasei W72
Lactobacillus rhamnosus W71

Enterococcus faecium W54
Lactobacillus salivarius W24
Lactobacillus plantarum W62
Bifidobacterium bifidum W23
Bifidobacterium lactis W18
Bifidobacterium longum W51

4.2. Methods

4.2.1. Clinical study

4.2.1.1. Study subject recruitment

Patients and controls were recruited at Vorsorgezentrum für Innere Medizin und Gastroenterologie, Dr. med. Albert Eimiller and Department of Internal Medicine II, Klinikum rechts der Isar, Technical University of Munich (TUM), Univ.-Prof. Dr. med. Roland M. Schmid. Study subjects were interviewed regarding their clinical data at least two days before their outpatient colonoscopy that was performed due to medical indications and routines (e.g. colon cancer surveillance). The study was approved by the ethics committee of the Medical Faculty of Klinikum rechts der Isar, TUM (AZ 297/16S). Suitable patients had to fulfill all inclusion criteria corresponding to each group:

- **Group A:** patients as healthy controls
  - planned for prevention colonoscopy
  - no internal pre-existing conditions
- **Group B**: patients with metabolic syndrome
  - impaired glucose tolerance/ type 2 diabetes mellitus
  - arterial hypertension (≥ 140/90 mmHg)
  - abdominal obesity (BMI > 30 kg/m^2)
- **Group C**: patients with ulcerative colitis
  - diagnosis based on the DGVS-criteria for ulcerative colitis
  - actual mild/moderate disease activity

The following contraindications were considered:

- age < 18 y
- pregnancy/while breast-feeding
- antibiotic treatment in the past two months
- actual/pre-existing colon cancer
- lacking capacity for consent.

In case of fulfilling all criteria, the patients were informed about all additional risks of participating in this clinical trial (e.g. gastrointestinal bleeding, perforation) by the attending physician and had to give their written consent to participation. Additionally, included patients were asked to give several information about their clinical (pre-) conditions and dietary habits (see Figure 7 and 8).

4.2.1.2. Sample collecting and processing

The samples were always transported on ice. All generated data and tissue samples were blinded and randomized.

a) Stool samples

The patients were asked for one stool sample before starting with the laxative treatment for the endoscopy. The stool samples then were transported on ice and frozen at -80 °C within 24 h. The 16S RNA sequencing was performed as described in 4.2.4.

b) Tissue for qPCR analysis

For the study's purpose the examining physician took five additional biopsies from normal mucosa of the sigmoid colon during colonoscopy. For RNA extraction four tissue samples were kept at -80 °C without solution. The process from RNA extraction to quantitative real-time PCR was done as described in 4.2.3.

c) Tissue for histological analysis

One biopsy was fixed in 4% formalin solution at 4 °C for a maximum of 4 d, cooling it for another 7 - 14 d the medium was changed to PBS. For histological purposes this biopsy underwent standard dehydration and following paraffin embedding. In a final step the samples were cut into 4 µm sections. The HE-staining and the IHC for Ki-67 were performed as described in 4.2.2.1. and 4.2.2.3.

4.2.1.3. Analysis of data from the clinical trial

The raw data from the quantitative real-time PCR analysis were processed as explained in 4.2.3.5.

4.2.2. Histology and immunohistochemistry

4.2.2.1. Hematoxylin and eosin staining

Slides were incubated at 60 °C for 20-30 min until the paraffin was melted. The hematoxylin staining was started with deparaffinization and rehydration of the sections. This procedure consists of putting the sections thrice in Roticlear solution and twice in absolute ethanol each

for 10 min. Continuing with bathing them consecutively in 90%, 70% and 30% ethanol in each case for 5 min. Afterwards they were immersed briefly in ddH_2O. Thereafter the sections were put in a hematoxylin solution (Hemalum solution acid according to Mayer) for 6 min and then washed in tap water. In the next step the sections were submerged in eosin (watery 1%) for 6 min and subsequently washed again in tap water. For dehydration they were immersed in 80% ethanol for 1 min. Finally, two times of washing in absolute ethanol, each for 5 min, and three times in Roticlear solution, each for 5 min were performed. In the final step the slides were mounted with coverslips by using one drop of the mounting media.

Based on: SOP-Hematoxylin-eosin staining; AG Gerhard Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, TUM; Stand: 27.01.2017, Autor: RML

4.2.2.2. Image acquisition and analysis of HE-staining

All pictures were visualized by a LEICA DMRBE microscope at 20 or 40-fold magnification and acquired by AxioCamMRm and Axio vision imaging software 4.8. Before analysis all sections underwent blinded randomization.

For every section, a histology score was evaluated by Prof. Dr. med. Michael Vieth (Institut für Pathologie, Klinikum Bayreuth). The score consists of the following five factors that can value between 0 and 3:

1. Lymphocytes	0 = no infiltration
	1 = some infiltration
	2 = massive infiltration in lamina propria
	3 = massive infiltration in lamina propria and muscle
2. Edema	0 = no edema
	1 = <50 µm
	2 = 50-100 µm
	3 = >100 µm
3. Ulceration	0 = no ulceration
	1 = ulceration
4. Crypt distortion	0 = no distortion
	1 = distortion
5. Neutrophilic granulocytes	0 = no infiltration
	1 = some infiltration
	2 = massive infiltration in lamina propria
	3 = massive infiltration in lamina propria and muscle

All the values were added to one cumulative score between 0 and 11.

As the tissue samples were extracted from macroscopically non-inflammatory areas, I observed microscopical inflammation in UC, but not in controls or MS patients.

4.2.2.3. Ki-67 immunohistochemistry

First, the sections were dewaxed by incubating them at 60 °C for 20-30 min. For the deparaffinization the slides were immersed in Roticlear solution three times each for 10 min. Continuing with rehydration the sections were put twice in absolute ethanol for 10 min and consecutively in 90%, 70% and 30% ethanol each for 5 min. This procedure was finished by washing in dH_2O. During the whole procedure the slides must be kept humid.

To unmask the antigen, the sections were heated in 10 mM sodium citrate buffer (11,764 g of tri-sodium citrate dihydrate in 4 l of dH_2O, stored at 4 °C; pH 6,0) by using a pressure cooker and a hot plate until the water boiled. Then slides were placed in the cooker for 5-10 min. Afterwards the hot plate was turned off and the sections were cooling down in the pressure cooker for 30 min.

For the inhibition of the tissue's endogenous peroxidase the slides were incubated in 3% hydrogen peroxide (30% H_2O_2 in dH_2O, diluted 1:10) at room temperature for 10 min. Then they were washed in dH_2O for 5 min. Subsequent the samples were washed in a buffer consisting of 60,5 g Tris (50 mM), 87,6 g NaCl (150 mM) and 1 ml Tween®20 dissolved in 1 l dH_2O (1 M HCL for adjusting the pH to 7,5), for 5 min. Afterwards each slide was covered with 400 µl blocking solution (normal goat serum diluted in the wash buffer, mixing ratio 1:20) and incubated at room temperature for 1 h. Then the blocking solution was removed and 400 µl of primary antibody targeting Ki-67 and diluted in SignalStain® Antibody Diluent (application ratio 1:400; Cell Signaling Technology) was added. Finishing the first part of the procedure the sections were incubated at 4 °C overnight.

The next day the antibody solution was removed, and the slides were washed four times in wash buffer each for 5 min. In the following, 300 µl of the secondary antibody (Anti-Rabbit IgG HRP Conjugate) mixed with Signal Stain® Antibody Diluent (1:200; Cell Signaling Technology) were applied to the tissue and incubated at room temperature for 1 h. Once more the sections were immersed into wash buffer for four times each for 5 min. Afterwards 30 µl from the mixture of DAB Diluent (Signal Stain® DAB Substrate Kit; Cell Signaling Technology) and DAB Chromogen Concentrate (Signal Stain® DAB Substrate Kit; Cell Signaling Technology) were added to each slide in application ratio 100:3. After 7 min, the sections were washed in dH_2O immediately. In addition, a hematoxylin counterstaining was performed. Therefore, the sections were in hematoxylin for 2 min and washed in tap water afterwards. Then the slides were

gradually immersed in 50%, 70% and 90% ethanol, each dehydrated for 5 min. Afterwards, they were twice in absolute ethanol each for 5 min and three times in Roticlear for 10 min per run. In the last step the slides were mounted (DPX Mountant for histology, slide mounting medium; SIGMA, Life Science) with coverslips.

4.2.2.4. Image acquisition and analysis of IHC

Image acquisition was performed the same way as described in 4.2.2.2 (HE-staining) and the sections were assessed after blinded randomization. In this analysis six intact crypts were detected in every section. In the crypts four different values were assessed:

- number of crypt cells
- number of Ki67 positive cells in the whole crypt
- number of base cells in the crypt
- number of Ki67 positive cells in the base of the crypt

From these values the proportion of Ki67 positive cells compared to the total number of cells was calculated for each crypt and its base. The obtained data were averaged and correlated to the cohorts. For this analysis 40-fold magnification was used.

4.2.3. Quantitative real-time PCR

4.2.3.1. RNA extraction

RNA extraction was performed using the GenElute™ Mammalian Total RNA (Sigma-Aldrich Chemie GmbH) according to the kit's experienced user protocol. 30-50 g of caecal or sigmoid tissue were used. To determine quality and the yield, RNA was measured using NanoDrop.

4.2.3.2. DNA digestion

Extracted RNA was gently diluted with 0,1 volume of 10x DNase I buffer (DNA-free™ DNA Removal Kit) and 1 µl rDNase I (DNA-free™ DNA Removal Kit) and incubated at 37 °C for 20-30 min. In the next step, 0,1 volume of resuspended DNase reactivation reagent (DNA-free™ DNA Removal Kit) was added and incubated at room temperature for 2 min while mixing it occasionally. After centrifugation, RNA was transferred to a new tube and the content of RNA was measured by a NanoDrop device. For the whole procedure RNA was kept on ice.

4.2.3.3. Reverse transcription

For transcription, extracted RNA was mixed with 1 µl random primers (150 ng/µl) and each sample was filled up with nuclease-free water to yield 15 µl. The varying amounts of extracted

RNA were unified to a total RNA content between 750 and 1000 ng. The probes were heated up to 70 °C and then cooled down on ice, each for 5min. Subsequently, 11 µl of a solution consisting of 5 µl reverse transcriptase buffer (5x), 1,25 µl dNTP mix (10 mM), 1 µl reverse transcriptase and 3,75 µl of nuclease-free water were added. In the following, the samples were incubated at room temperature for 10 min, then at 50 °C for 50 min and in a final step at 70 °C for 15 min. Finally, the concentration of cDNA was measured with the NanoDrop and the probes were frozen at -20 °C.

4.2.3.4. Quantitative real-time PCR protocol

For qPCR, the obtained cDNA was adapted to a concentration of 200 ng cDNA per µl. Then 6 µl of each sample were taken and pipetted into a FrameStar® 384-well PCR plate. Beside this, 6 µl of a mixture consisting of 0,5 µl forward primer, 0,5 µl reverse primer for the different markers and 5 µl Cybr Green were added to the plate. Experiment was performed in duplicates. The PCR plate was sealed with an optical adhesive film and centrifuged at 300 rpm for 1 min. In the final step the PCR plate was put in the Bio-Rad C1000 Touch™ Thermal Cycler with Bio-Rad CFX384™Real-Time System, performing the following amplification program:

Initial denaturation	95 °C	3 min	
Denaturation	95 °C	15 s	40x
Annealing	60 °C	45 s	
Melting curve	65-95 °C	0,5 °C/5 s	

4.2.3.5. Quantitative real-time PCR analysis

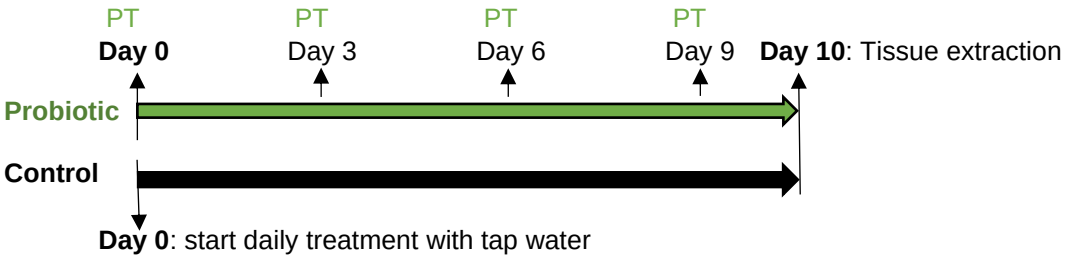
The raw data from qPCR were analyzed with the Bio-Rad CFX Manager software. The generated data were exported into excel and evaluated by using the ΔΔCq method. All mRNA expression levels were normalized to *GAPDH*.

4.2.4. 16S ribosomal RNA sequencing

OneStep-96 PCR Inhibitor Removal Kit was performed. 16S rRNA gene amplicons spanning the V4 – V5 hypervariable region were sequenced using a MiSeq system (Staffas et al., 2018). Sequencing data were compiled and processed using Mothur, then screened and filtered for quality. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity using NCBI blast classifier and the alignment hit with the highest score. To correct for very rare OTUs, which could bias downstream statistical analyses, I excluded OTUs with less than 10 counts in less than 5% of all samples.

4.2.5. Animal experiments

4.2.5.1. Influence of probiotic treatment on murine intestine



Ten animals (C57BL/6JOlaHsd) were divided into two groups of 5 mice. One group (Probiotic) received probiotic treatment (PT) consisting of one package of OMNi-BiOTiC® 10 (= 5 g) mixed with 100 ml tap water every 3 d for 10 d. The remaining 5 animals (Control) were treated with tap water for 10 d. At day 10 all laboratory mice of this experiment were sacrificed.

4.2.5.2. Rifaximin effects on the murine intestinal mucosa

Two cohorts of C57BL/6JOlaHsd rodents (6 mice per group) were administered orally either with rifaximin (RFX: Rifaximin 150 mg/l) or with tap water (Control) for 14 d until they were sacrificed.

4.2.5.3. Impact of antibiotic treatment on murine intestinal mucosa

6 mice (C57BL/6JOlaHsd) underwent a treatment of an antibiotic mixture (AMT: Metronidazole 1 g/l, Ampicillin 1 g/l, Vancomycin 0,5 g/l and Neomycin sulfate 1 g/l) for 14 d. The control group (6 mice) received tap water for 14 d. At day 14 all 12 rodents were sacrificed. In another experiment with C57BL/6JOlaHsd mice we compared the RFX treatment (Rifaximin 150 mg/l) on 6 mice, while the other groups received either AMT (6 rodents) or tap water (6 mice), each for 14 d. All mice were sacrificed at day 14.

4.2.5.4. ISC markers in Myd88 knock-out and wild type mice

Expression levels of intestinal stem cell markers were compared in normal wild type mice (C57BL/6JOlaHsd) with MyD88 knockout mice (C57BL/6JOlaHsd, bred in-house, MIH, kindly provided by Dr. Thorsten Buch). The rodents were killed at age of 6-10 weeks.

4.2.6. Murine tissue collection and processing

First the mice were subjected to isoflurane narcotization and were sacrificed by cervical dislocation. For the following RNA extraction, the Caecum and stool content were removed and stored at -80 °C. For histological assessments a part of the Caecum and a part of the small intestine were fixated in 4% formalin solution for a maximum of 4 d. Then a standard procedure of dehydration and paraffin embedding was executed. Afterwards the tissue was sectioned into 4 µm thin cuts.

4.2.7. Analysis of data from animal experiments

4.2.7.1. Image acquisition and analysis of HE-staining

All pictures were visualized by a LEICA DMRBE microscope at 20 or 40-fold magnification and acquired by AxioCamMRm and Axio vision imaging software 4.8. Before analysis, all sections underwent blinded randomization. Each slide was analyzed by measuring the thickness of the mucosa, the length of the villus and the depth of crypts in ten different areas. The measurements of each category were averaged and correlated to the categories in the three different mouse groups.

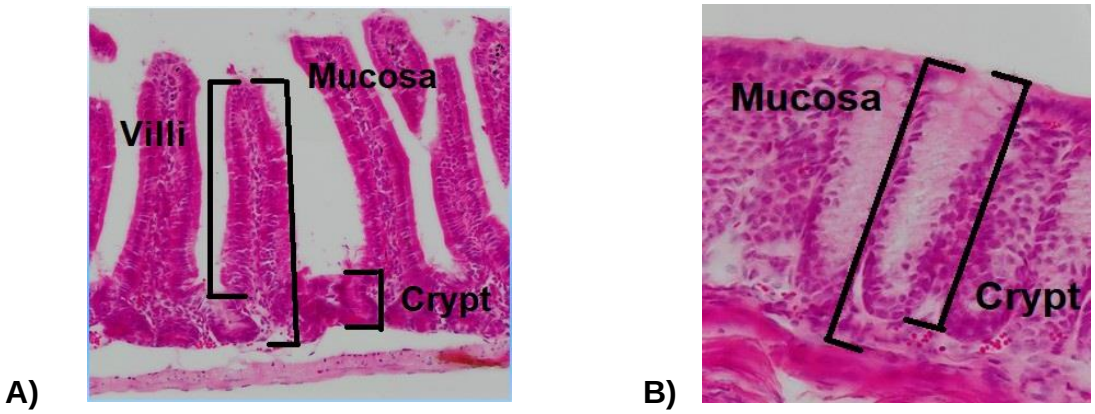


Figure 5: Measurement methods

in **A)** Small intestine and **B)** Colon

4.2.7.2. Quantitative real-time PCR analysis

The data from the qPCR analysis were analyzed as explained in 4.2.3.5.

4.2.8. Statistics

All statistical analysis were done with the help of GraphPad Prism 5 or 9 software. Data were analyzed by Kolmogorov-Smirnov test to check for a Gaussian distribution. In the case of normally distributed data unpaired t-tests were applied for 2-group comparisons or one-way ANOVA with consecutive Bonferroni test for 3 or more group comparisons. Non-normally distributed data were either analyzed by nonparametric Mann-Whitney test for 2-group comparison or by Kruskal-Wallis test with consecutive Dunns test for 3 or more group comparisons.

Assessing the microbiota data, alpha diversity was calculated based on species richness and Shannon effective counts. Taxonomic results were explored descriptively based on relative abundances at different phylogenetic levels. Overall, results with p-value < 0.05 were considered as significant.

5. Results

5.1. Overview of clinical characteristics in the human study

The main part of this work was aimed to find out whether colonic crypts of patients with CRC-disposing diseases show different trends or patterns in the ISC-marker expressions and in proliferation activity compared to healthy study subjects. In addition, I analyzed the microbial composition of the participating patients in search of alternations linked to CRC development. Subjects were assigned to either the CTR, MS or UC group corresponding to inclusion criteria. Participants were asked to complete a questionnaire concerning their past medical history, dietary habits, gastrointestinal symptoms, and basic clinical data to assess possible confounders. Clinical diagnoses provided should be medically confirmed. All data were analyzed and indicated as percentage of positive answers related to all patients of the corresponding group. The questionnaire for symptoms and dietary habits covered the last four weeks prior to colonoscopy. The analysis showed a similar age distribution of all participants. According to the typical diseases onset at higher age that are defining the metabolic syndrome only MS patients were slightly older (Figure 6). In this group, I also observed a higher incidence of other disorders such as coronary heart disease (29%), rheumatological disorders (29%), cancer (14%) and chronic renal insufficiency (14%) compared to the other groups. 87% of MS probands were under regular medication, especially analgesics (57%), proton pump inhibitors/H2-blocker (43%) and others (43%). The cohort with ulcerative colitis showed 89% of patients with a regular intake of medication, particularly immunosuppressants (67%), probiotics (33%) and analgesics (22%). In patients with UC low incidence of food intolerance was seen with the corresponding alimentary forgoing (22%). All cohorts showed a varying incidence of *Helicobacter pylori* infection (11-30%), allergies (30-57%), number of smokers (11-20%) and consumption of alcohol (43-50%).

	CTR	**MS**	**UC**
Number of probands	(10/26)	(7/26)	(9/26)
Age (y)	53 ± 11	66 ± 9	52 ± 9
Sex (f/m)	(6/4)	(2/5)	(3/6)
BMI (kg/m²)	26 ± 3	32 ± 3	26 ± 3
Pre-existing conditions:			
Arterial hypertension (> 140/90 mmHg)	40% (4/10)	100% (7/7)	22% (2/9)
Coronary heart disease	10% (1/10)	29% (2/7)	0% (0/9)

Metabolic disorders	20% (2/10)	100% (7/7)	11% (1/9)
Obesity (BMI ≥ 30 kg/m²)	20% (2/10)	100% (7/7)	0% (0/9)
Cancerous disease	10% (1/10)	14% (1/7)	0% (0/9)
Chronic inflammatory bowel disease	0% (0/10)	0% (0/7)	100% (9/9)
Rheumatological disorder	0% (0/10)	29% (2/7)	0% (0/9)
Chronic hepatic/pancreatic disease	10% (1/10)	0% (0/7)	22% (2/9)
Chronic renal insufficiency	0% (0/10)	14% (1/7)	0% (0/9)
Helicobacter pylori infection	30% (3/10)	29% (2/7)	11% (1/9)
Others	10% (1/10)	29% (2/7)	11% (1/9)
None	30% (3/10)	0% (0/7)	0% (0/9)
Medication			
Antibiotic treatment (within the past 2 months)	0% (0/10)	0% (0/7)	0% (0/9)
Probiotic treatment (within the past 2 months)	0% (0/10)	29% (2/7)	33% (3/9)
Immunosuppressants	0% (0/10)	0% (0/7)	67% (6/9)
Proton pump inhibitor/H2-blocker	10% (1/10)	43% (3/7)	11% (1/9)
ASS/voltaren/opiates/other analgesics	20% (2/10)	57% (4/7)	22% (2/9)
Marcumar/others	0% (0/10)	29% (2/7)	0% (0/9)
Chemo-/radiotherapy	10% (1/10)	0% (0/7)	0% (0/9)
Others	60% (6/10)	43% (3/7)	0% (0/9)
None	30% (3/10)	14% (1/7)	11% (1/9)
Allergies	30% (3/10)	57% (4/7)	44% (4/9)
Pregnancy/lactation period	0% (0/10)	0% (0/7)	0% (0/9)
Smoker	20% (2/10)	14% (1/7)	11% (1/9)
Alcohol intake	50% (5/10)	43% (3/7)	44% (4/9)
Assured food intolerance:			
Lactose intolerance	0% (0/10)	0% (0/7)	0% (0/9)
Fructose intolerance	0% (0/10)	0% (0/7)	22% (2/9)
Sorbitol intolerance	0% (0/10)	0% (0/7)	11% (1/9)
Histamine intolerance	0% (0/10)	0% (0/7)	0% (0/9)
Others	10% (1/10)	0% (0/7)	0% (0/9)
None	90% (9/10)	100% (7/7)	78% (7/9)
Actual alimentary forgoing (past 4 weeks)			
Lactose	0% (0/10)	0% (0/7)	11% (1/9)
Dairy products	0% (0/10)	0% (0/7)	11% (1/9)
Fructose	0% (0/10)	0% (0/7)	22% (2/9)

Sorbitol	0% (0/10)	0% (0/7)	11% (1/9)
Gluten	0% (0/10)	0% (0/7)	11% (1/9)
Wheat	0% (0/10)	0% (0/7)	11% (1/9)
Histamine	0% (0/10)	0% (0/7)	11% (1/9)
Fish/meat containing food (vegetarian)	10% (1/10)	0% (0/7)	11% (1/9)
Animal products (vegan)	0% (0/10)	0% (0/7)	11% (1/9)
Others	0% (0/10)	0% (0/7)	0% (0/9)
None	90% (9/10)	100% (7/7)	78% (7/9)

Figure 6: Basic clinical data from the human study

Each line shows the number of subjects, in relation to the total number of individuals in each group (%), who were positive for the listed criteria on the left side. The subjects were assigned to the control group (CTR), the metabolic syndrome group (MS) or the ulcerative colitis group (UC).

I assessed gastrointestinal symptoms of the subjects and found that the majority (71%) of the MS group suffered from gastrointestinal symptoms, especially from flatulence (71%), diarrhea (43%) and acid indigestion/gastroesophageal reflux (43%). In comparison, the UC cohort showed a lower, but also high incidence of gastrointestinal problems (67%). In these patients, stomach pains/cramps (44%), flatulence (44%) and diarrhea (22%) or other symptoms (22%) occurred more often (Figure 7). In the CTR group 40% reported gastrointestinal symptoms. Moreover, I did not observe any patient with UC undergoing a gastrointestinal surgery before this colonoscopy to 14% of MS cohort receiving surgical interventions prior to this colonoscopy. Anamnestic polyps occurred more often in patients with UC (89%) compared to the other cohorts (60 – 70%) (Figure 7).

	CTR	MS	UC
Number of probands	(10/26)	(7/26)	(9/26)
Abdominal symptoms (past 4 weeks)			
Stomach pains/cramps	20% (2/10)	29% (2/7)	44% (4/9)
Diarrhea	10% (1/10)	43% (3/7)	22% (2/9)
Flatulence	20% (2/10)	71% (5/7)	44% (4/9)
Nausea	0% (0/10)	14% (1/7)	0% (0/9)
Vomiting	0% (0/10)	14% (1/7)	0% (0/9)
Constipation	20% (2/10)	29% (2/7)	0% (0/9)
Itching/rash	10% (1/10)	14% (1/7)	11% (1/9)
Acid indigestion/gastroesophageal reflux	20% (2/10)	43% (3/7)	11% (1/9)
Others	0% (0/10)	14% (1/7)	22% (2/9)
None	60% (6/10)	29% (2/7)	33% (3/9)
Gastrointestinal surgery in the past	10% (1/10)	14% (1/7)	0% (0/9)
Anamnestic polyps	60% (6/10)	71% (5/7)	89% (8/9)

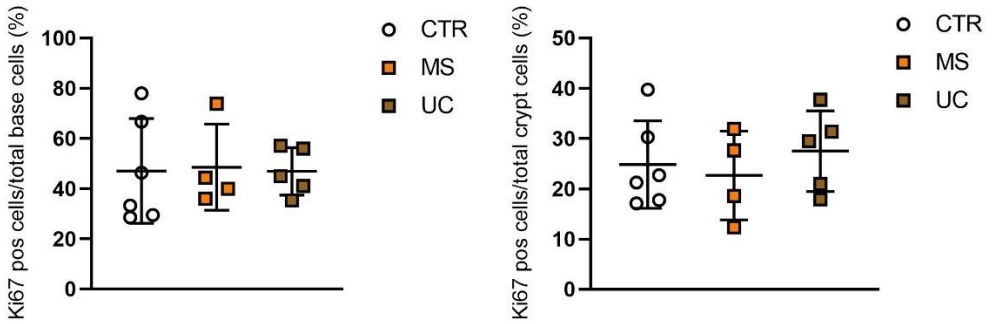
Figure 7: Gastrointestinal symptoms in probands from the human study

Each line shows the number of subjects, in relation to the total number of individuals in each group (%), who were positive for the listed criteria on the left side. The subjects were assigned to the control group (CTR), the metabolic syndrome group (MS) or the ulcerative colitis group (UC).

5.2. Mucosal proliferative activity appears at a similar rate in all study groups

I histologically analyzed the sigmoid tissue of all probands concerning their crypt cell proliferative activity by Ki67 staining. This revealed a tendency towards more proliferation in the entire crypt in patients with UC compared to the controls (Figure 8), but this was not statistically significant. Additionally, when investigating only the number of proliferating cells in the crypt base, I did not detect any difference (Figure 8).

A)



B)

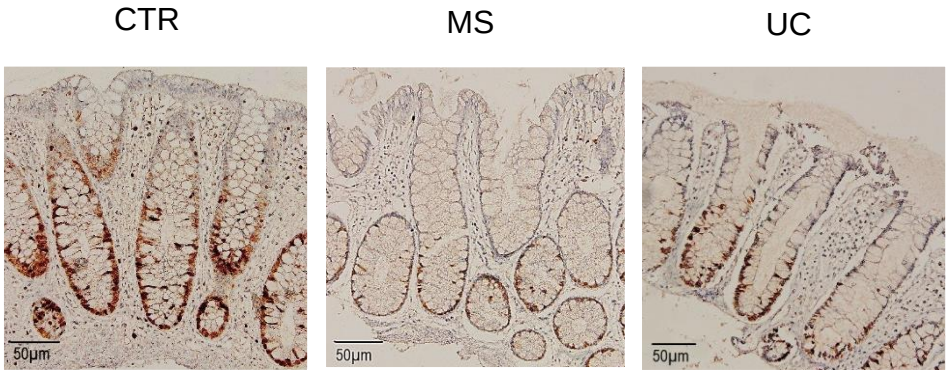


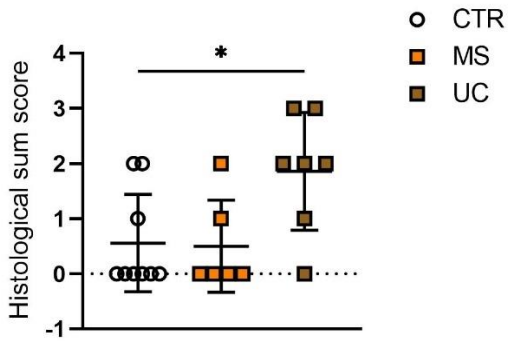
Figure 8: Proliferation activity in probands from human study

A) Ki-67 positive cells in relation to the total of crypt cells and to the total of base cells in probands from MS- or UC-group compared to the CTR group. Sections from the colonic mucosa were stained for Ki-67 by IHC and analyzed (high power field at 40-fold magnification). Kolmogorov-Smirnov test was followed by unpaired t-test. Horizontal lines depict medians, and every dot represents one subject. **B)** Representative images of Ki-67 IHC stained sections from the colonic mucosa of individuals from the CTR, MS or UC group (20-fold magnification).

5.3. Higher inflammation in sigmoid tissue from subjects with UC

I further evaluated colonic inflammation using a histological score assessed on HE stained biopsies taken during colonoscopy (see 4.2.2.2.). This score consists of inflammatory criteria like edema, lymphocytic infiltration, neutrophil infiltration, crypt distortion and ulceration. As shown in (Figure 9) a significant higher sum score was found in individuals from the UC group. This indicates that these patients have higher inflammation in their sigmoid mucosa compared to the other groups.

A)



B)

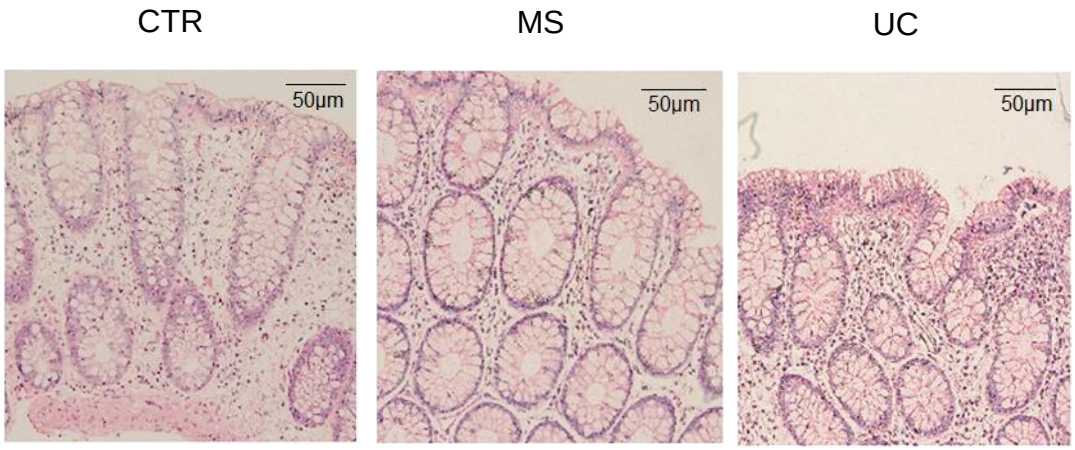


Figure 9: Histopathological inflammation is higher in ulcerative colitis patients

A) Sections from sigmoid colon stained with hematoxylin and eosin were analyzed with a histological sum score to evaluate the histopathological inflammation state. Resulting sum scores were assigned to controls (CTR), metabolic syndrome group (MS) or ulcerative colitis group (UC). Kolmogorov-Smirnov test was followed by unpaired t-test; *p<0,05. Large horizontal lines are medians, each dot represents one individual. **B)** Representative images from sigmoid colon stained with hematoxylin and eosin from subjects of control group (CTR), metabolic syndrome group (MS) or ulcerative colitis group (UC) (20-fold magnification).

5.4. Assessment of expression of ISC and inflammation markers

In the next step, I assessed the expression levels of intestinal stem cell markers and pro-inflammatory cytokines in sigmoid biopsies of all subjects. This revealed a distinct, non-significant upregulation of LGR5 in the MS group, and a significant upregulation of OLFM4 in UC patients (p-value = 0,036; Figure 10). No significant differences in the mucosal expression levels of TNFα or IL-6 could be observed.

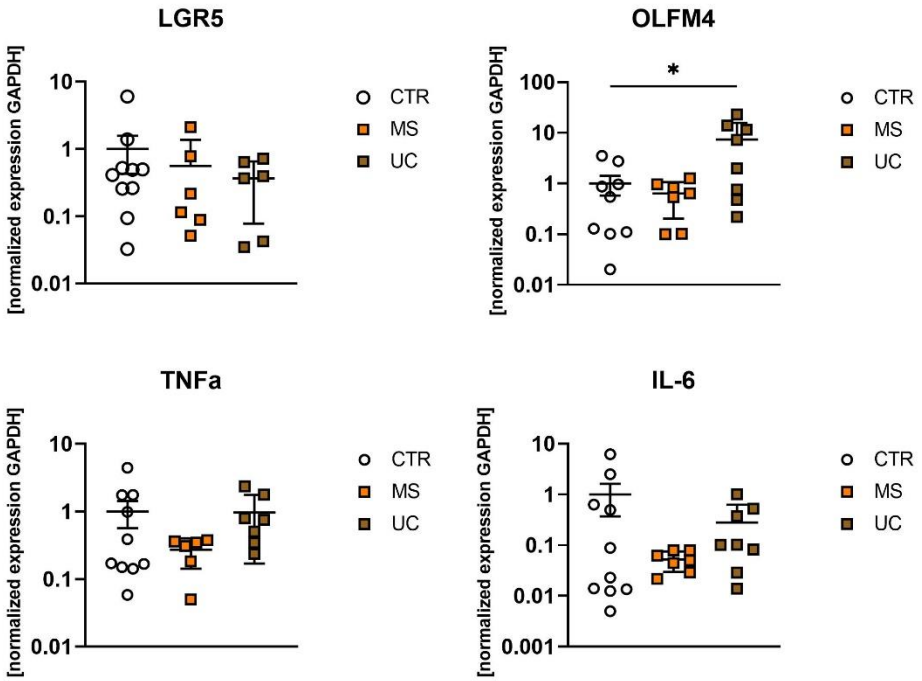


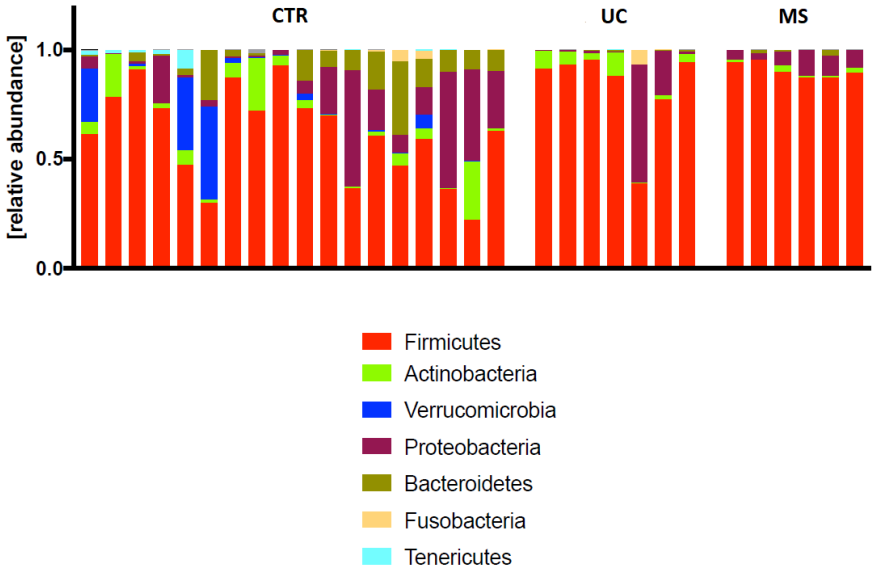
Figure 10: Upregulation of OLFM4 in ulcerative colitis patients and a trend towards higher expression levels of LGR5 in metabolic syndrome group.

Quantitative PCR analysis of LGR5, OLFM4, TNFα and IL-6 mRNA levels in sigmoid colon of individuals from control group (CTR), metabolic syndrome group (MS) or ulcerative colitis group (UC). All mRNA values were normalized to GAPDH, and each sample was quantified in duplicate. Pairwise comparison was done. Kolmogorov-Smirnov test was followed by unpaired t-test, *p<0,05. Horizontal lines represent medians; each dot represents one subject.

5.5. Investigating the gut microbiome in controls and patients

Last, I analyzed the microbial composition of every participant by 16S RNA gene sequencing of stool samples collected one or two days before colonoscopy. I detected the most frequent bacterial phyla and families in each group and compared the cohort's intestinal microbiomes (Figure 11). Analyzing the occurrence of different phyla in the individuals, I found significant deviations in patients with MS or UC compared to controls. In both groups a similar shift towards more abundant *Firmicutes* was detected. Beside this, in the UC group, only *Actinobacteria*, *Proteobacteria* and *Fusobacteria* were present. In individuals from the MS cohort also *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* were found, but to a much smaller extent than in the controls. The variability of phyla was significantly reduced in the patients with MS or UC. Next, I analyzed the stool for the presence of several bacterial families. In patients with UC the analysis revealed a significant decrease of *Ruminococcaceae*, a reduction of *Bifidobacteriaceae* and an increase of *Streptococcaceae*, compared to the CTR cohort. In the group of MS I found more *Streptococcaceae* and notable less *Enterobacteriaceae*, *Ruminococcaceae* and *Bifidiobacteriaceae*, than in controls. Both groups, UC and MS, showed a lack of *Bacteroidaceae*, *Verrucomicrobiaceae* and *Veillonellaceae*. Moreover, an increase of *Enterococcaceae* and *Lactobacillaceae* was seen, compared to the CTR group. I also observed a slight decline of *Lachnospiraceae* and *Clostridiaceae* in the cohorts of UC and MS.

A) Phylum



B) Family

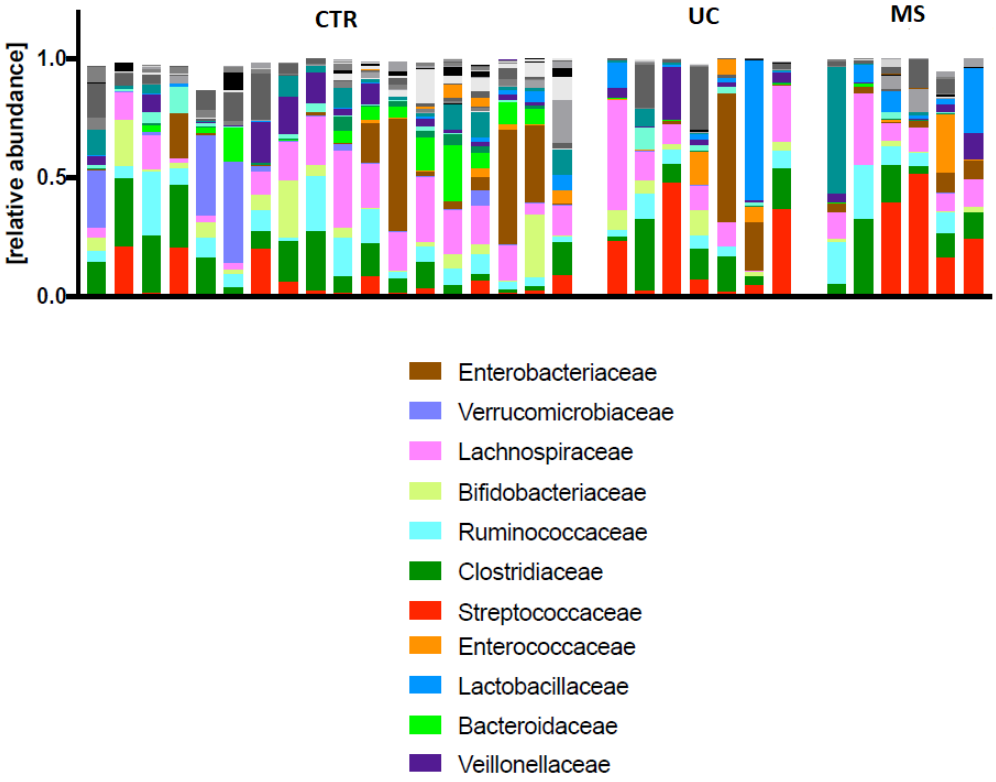


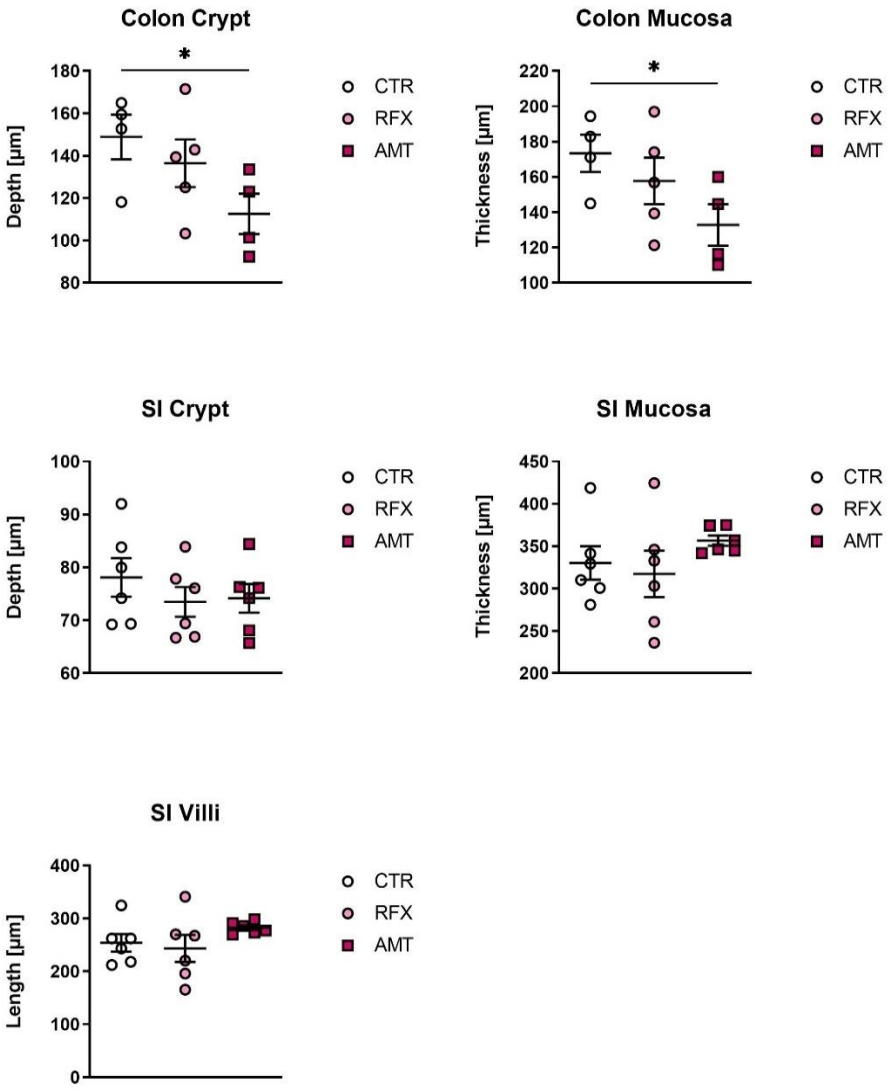
Figure 11: Decreased variability of the microbiome and changed microbial profiles in patients with metabolic syndrome or ulcerative colitis

A) Abundance of several bacterial phyla. Each bar represents one individual from the control group (CTR), the ulcerative colitis group (UC) or the metabolic syndrome group (MS). The distribution of colors in one bar displays the mRNA levels of one abundant phylum in relation to all evaluated phyla. **B)** Abundance of different bacterial families. Each bar represents one individual from the CTR, the UC or the MS group. The distribution of colors in one bar displays the mRNA levels of one occurring bacterial family in relation to all analyzed bacterial families.

5.6. Antibiotic treatment affects the thickness of the colonic mucosa in mice

The colonic stem cells are located at the base of the crypt unit in the intestinal mucosa. It is known that the colonic CBCs are responsible for the maintenance of the intestinal epithelium. As the surface of the epithelium gets in contact with many different substances, its function and structure are exposed to many challenging influences from the intestinal lumen. Current research focuses on the microbiota-host interaction to better understand how microbial metabolites interact with their environment. It was shown that commensal bacteria are essential for a healthy gastrointestinal tract (Guarner & Malagelada, 2003). First, I was interested to learn whether antibiotic treatment in general affected the morphology of the mucosa. To this end one group of animals received AMT, which is a mixture of Metronidazole, Ampicillin, Vancomycin and Neomycin sulfate. Another group of mice was treated with Rifaximin, a poorly absorbable antibiotic that is known to cause dysbiosis (Bajic et al., 2020). These mice were compared to the control group treated with tap water. To assess the impact of the treatments on intestinal architecture I determined the thickness of the entire mucosa and its units, the length of the villi and the depth of its crypts, in the small intestine and colon. The measurements revealed significant changes in the colonic mucosa of mice receiving AMT. These mice showed a thinner layer of mucosa as well as flattened crypts (Figure 12). The small intestine of the same group and the RFX-treated cohort did not show any alternation of mucosal thickness compared to the controls. This experiment also suggests that AMT is likely to induce a loss of goblet cells in the colonic crypts as well as AMT-treated mice macroscopically showed a remarkable widening of the large bowel, both observations need to be investigated in the future. Measurements were performed as described in 4.2.7.1.

A)



B)

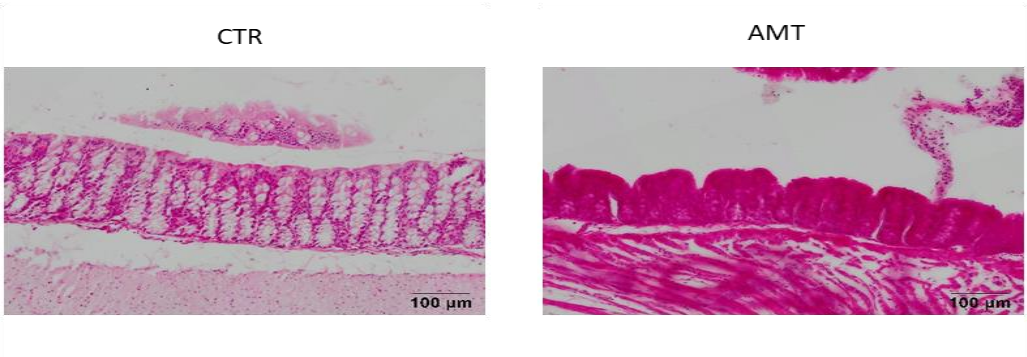


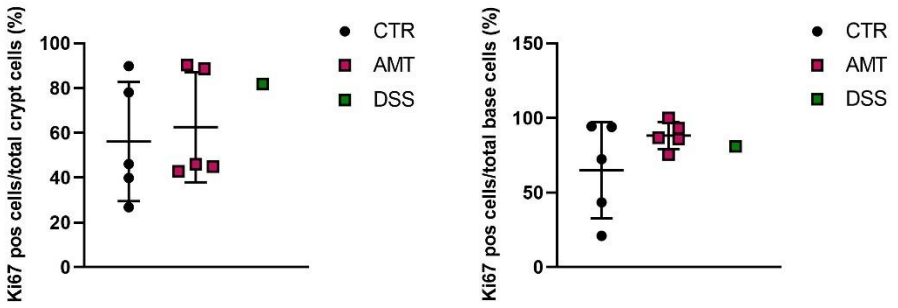
Figure 12: Antibiotic treatment changes the mucosal morphology of the colon

A) Measurement of mucosal thickness, length of villi and depth of crypt in HE stained sections of the small intestine (SI) or colon of mice. The mice received tap water (CTR), rifaximin (RFX) or antimicrobial treatment (AMT). All values ware measured in high power field at 40-fold magnification. Kolmogorov-Smirnov test was followed either by unpaired t-test or by Mann-Whitney test. Horizontal lines indicate medians, *p<0.05, each dot represents one mouse.
B) Representative images of HE stained sections of the colon from mice in the control group (CTR) compared to mice after antibiotic treatment (AMT) (20-fold magnification).

5.7. Antibiotic treatment does not affect proliferation within colonic crypts

As antibiotic therapy induces morphological changes in the colonic mucosa, I hypothesized that AMT enhances the regenerative activity of CBC stem cells at the crypts base of Lieberkühn. Ensuring the homeostasis of the intestinal mucosa, the epithelium shows high turnover rate of 4 – 5 d in small intestine, the fastest renewing tissue in mammals (Beumer & Clevers, 2016) and 5 – 6 d in the colon (Arike et al., 2020). Due to many different intrinsic or extrinsic factors the CBC stem cells are in a permanent process of dividing to produce enough daughter cells replacing damaged differentiated cells. This division activity can be analyzed by the expression level of Ki-67, a representative immunohistochemical marker for cell proliferation. In this experiment I investigated whether AMT or treatment with dextran sodium sulfate (DSS) as colitis causing substance, induce proliferative activity in the intestinal stem cell compartment. I performed Ki-67 immunostaining in colonic tissue and counted the positive cells in relation to the total number of crypt cells or of base cells. This analysis revealed no difference in proliferative activity in the colon of the cohort treated with antibiotics, especially at the crypt's base. Because of technical problems only one mouse with DSS-colitis was tested in this experiment.

A)



B)

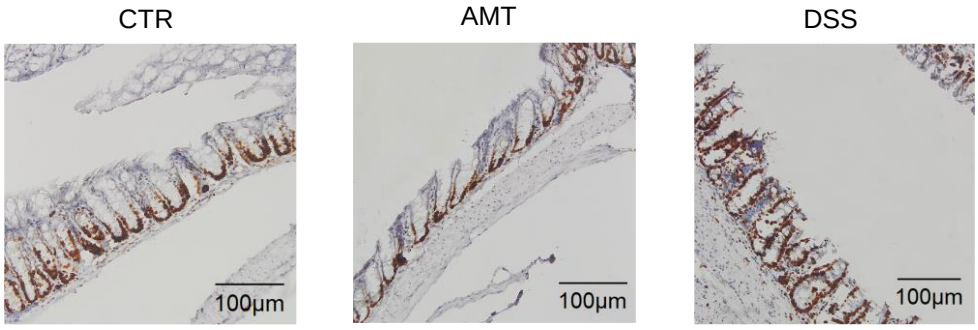


Figure 13: Proliferative activity in colonic crypts is not affected by antibiotic treatment

A) Number of Ki-67 positive cells in relation to the total of crypt cells and to the total of base cells in AMT- or DSS-treated mice compared to CTR group. Sections from the colonic mucosa were stained for Ki-67 by IHC and analyzed (high power field at 40-fold magnification). Kolmogorov-Smirnov test was followed by unpaired t-test. Horizontal lines indicate medians, and every dot represents one mouse. **B)** Representative images of Ki-67 IHC stained sections from the colonic mucosa in mice after AMT- or DSS-treatment compared to CTR (20-fold magnification).

5.8. Antibiotic treatment reduces Lgr5 and Rnf43 mRNA expression in the murine colon

Next, the impact of antibiotic treatment on the expression levels of colonic stem cell markers was analyzed. Therefore, the expression of Lgr5, Ascl2, Olfm4 and Rnf43 in murine colonic tissues was measured by qPCR. A significant downregulation of Lgr5 and Rnf43 expression was observed in the AMT-administered cohort compared to controls. Simultaneously, the same group showed increased levels of Ascl2 and Olfm4 in relation to the control cohort. RFX had no effect on stem cell marker expression.

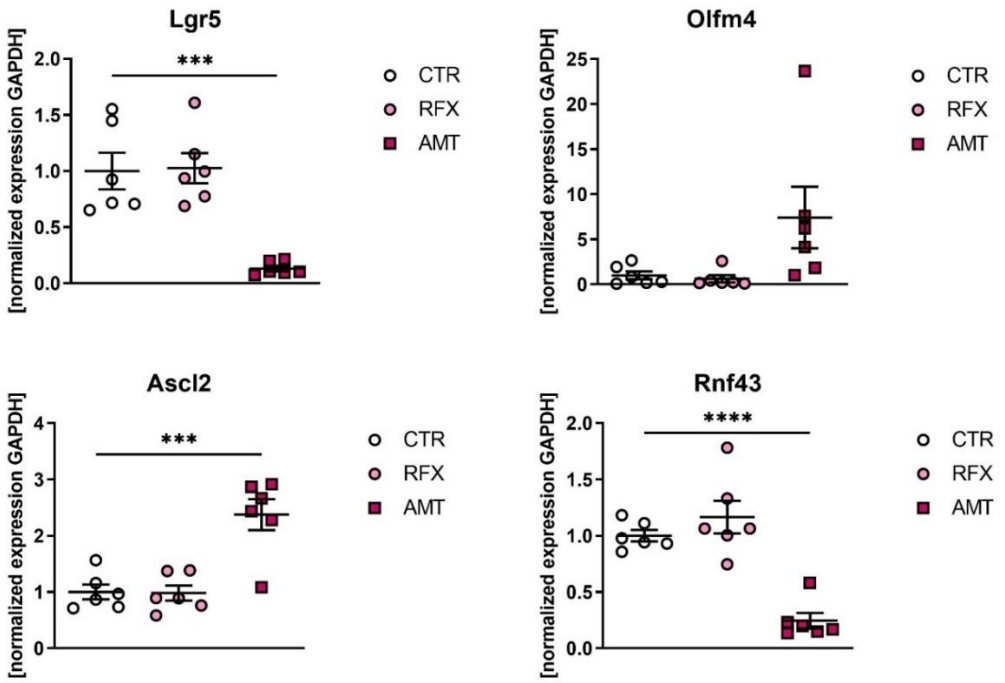


Figure 14: Downregulation of Lgr5 and Rnf43 and upregulated Ascl2 mRNA expression levels in murine colon after antibiotic treatment

Lgr5, Ascl2, Olfm4 and Rnf43 mRNA levels in murine colon. All mRNA values were normalized to GAPDH, and each sample was quantified in duplicate. Pairwise comparison was done. Kolmogorov-Smirnov test was followed by unpaired t-test. Horizontal lines represent medians; *p<0,05; each dot represents one mouse, n = 6 mice per group.

5.9. ISC marker expression in Myd88 – KO mice

As the TLR/Myd88-signaling pathway is discussed to be involved in ISC regulating mechanisms of the innate immune system (Moossavi, 2014), I was interested in its impact on different ISC markers. Therefore, I assessed the expression levels of Lgr5, Olfm4, Ascl2 and Rnf43 in mice genetically deficient for Myd88, that results in abrogated TLR signaling pathway. This experiment revealed only trends towards higher expression of Lgr5 and Olfm4 in the Myd88-KO mice compared to the wild type mice. No group differences were observed for Ascl2 and Rnf43 expression levels.

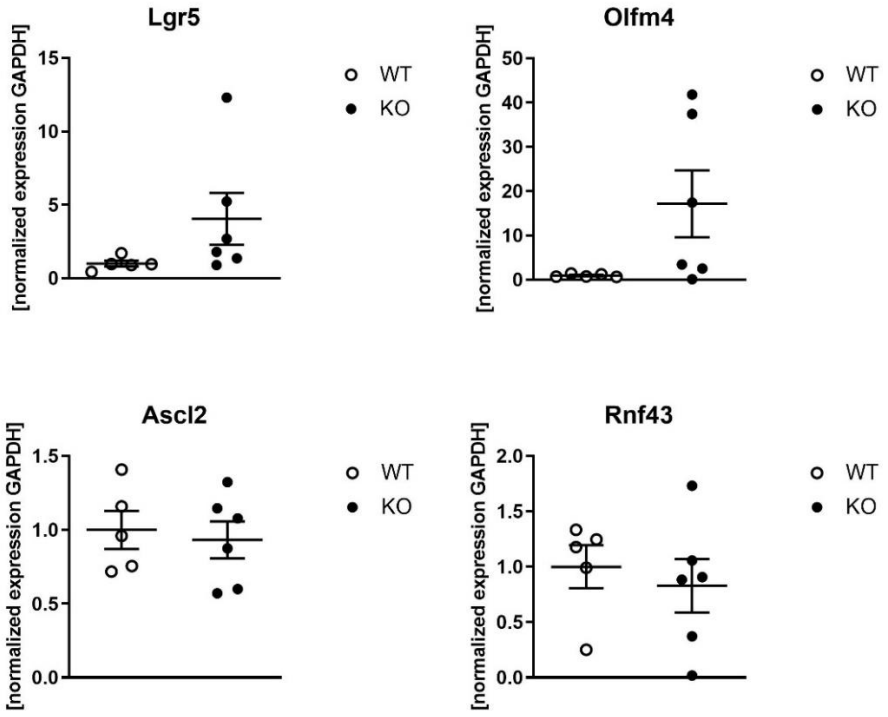


Figure 15: ISC marker expression levels in Myd88 – KO mice compared to wild type mice

Quantitative PCR analysis of Lgr5, Ascl2, Olfm4 and Rnf43 mRNA levels in colon of wild type (WT) or Myd88 – KO (KO) mice. All mRNA values were normalized to GAPDH, and each sample was quantified in duplicate. Pairwise comparison was done. Kolmogorov-Smirnov test was followed by unpaired t-test. Horizontal lines represent medians; each dot represents one mouse, n = 5 mice in WT and n = 6 in KO.

6. Discussion

6.1. Are metabolic syndrome and ulcerative colitis affecting the intestinal stem cells in a pre-oncogenic way?

In the main project, I performed a clinical study focusing on altered microbial compositions in connection with divergent ISC marker expression levels in subjects with CRC predisposing diseases. Patients suffering either from metabolic syndrome or ulcerative colitis were checked for characteristic alterations in their intestinal microbiota on the one hand, and for abnormal expression levels of several ISC markers on the other hand. Additionally, the tissue obtained from the colonic mucosa through colonoscopy was screened for inflammation and proliferative activity of the crypt cells. No group differences concerning the proliferative activity of crypt cells in patients with MS or UC compared to healthy controls were found. Despite the lack of signs for macroscopic inflammation, study subjects from the UC cohort show higher microscopic inflammation than controls or MS patients. Furthermore, a significant upregulation of OLFM4 in colonic tissue samples from participants with UC was observed. As previously shown by Gersemann et al., a combination of microscopically active inflammation and upregulated OLFM4 expression points out a more active UC. Additionally, the expression of OLFM4 was shown to expand throughout the colonic crypts as well as its secretion into the mucus slightly reduces the antimicrobial activity of defensins (Gersemann et al., 2012). Moreover, OLFM4 was demonstrated to play a crucial anti-inflammatory role since the colon of Olfm4-deficient mice showed serious inflammation and mucosal destruction (W. Liu et al., 2016). OLFM4 is known to be regulated by Notch pathway, NF-κB and Wnt signaling (X.-Y. Wang et al., 2018). However, its either protective or supporting role in colorectal carcinogenesis needs to be investigated more precisely. Furthermore, it is still unsolved whether the increase of OLFM4 depends on the immune system, altered microbiota, or on other factors. Using 16S rRNA sequencing, I determined the profiles of intestinal microbiota in the study subjects by screening for certain bacterial phyla and families. The results reflect previous findings of reduced microbial diversity in individuals with MS (Le Chatelier et al., 2013) or UC (Lepage et al., 2011) including the expansion of certain bacterial genera or species. Higher abundances of *Firmicutes* were observed, especially of *Streptococcaceae* in patients with MS, while *Bacteroidetes* nearly disappeared in both, the UC and MS group. In contrast, a healthy gut microbiota is considered to predominantly consist of *Firmicutes* and *Bacteroidetes* (Jandhyala et al., 2015) as confirmed by my results from the control group. It is known that these phyla can exert a protective function such as colonization resistance which is a known strategy to maintain a healthy balance of intestinal microbiota. Ishikawa et al. developed a model which highlights the therapeutic potential of FMT, particularly containing *Bacteroidetes* species. In their study, pretreatment with antibiotics improved the efficiency of FMT with live *Bacteroidetes* cells by enhancing their

capability to promote colonization resistance. The concept of malignant transformation-promoting alternations of the microbiome which is displayed by more abundant *Firmicutes* or lacking *Bacteroidetes*, represents an interesting model for further investigations. For example, in a retrospective clinical study Kumar et al. detected an association between the strains *Streptococcus gallolyticus* subsp. *gallolyticus* (*Sg*) and the development of CRC. Strikingly, this study revealed variations between certain Sg strains concerning their ability to promote tissue proliferation (Kumar et al., 2018). These findings point to a multifactorial process that enables tumor growth in the presence of distinct bacteria and further investigations need to scrutinize all involved factors more precisely.

6.2. Consequences of antibiotic treatment in the mouse model

To examine the consequences of extensive antibiotic treatment on the mucosal morphology of the small intestine and the colon, laboratory mice were treated either with AMT (metronidazole, ampicillin, vancomycin, and neomycin sulfate) or with RFX for two weeks. Both treatments are known to cause dysbiosis (Bajic et al., 2020) and therefore were used to investigate the consequences of microbial shifts on intestinal mucosa. In this context, I detected morphologic changes in the colonic mucosa of mice after AMT. Measurements revealed a thinning of the mucosa layer in the murine colon, including more plane crypts after extensive antibiotic treatment which possibly facilitates invasion of pathogens. These observations were accompanied by a significant downregulation of Lgr5 and Rnf43, whereas Ascl2 was significantly upregulated in mice receiving AMT. In their recent work Murata et al. described the influence of Ascl2-deficiency on the regeneration process after irradiation or after Diphtheria toxin-mediated Lgr5+ ISC depletion. After ablation of Lgr5+ ISCs in the colon, they observed that differentiating progenitor cells in the middle of the crypts express higher levels of Ascl2, dedifferentiate and migrate back to the base niche where they regain Lgr5+ stem cell state (Murata et al., 2020). In the light of these findings, my results suggest a similar model of AMT-induced tissue injury represented by a thinner mucosa layer and reduced levels of Lgr5 and Rnf43, both part of the Wnt signaling pathway, the most important promoter of epithelial homeostasis in the intestine (Krausova & Korinek, 2014). Correspondingly, high Ascl2 concentration in the colonic mucosa supports the idea of an active regeneration process 14 days after starting AMT. The divergence in ISC marker profiles can be regarded either as direct consequence to pharmacologically induced epithelial destruction or as a result of a disrupted microbial ecosystem due to antibiotics-induced dysbiosis or both. Furthermore, I hypothesized changing proliferative activity in the colonic crypts of mice after AMT which was not confirmed by my measurements. In addition, the DSS-colitis-induced damage of intestinal epithelium also suggests higher proliferation in colonic crypts due to regenerative processes that need closer examinations.

In another experiment it was assessed whether mice with genetic deficiency for Myd88 show deviations in ISC marker expression levels, as the Myd88/TLR signaling pathway is known to be involved in the development of sporadic intestinal cancer (Rakoff-Nahoum & Medzhitov, 2007). No significant changes were observed, although Myd88 as part of the TLR signaling is discussed to represent an important junction between the innate immune system and ISC regulating mechanisms. My results point out that the depletion of Myd88 does not exclusively disturb the homeostatic ISC balance in the colonic epithelium.

6.3. Conclusion and outlook

A broad spectrum of factors is known to cause gastrointestinal diseases, accompanied by either causative or consecutive dysbiosis, which can culminate in the development of CRC. Future research needs to gain a better understanding of molecular processes occurring in the ISC niche and how these mechanisms interact with the surrounding ecosystem in health and disease. On the one hand it is of great importance to analyse the diversity of microorganisms inhabiting the human gut, on the other hand all substances, including MAMPs or microbial metabolites, that are involved in the host-microbiota interactions need to be elucidated. Growing knowledge about ISC regulating mechanisms that represent an attractive target for therapeutic approaches, is essential for the treatment of intestinal diseases and particularly for the prevention of CRC development. Considering advances in microbial profiling, prospective personalized therapies such as FMT represent promising strategies to enable optimized anti- or probiotic treatments with less side effects such as antibiotic resistances or overgrowth of pathogens. Additionally, future work needs to focus on key processes that are responsible for maintaining mucosal homeostasis and mucosal barrier function as a result of a co-evolutionary symbiosis between the host and intestinal microbiome.

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