Neural Influence on Colorectal Carcinogenesis

Summary

When starting this PhD project, in 2020, gastrointestinal (GI) cancers, including those affecting the esophagus, stomach, and intestine, accounted for a significant health burden, with over 3.6 million diagnoses and 2.2 million related deaths worldwide. This represents approximately 22.5% of all cancer-related deaths globally. Managing these cancers is particularly challenging due to the intricate organization of the GI tract, which comprises a repertoire of different cell types such as epithelial, smooth muscle and immune cells, luminal contents, microbiota and extracellular matrix (ECM) components. Each of these elements can contribute to the development of GI cancers, together forming what is known as the tumor microenvironment (TME). While immune cells and fibroblasts within the TME have long been acknowledged for their role in cancer progression, the involvement of the nervous system has only recently garnered attention. Both extrinsic innervation and signals from the gut's intrinsic innervation, the enteric nervous system (ENS), can influence the development of GI cancers. However, understanding of their role is still limited, which is due to the novelty of the field and because models to study this crosstalk are missing or need to be optimized.

The goal of this thesis was to enhance our understanding of the neural aspects of colorectal cancer (CRC), by exploring potential models to study this crosstalk and investigating neural signatures as well as interactions, between tumors and ENS cells. To achieve these objectives, current *in vitro* models were reviewed in Chapters 2 and 3. **Chapter 2** addresses the utilization of organoids for modeling CRC, which offer improved *in vitro* platforms for studying cancer development and can be used to explore potential treatment strategies. It also provides an overview of both normal and CRC intestinal organoid models, emphasizing the importance of incorporating TME components into CRC modeling to address translational gaps in this field. Furthermore, it explores the use of CRC organoids derived from pluripotent stem cells and discusses their potential applications but also limitations, for cancer research.

In **Chapter 3**, we list the protocols for generating primary ENS cells from adult mice, detailing every stage of the process, including intestinal segment dissection, advanced enzymatic digestion techniques, specialized surface coatings, and tailored culture media. We also discuss the emerging potential of human ENS cell cultures, specifically those derived from pluripotent stem cells, paving the way for advancements in research and therapeutic applications.

In **Chapter 4,** we summarize the evidence for the crosstalk between GI cancers and the nervous system. We discuss the impact of neural influences on GI cancer hallmarks and identify neural signatures specific to this type of cancer. Additionally, potential neural-related therapies for managing GI cancers are highlighted.

Neural-related DNA methylation signatures are further investigated in **Chapter 5**. Previously, our research identified a neural-related DNA hypermethylation fingerprint in colon cancer, characterized by hypermethylation and downregulation of genes with known functions in the nervous system. To assess the presence and relevance of this signature in carcinogenesis across various cancer types, we analyzed data from The Cancer Genome Atlas (TCGA). Our

findings reveal significant enrichment of neural-related genes among hypermethylated genes in all tested cancers. Remarkably, this signature is evident even in premalignant tissue types and could not be attributed to potential co-founders, such as bivalency status or tumor purity. Further characterization of the neural-related DNA hypermethylation signature in colon cancer demonstrated a specific enrichment for genes overexpressed during neural differentiation. Moreover, analysis of upstream regulators highlighted the RE1-Silencing Transcription factor (*REST*) as a potential mediator of this DNA methylation signature. Our results underscore the presence of a neural-related DNA hypermethylation fingerprint in various cancers, suggesting an involvement of DNA hypermethylation in maintaining neural stemness in cancer cells.

Chapter 6 of the thesis focuses on the influence of enteric nerves on the cellular and molecular environment of CRC. To do so, we utilized mouse models with reduced enteric innervation, alongside control mice, both subjected to a colitis-associated CRC protocol. We monitored tumor initiation, growth, and burden using computed tomography scans, and analyzed tumor and stool samples. Despite the observed decrease in enteric neurons, the hypo-innervated mice exhibited similar tumor initiation, growth, and burden compared to the control group. Analysis of tumor RNA sequencing highlighted enrichment of cancer hallmarks, such as 'avoiding immune destruction' and 'deregulating cellular energetics' when comparing both genotypes. In addition, a reduction in the population of B cells was observed, particularly germinal center B cells and immature B cells, in the cancerous colon of hypo-innervated mice. Alterations in immunoglobulin presentation by B cells and upregulation of antigen presentation by macrophages were also noted. Based on these findings, we concluded that while reduced numbers of enteric neurons do not directly impact murine tumor growth, they do affect the immune landscape of CRC.

As shown by us and others, it is now established that ENS cells play a role within the tumor microenvironment. However, knowledge of their origin and molecular characteristics is still lacking. In **Chapter 7** we aimed to study the molecular profile of ENS cells within the CRC microenvironment, using single-cell RNA sequencing. To achieve this, we utilized fluorescence-activated cell sorting (FACS) to isolate ENS cells from both tumor tissue and adjacent normal tissue, obtained from CRC patients. Subsequently, employing the 10x Genomics platform, we conducted single-cell RNA sequencing. Doing so, we were only able to capture enteric glial cells, while enteric neurons were lost in our analysis. Our transcriptional profiling unveiled the presence of seven distinct enteric glia subtypes (n = 51,715 glial cells) in both normal and adjacent colon cancer lesions. Notably, most of the captured enteric glia were enriched in ferritin genes, *FTH1* and *FTL*, previously recognized as mucosal enteric glia. Conversely, enteric glia enriched for *NRXN1* and *CADM2*, markers for intraganglionic glia, constituted less than 5% of the population. By comparing our data to published and unpublished datasets, we observed a diminishing contribution of cells with a transcriptomic profile associated with intraganglionic enteric glia, relative to the total enteric glia cell population in the colon with age progression, from fetal to pediatric, to adult-aged colon. Subtle transcriptional changes were noted within each enteric glia cluster, when comparing cells isolated from healthy versus CRC tissue. However, a significant shift in the distribution of

enteric glia among these clusters was observed in tumor samples, favoring antigen-presenting specialized glia, indicative of the immunoreactive characteristics of cancer. Similar to previous studies investigating the human ENS, we were unable to detect *GFAP* transcripts in the captured enteric glial cells. For elucidating the precise function of enteric glia in the context of CRC, future investigations to figure out the reason for this observation and to profile *GFAP*$^{+}$ glia in this disease, are imperative.

Finally, **Chapter 8** summarizes and discusses the significance of the results presented in this thesis. Specifically, our findings provide further support to the emerging evidence pointing to the involvement of the ENS and neural signaling pathways in general, in CRC. Overall, this thesis underscores the contribution of enteric neurons and glia to CRC by influencing the immune system in the gut. These insights may pave the way for the identification of neural targets for potential use in CRC therapy.