Exploring available models to investigate the brain of the gut and its role in colorectal cancer

Summary

Globally, colorectal cancer (CRC) is the second deadliest cancer and the third most prevalent cancer type. The health burden is expected to further increase due to aging, population growth, and the growing number of countries with a high human development index. Early detection of CRC can significantly improve survival, which can be achieved by population-wide screening. Previously, we identified N-myc downstream regulated gene 4 (*NDRG4*) DNA methylation as biomarker for CRC, which has been incorporated in an FDA-approved multi-target stool test for early detection of CRC (Cologuard®), that is currently used in the USA. Interestingly, *NDRG4* is specifically expressed by neurons of the gut's intrinsic nervous system, the enteric nervous system (ENS). The ENS, also referred to as 'the brain of the gut', is an extensive neural network consisting of neurons and glia that runs throughout the entire length of the gastrointestinal tract. It is important for gut functioning, by regulating gut motility, mucosal secretion, immune response and local blood flow. ENS dysfunction is linked with a plethora of (gastrointestinal) diseases, including neuropathies such as Hirschsprung disease, inflammatory bowel disease, disorders of gut-brain interaction, and neurodegenerative disorders. How enteric neurons, and NDRG4 in particular, influence CRC is not completely understood. Therefore, the overall aim of this thesis was to investigate how the ENS is involved in CRC development and progression.

In **Chapter 2**, we reviewed the current knowledge on the role of nerves and glia in CRC. Not only the contribution of the ENS was considered, but also the existing literature on the role of extrinsic innervation from the sympathetic and parasympathetic nervous system was reviewed. In this context, perineural invasion, the migration of cancer cells along nerves, which is correlated with adverse prognosis in CRC, is the most well-known interaction between both cell types. Recently, active crosstalk between cancer and neurons has been described for several types of cancer, but in the case of CRC, this bidirectional communication is yet to be fully explored. Neurotransmitters and neuropeptides, such as serotonin, gamma-aminobutyric acid (GABA), and neuropeptide Y (NPY) can affect CRC progression. At the same time, CRC cells can secrete growth factors to stimulate nerve growth. Enteric glial cells seem to have a pro-tumorigenic role in animal models, while their presence in human CRC seems to be protective. It remains to be elucidated how intrinsic and extrinsic neural input balances in colorectal carcinogenesis, but there are strong indications that nerves are important for CRC.

Subsequently, we explored two methodologies to study the ENS in more detail. As for other biomedical research areas, *in vitro* techniques are instrumental for the simplified investigation of ENS function. Methods to isolate and culture murine primary enteric neurons and glia were compared and critically reviewed in **Chapter 3**. Currently, the majority of protocols relies on a similar approach consisting of four main steps that involve dissection and isolation of the longitudinal muscle-myenteric plexus (LMMP) preparations, their dissociation and digestion by a combination of mechanical disruption and enzymes, cell plating on coated surfaces, and their maintenance in specific culture media. However, the protocols differed considerably in every step, for example in the types of reagents, enzymes, and growth factors that are used. Furthermore, the comparison between the different methods is hampered because different outcome measurements are used. Primary ENS cells of human origin can also be cultured, but this is currently only established for enteric glial cells. For the culture of human enteric neurons, current methods rely on the differentiation of human pluripotent stem cells.

In vivo methods to study the ENS mostly assess its functional output, the most common example of that being gastrointestinal motility. In **Chapter 4**, we optimized and refined the widely used method for whole-gut transit time assessment in mice to reduce animal discomfort, decrease variability and allow physiological measurements. At present, whole-gut transit time is usually assessed by orally administrating mice with a colored dye and measuring the time until expulsion during light conditions. However, because mice are nocturnal animals, this hampers the physiological assessment of gastrointestinal motility. Our refined whole-gut transit assay is based on UV-fluorescent DETEX® to enable measurements in the dark, and allows social housing in the home cage to reduce stress. When compared to the standard method in presence and absence of loperamide, both techniques were equally reliable to detect the loperamide-induced delay in transit time. Importantly, the refined method resulted in less variation compared to the standard method. Thus, we established an optimized method to measure whole-gut transit time in mice that improves animal well-being and reduces variability.

To learn more about the expression and role of NDRG4 and its family members in the nervous system, we performed a literature search and analyzed different *in silico* datasets (**Chapter 5**). We found that NDRG1 is mainly expressed in myelinating glial cells, NDRG2 in astrocytes, and NDRG3 and NDRG4 in neurons. The NDRGs share some functionalities in neural cells, like a role in vesicle trafficking and stress response, but mostly have unique functional

properties, like a role in myelination (NDRG1), proliferation and apoptosis (NDRG2), ischemia (NDRG3), and neuronal differentiation (NDRG4). It turns out that all NDRGs, except for NDRG3, are involved in nervous system cancers, like glioma, neuroblastoma, and meningioma, where they are described as tumor suppressor genes.

Next, we investigated the role of the (enteric) neuronal protein NDRG4 in CRC in **Chapter 6**, in *Ndrg4*$^{-/-}$ mice using a genetic (*APC*$^{Min/+}$) and azoxymethane (AOM)-induced CRC model. Although the number of tumors was not significantly altered, *Ndrg4* deletion resulted in enlarged and more aggressive tumor formation, as quantified by higher levels of nuclear β-catenin immunoreactivity. In addition, we explored the role of Ndrg4 in an indirect co-culture of primary ENS cells and intestinal organoids, and observed that the secretome of *Ndrg4*$^{-/-}$ ENS cells, which was enriched for the extracellular matrix proteins nidogen-1 and fibulin-2, stimulated organoid growth. Nidogen-1 and fibulin-2 protein expression was confirmed in the mouse and human ENS and addition of these proteins to the CRC cell lines HCT116 and Caco-2 enhanced their migration capacities. Moreover, nidogen-1 and fibulin-2 were enriched in human CRC secretomes as well. Thus, alterations in the ENS, exemplified by ablation of the enteric neuron-specific protein Ndrg4, can influence colorectal carcinogenesis.

To further investigate the role of the ENS in CRC more generally, we induced CRC with AOM and dextran sodium sulfate (DSS) in a hypo-innervated mouse model, the *Hand2*$^{fl/+}$*;Wnt1Cre2* mice (**Chapter 7**). The reduced enteric neuron density in these mice did not alter tumor initiation, growth and burden, but transcriptomic analysis on tumors revealed upregulation of immunoglobulin- and immune response-related genes. Gene set enrichment analysis suggested association of differentially expressed genes with the cancer hallmarks 'avoiding immune destruction' and 'deregulating cellular energetics'. When exploring the intestinal immune system of these AOM/DSS-treated mice in more depth, we observed a decrease in the proportion of B lymphocytes in the colon lamina propria, as well as increased activation of macrophages. These findings suggest that enteric neurons (indirectly) influence CRC by affecting the CRC immune landscape.

In **Chapter 8**, we aimed to study the effects of a hyper-innervated bowel on CRC by using the *NSE-Noggin* mouse model. However, these mice did not present with an increased density of enteric neurons in the colon, in contrast to previous studies in another institute. Transgene presence was confirmed by genotyping (q)PCR and by altered fur appearance, a phenotypic characteristic of *NSE-Noggin* mice. Overall enteric neuron density and the proportions of specific enteric neuron subsets (calbindin, calretinin, and serotonin) were similar in transgenic and

wild-type mice, rendering them unsuitable as hyper-innervated model. This apparent loss of phenotype might be related to alterations in the microbiome or other environmental factors, and highlights the importance of animal model validation.

Finally, in **Chapter 9**, the significance of the results in this thesis is summarized and discussed. Our findings support the growing evidence that nerves, with specific focus on the ENS, play a role in CRC. After identifying knowledge gaps in the neuron-cancer field, we reflected on different models and methodologies to study the ENS in the context of CRC, with suggestions for the use of novel tools to further unravel the mechanisms underlying ENS-CRC interaction. All in all, this thesis illustrates that enteric neurons contribute to CRC by actively interacting with tumor cells and by influencing the immune system in the gut, which might provide a first stepping stone for the future discovery of neural targets that can be used in CRC therapy.