Between cancer and therapy: Studies of the colon
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SUMMARY
In this thesis, we have investigated factors that influence the initiation of colorectal cancer (CRC), as well as mechanisms that manipulate resistance to conventional chemotherapy in established CRC. Increasing our knowledge of both processes is instrumental to combat cancer better. Collectively, our results put forward novel candidates that play critical roles in these processes and may therefore serve as attractive targets in the development of new therapies that may ultimately prevent or treat CRC.

In chapter 3 and 4, we investigated the role of sex hormones on CRC development. Epidemiologic studies have shown that females have a lower incidence and delayed onset of CRC compared to males. Additionally, two very large randomized controlled trials performed by the Women’s Health Initiative (WHI) examined the effects of hormonal replacement therapy in postmenopausal (PMP) women. The first study showed that combined treatment with both equine estrogen (E2) and medroxyprogesterone acetate (MPA) substantially reduced the risk of CRC cancer compared to placebo with 37%. This protection was not found in a second randomized controlled trial among women that were treated with equine estrogen alone. Thus large clinical studies suggest that MPA is protective against CRC. The gender disparity in CRC incidence was therefore generally attributed to a protective effect of female hormones.

Chapter 3
The data presented in this chapter support a paradigm shift, namely that gender disparity in sporadic CRC depends on male hormone tumor promotion instead of female hormone protection. In careful analysis of two established animal models for colonic adenoma formation, we found that castration reduced, and testosterone supplementation restored, the number of adenomas in the male rat and mouse colon. In contrast, ovariectomy and replacement of female hormones had no measureable effect on colonic adenoma formation. In Apc\textsuperscript{min/+} mice, where most of the tumors arise in the small intestine, this testosterone-dependent sexual dimorphism was specific to the colon. Mechanistically we demonstrated that the androgen receptor (AR) is not expressed in the intestinal or colonic epithelium indicating that testosterone promotes adenoma formation through an indirect mechanism.

Chapter 4
Here, we further investigated the apparent discrepancy between findings in animal models and the clinical trials from the WHI. In previous animal studies conducted by us and others, the postmenopausal hormone status was mimicked by performing ovariectomies which leads to attenuated production of all female and male hormones. Indeed in humans, the production of female hormones is substantially reduced after the menopause. However postmenopausal ovaries are still capable of producing other substances that have oncogenic effects, such as androgens. Therefore we tested the effect of MPA on adenoma formation in a mouse model of menopause. In concordance with previous reports using ovariectomized mice, we confirmed that MPA does not reduce adenoma formation in fertile mice. Strikingly, induction of menopause significantly increased the number of adenomas. In contrast to
fertile mice, MPA protected against adenoma formation in postmenopausal mice and reduced the number of adenomas to similar numbers as those in fertile mice. These data argue that the protective effect of MPA is critically dependent on postmenopausal hormone status and suggest that in contrast to postmenopausal women, fertile women may not benefit from progestins.

Chapter 5

In contrast to the protective effect on CRC, treatment with female hormones increases the risk of developing ulcerative colitis. As chronic intestinal inflammation predisposes to colon cancer development, we investigated the effects of female hormones on colitis-associated cancer. To that end, we performed ovariectomies in a well-established mouse model of inflammation associated colon carcinogenesis and found that, in contrast to sporadic colorectal carcinogenesis, in the context of chronic inflammation female hormones promote tumorigenesis. Using hormone replacement, we subsequently identified estrogens (E2) but not progestins (MPA) to be responsible for this tumor-promoting effect. E2-treated animals showed increased clinical symptoms and Il-6 production upon DSS-induced colitis and treatment with E2 did not only increase the numbers of adenomas but also strongly promoted tumour progression with all E2-treated animals developing at least one invasive adenocarcinoma, whereas placebo-treated animals developed adenomas only. Using Erα mutant mice, we found that the pro-tumorigenic effect of oestrogens depends on both Erα and Erβ.

Chapter 6

Azathioprine and other thiopurines are an important treatment modality in patients that suffer from inflammatory bowel disease. In these patients, treatment with thiopurines has been associated with a reduced risk of developing CRC. Additionally, the molecular target of azathioprine, Rac1 has recently been implicated as a critical player during intestinal tumorigenesis. We hypothesized that Azathioprine may thus be a candidate for chemoprevention of CRC in patients that have increased risk for developing this disease. We tested this hypothesis in the ApcMin/+ mouse model of sporadic intestinal tumorigenesis. Even though all azathioprine treated mice showed signs of drug-associated toxicity, it did not reduce tumor formation. We therefore concluded that in the absence of inflammation azathioprine does not affect intestinal tumorigenesis.

Chapter 7

Cancer stem cells (CSCs) are more resistant to conventional chemotherapy than differentiated cancer cells and may therefore play an important role in post-therapeutic tumor relapse. In order to improve the rate of sustained response to conventional chemotherapy, novel approaches are warranted that specifically sensitize these colon-CSCs to therapy. In this chapter, we report that induction of ER-stress leads to phenotypic differentiation of colon-CSCs and a suppressed clonogenic potential in vitro and in vivo. ER-stress induced differentiation resulted in enhanced sensitivity to several conventional chemotherapies in vitro and suppressed the growth of subcutaneous xenografts when combined with chemotherapy. Our data thus provide a novel approach to force the differentiation of cancer stem cells and sensitize these cells to conventional therapy.
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DISCUSSION AND FUTURE PERSPECTIVES

All experimental chapters are discussed at the end of the chapter itself. In this section we will discuss unanswered questions and elaborate on how future experiments are directed to answer these questions. In particular we will further investigate the underlying molecular mechanism by which androgens promote colorectal tumorigenesis. In the last part of this chapter we will elaborate on how ER-stress may cause differentiation mechanistically and how our findings may be applied to the clinic.

Androgen receptor

The receptor for testosterone, the androgen receptor (AR), is not expressed in the intestinal and colonic epithelium, which indicates that its androgen-dependent tumor promotion is effected in an indirect manner. We have shown that the androgen receptor is highly expressed in brain and liver and are currently investigating how these tissues modulate adenoma formation in response to androgens by using tissue specific deletion of the AR-gene.

Testosterone and the brain

The brain produces several factors that may promote colorectal cancer (CRC) development. Human Growth Hormone Releasing Hormone (GHRH) is a hypothalamic decapeptide which binds to the Growth Hormone Releasing Hormone Receptor (GHRHR) in the pituitary gland, resulting in the production and release of Growth Hormone (GH) into the circulation and exerts its effects either directly by binding to the Growth Hormone Receptor (GHR) or indirectly through its tissue mediated Insulin Like Growth Factor 1 (IGF-1).

A tumor-promoting role of GH is suggested by the observation that patients with acromegaly have increased risk of developing CRC 1,2. These patients suffer from excessive production of GH and IGF, most commonly due to pituitary adenoma’s. IGF-1 has anti-apoptotic properties and the mRNA of IGF-1 and its receptor are expressed in colon cancer cell lines 3,4. Epithelial proliferation is the colon is increased in acromegaly patients and correlates with the levels of circulating IGF-1 5. Conversely, treatment with antagonists of GHRH results in cell cycle arrests in colon cancer cells and delayed growth of subcutaneous xenografts in mice 6,7.

Thus far, the relationship between circulating androgens and release of GH and IGF-1 is poorly investigated. Elevated levels of IGF-1 are observed in bodybuilders abusing androgen anabolic steroids 8, however if this finding can be extrapolated to physiologic differences in androgen levels between male and female patients is uncertain, let alone if brain-derived factors can explain gender differences in CRC.

The microgenderome

Although not investigated in this thesis, the role of gut microbiota has received much attention in the last decade. Gender bias is observed in numerous autoimmune diseases, many of which are more prominent in women. As such, male gender protects against disease susceptibility and severity of
type I diabetes mellitus (T1D) in the Non Obese Diabetic (NOD) mouse model. This protective effect was shown to be dependent on circulating androgen levels. The composition of intestinal gut microbiota is clearly different in males compared to females. An important role of microbiota on T1D development is suggested by the observation that gender difference disappears when NOD mice are housed under germ free conditions. Markle et al. demonstrated that removal of the gut microbiota increased circulating testosterone concentrations in female mice but decreased them in male mice. Furthermore, transfer of gut microbiota from adult males to immature female resulted in elevated serum testosterone levels and robust protection against T1D. All these effects were dependent on androgen receptor activity. Together these data suggest that gender-specific composition of microbiota modulates circulating testosterone levels, resulting in relative protection against T1D. Yurkovetskiy et al. demonstrated that the crosstalk between microbiota and testosterone is not unidirectional and that in fact testosterone itself can also modulate microbiota composition.

How intestinal microbiota influences the development of colon cancer is incompletely understood, however accumulating evidence shows that colorectal carcinogenesis is accompanied by microbial dysbiosis. There is no single causative organism identified in CRC; however, some bacteria such as Fusobacteria, Alistipes, Porphyromonadaceae, Coriobacteriidae, Staphylococcaceae, Akkermansia spp. and Methanobacteriales are consistently augmented in patients with colorectal cancer whereas others are constantly diminished such as Bifidobacterium, Lactobacillus, Ruminococcus, Faecalibacterium spp., Roseburia and Treponema. Moreover, bacterial metabolites amino acids are increased and butyrate is decreased throughout colonic carcinogenesis. Which of these alterations is truly responsible for (anti-) oncogenic effects remains to be elucidated. Future experiments in our laboratory shall investigate whether hormonally regulated differences in intestinal microbiota may account for the gender difference colonic adenomas.

Testosterone and the Liver
How testosterone modulates the intestinal microbiota composition is thus far incompletely understood. The androgen receptor is highly expressed in the liver so potentially testosterone influences the composition of bile acids, ultimately resulting in alteration of microbial composition. Vice versa, as a result of the enterohepatic bile circulation, alterations in microbiota may influence the composition of bile acids. Altered bile acid composition has been suggested to exert direct oncogenic effect on the colonic epithelium. As such, relatively high levels the bile acids lithocolic acid and deoxycolic acid are found in persons with CRC, whereas ursodeoxycholic acid is linked to a lower incidence in CRC.

Furthermore, sex steroids are important regulators of adipose tissue metabolism in the liver. It was shown that treatment of orchidectomized mice with dihydrotestosterone (DHT) results in obesity, associated with reduced energy expenditure and fat oxidation, whereas in contrast, DHT did not affect food consumption or locomotor activity. Another study revealed a reduction in high-density lipoprotein-cholesterol in primates upon DHT treatment.
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It must be stated that all the above suggested involvements of the androgen receptor in colonic carcinogenesis are highly speculative. The first step towards revealing the oncogenic mechanism of testosterone is by systematic testing which organ systems are involved. This can and will be investigated by tissue-specific deletion of the androgen receptor.

**Tissue-specific deletion of the androgen receptor**

The target cell by which testosterone promotes adenoma formation remains yet to be elucidated. To investigate this, we will perform genetic experiments using Cre-LoxP mediated tissue specific deletion of the androgen receptor (AR). We are currently breeding a genetic mouse model in which exon 2 of the AR gene is flanked by LoxP sites. The presence of LoxP sites itself does not influence the functioning of the AR and thus these mice can functionally be seen as AR-wild type. Expression of the enzyme Cre-Recombinase (Cre) results in excision of the DNA fragment that is flanked by the LoxP sites and thus leads to a functional knock-out phenotype of the androgen receptor in all cells expressing Cre. Crossing AR-Flox mice into mouse strains that express Cre under tissue specific promoters will allow us to selectively delete the androgen receptor in either the whole body, the brain, the liver or the intestinal epithelium.

**Mechanism of ER-stress induced differentiation**

In an attempt to elucidate the underlying molecular mechanism of stem cell differentiation by ER-stress, we performed STORM-analysis on the list of downregulated genes by SubAB treatment in chapter 7. The STORM software (Search Tool for Occurences of Regulatory Motifs) is designed to scan through sequences in search of binding sites that match given motifs which allows prediction of transcription factors that may be responsible for a difference in gene expression upon a certain treatment (Figure 1a). This analysis revealed involved of the Nuclear Factor κ-light-chain-enhancer of activated B-cells (NF-κB) pathway after SubAB treatment, as binding sites for both NF-kB as well as NFKB1 were among the 15 most abundant transcription factors of all SubAB-downregulated genes. The NFKB1 gene encodes for Nuclear factor NFkB p105 subunit which can undergo cotranslational processing by the 26S proteasome to produce the NFkB subunit p50.

NF-κB is a transcription factor consisting of the subunits p65 (also called RelA) and p50. NF-kB signaling is activated by numerous processes such as inflammation, damage by oxidant-free radicals, ultraviolet irradiation, and is activated by cytokines, chemokines, adhesion molecules and bacterial or viral products. In the absence of activating stimuli, NF-kB is located in the cytoplasm and bound to NF-kB inhibitor α (IκBα) where IκBα prevents nuclear translocation of NF-kB. Upon activation, NF-kB inducing kinase (NIK) phosphorylates the IκBα kinase (IKK) complex. This complex phosphorylates IκBα, marking it for ubiquitination and subsequent 26S proteasome-mediated degradation. As a consequence of IκBα degradation, NF-kB translocates to the nucleus where it causes transcription of its target genes. NF-kB plays a key role in several cellular functions such as inflammation, apoptosis, cell survival, proliferation, angiogenesis and innate and acquired immunity. Additionally, NF-kB has recently been implicated to play a crucial role in stem cell proliferation and colorectal cancer.
Figure 1. UPR-induced differentiation is mediated by inhibition of NFkB signaling. (A) Schematic representation of STORM analysis (Search Tool for Occurrences of Regulatory Motifs) of differentially expressed after SubAB treatment. Pre-scanned human Refseq promoter regions were used from the Transfac Professional database (-1500 bp to +500 bp from the transcription start site (TSS). (B) Geneset Enrichment Analysis of a previously described NF-kappaB Geneset\(^1\) using GSEA software from the Broad Institute (http://www.broadinstitute.org/gsea). (C) Quantitative RT-PCR for LGR5 relative to GAPDH of GTG7 colon cancer stem cells after 24 hours treatment with NF-kappaB inhibitor JSH40 and BMS at indicated doses. Experiments were set up in a similar manor as described in chapter 7 of this thesis. Data are representative of three independent experiments. Values mean ± SEM, significance was measured by Student’s t-test where *p<0.05, **p < 0.01, ***p < 0.001.
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initiation. Upon activation of the NF-kB signaling pathway, nonstem cells were shown to undergo dedifferentiation and acquire tumor-initiating capacity.\textsuperscript{23,24}.

Geneset Enrichment Analysis using a previously described NF-kappaB geneset\textsuperscript{25} confirmed inhibition of NF-kB signaling by SubAB treatment (Figure 1b). In order to investigate if inhibition of NF-kB signaling is sufficient to induce stem cell differentiation we treated GTG7 colon cancer stem cells with NF-kB inhibitors BMS345541 (BMS) and JSH-23 (JSH). BMS is an IKK inhibitor and prevents phosphorylation of IκBα. JSH interferes with nuclear translocation of NF-kB, without affecting the process of IκB degradation. Both JSH and BMS were equally effective in downregulating stem cell marker LGR5 (Figure 1b). Although we did not perform rescue-experiments to investigate if the effect of ER-stress would be neutralized by simultaneous NF-kB activation, these data suggest that ER-stress induced differentiation is mediated by NF-kB inhibition.

Clinical opportunities of ER-stress

The data described in chapter 7 provide a novel approach to force the differentiation of cancer stem cells and sensitize these cells to conventional therapy. The compounds described in this chapter however are not suitable for treatment of patients. SubAB and thapsigargin are potent activators of the UPR, but are highly toxic \textit{in vivo} and rapidly lead to death after injection in rodents\textsuperscript{26,27}. In our experiments we did not observe any signs of toxicity of Salubrinal treatment \textit{in vivo}, however this compound has thus far not been tested in humans.

In an effort to find a safe treatment, registered for patients, we tested the efficacy of the proteasome inhibitor bortezomib. Although the mechanism of action of bortezomib is incompletely understood, inhibition of the NF-kB is suggested to play an important role due to a reduction of IκBα degradation\textsuperscript{28}. We hypothesized that proteasome inhibition would lead to an accumulation of unfolded and misfolded proteins (ER stress) resulting in activation of the UPR. Indeed, treatment of LS174T colon cancer cells with bortezomib caused upregulation of the endoplasmic reticulum chaperone GRP78/BiP and the downstream transcription factor CHOP (Figure 1a-b). UPR activation by bortezomib was accompanied with downregulation of Intestinal Stem Cell marker OLFM4 and LGR5 (Figure 2c) and a loss of self-renewal capacity (Figure 2d). Interestingly, low dose bortezomib of 5nM showed differentiation in the absence of UPR activation, leaving the option open that bortezomib-induced differentiation may not directly result from UPR activation but from another stimulus such as inhibition of NF-kB signaling.

Currently it is not completely clear if combined treatment of bortezomib with standard therapy is safe and effective in the treatment of colon cancer. Two fase-i trials have thus far shown contradicting results. Cohen et al. reported that combined treatment of capecitabine and oxaliplatin with bortezomib was safe in a fase-i trial for solid tumors\textsuperscript{29}. A later fase-I study for the treatment of advanced or metastatic rectal cancer however showed that the maximal tolerable dose of bortezomib in combination with standard 5-fluorouracil and external beam radiation therapy may be below a clinically relevant dose, limiting the clinical applicability of this combination\textsuperscript{30}.
In conclusion, forced differentiation of therapy resistant colon cancer stem cells is an attractive and feasible avenue which may optimize the sensitivity to chemotherapy and improve outcome in patients with colon cancer; however this aim is currently retained from the lack of safe and effective compounds that are registered for human applications. Therefore future research is warranted for the development of new compounds or the identification of existing agents that may be effective in achieving this aim.

Figure 2. Bortezomib activates the UPR and causes intestinal stem cell differentiation. Effects of 24 hours treatment with bortezomib at indicated doses of LS174T colon cancer cells. (A) Quantitative RT-PCR of components of the Unfolded Protein Response CHOP and GRP78 relative to GAPDH. (B) Westernblot analysis of endoplasmic reticulum- chaperone GRP78 after bortezomib treatment. (C) Quantitative RT-PCR of Intestinal Stem Cell markers OLFM4 and LGR5 relative to GAPDH. (D) Percentage of colony forming cells after bortezomib treatment, assessed by limiting dilution assay. Assessment of clonal frequencies and statistical analysis was evaluated with the Extreme Limiting-dilution Analysis (ELDA) ‘limdil’ function (http://bioinf.wehi.edu.au/software/elda/index.html). Exclusion of dead cells was performed with propidium iodide (PI). Experiments were set up in a similar manor as described in chapter 7 of this thesis. All data are representative of three independent experiments. Statistical significance of (A, C) was determined by one way ANOVA with a Bonferroni post-test. Values mean ± SEM (A,C) or mean with 95% CI, *p<0.05, **p < 0.01, ***p < 0.001.
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REFERENCES


Summary and Discussion


