CHAPTER 1: INTRODUCTION

1.1 Background

To date, colorectal cancer (CRC) remains as the third most prevalent and second most lethal malignancy worldwide (Bray et al., 2018). Majority of the mortalities are primarily due to cancer metastasis, a process referring to the dissemination of cancer cells from the primary site in the colorectum to distal organs. The most frequent site of metastasis in patients with CRC is the liver, historically attributable to the haemotogenous route of CRC metastasis and organotropism (Leporrier et al., 2006). Metastasis itself is a complex series of interrelated and sequential steps, commonly referred to as the metastatic cascade, originating from the invasion of primary tumor cells through the basal membrane. It is followed by intravasation into the vasculature as circulating tumor cells, and evade cell death due to mechanical shearing and/or immune attack. Cell aggregates will adhere to a distant endothelial bed, extravasate, and enter the organ parenchyma, establishing a micrometastasis niche. Thereafter, most of the clones undergo cell death or may remain in a dormant state, in which a fraction of these cells are eventually able to grow and colonize the organ (Anderson et al., 2019). Gradual colonization may then perturb normal organ function. Consequently, metastasis is associated with poor overall survival and is the foremost cause of CRC-related death due to refractory towards various therapies (Fidler & Kripke, 2015). Yet, no current therapies specifically target CRC liver metastases, posing the need for a better understanding of the biology of metastasis and the identification of druggable candidates to restrict metastatic growth.

Development of micrometastases into overt, clinically detectable macroscopic tumors termed as colonization requires viability and proliferative exigencies, thus identification of molecular determinants that dictate these processes is vital. This is driven by the concept that metastasis formation is non-random, but instead, an outcome of selective survival of specific tumor subpopulations that possess intrinsic cellular properties enabling them to complete the metastatic

cascade through the acquisition of genetic and epigenetic alterations (Anderson et al., 2019). Metabolic reprogramming is unequivocally a salient adaptation mechanism exploited by cancer cells to drive accelerated growth. As metabolic products accessible to cancer cells depend on the environment in which they reside, its metabolic dependencies may also vary based on the site of metastasis. The liver – the central metabolic organ – is composed of oxygen gradients and metabolic zonation by segregated hepatocytes (Ma et al., 2020; Planas-Paz et al., 2016). CRC cells metastasizing to the liver therefore experience intense metabolic stress. Genes involved in oxidative stress and metabolism such as aldehyde dehydrogenase 1 family member L2 (ALDH1L2), methylenetetrahydrofolate dehydrogenase (MTHFD1), aldolase B (ALDOB), and creatine kinase Brain-type (CKB) have been reported to be enriched in tumors with liver tropism, regardless of histological origin (Bu et al., 2018; Loo et al., 2015; Piskounova et al., 2015; Smith & Kang, 2017).

Prior to initiating the current study, preliminary studies investigated the transcriptomic signature of metastatic CRC through multiple models (unpublished data). Utilized samples include patient-derived CRC cell lines with different metastatic capabilities based on the rate of liver colonization in immunodeficient mouse models (highly, moderately, and poorly metastatic), clinical datasets from patients with metastatic CRC compared to primary CRC and normal colon, as well as *in vivo* selected highly metastatic cell population compared to their poorly metastatic parental cell populations. Convergence of data from the multiple datasets ensures that the data is robust and augments patient stratification. Aside from the accurate information of actual patients' biological condition that clinical samples are able to show, it reveals inter-patient heterogeneity. Meanwhile, through analysis of the *in vivo* selected models, intra-patient heterogeneity can be observed, implying that genetic alterations that occur during the acquisition of the metastatic phenotype can be selected for. Propagation of the cells and subsequent reinjection into mice displayed enhanced metastatic capacity, marked by faster rate of liver colonization than the original parental cell counterparts. All in all, the novel approaches employed will elicit high confidence gene candidates to be experimentally validated. Gene set enrichment analysis demonstrated upregulation of genes in multiple metabolic

pathways in metastatic CRC cells including genes annotated in cholesterol homeostasis, fatty acid metabolism, glycolysis, mammalian target of rapamycin complex 1 signaling, adipogenesis, and oxidative phosphorylation. In particular, a gene known as vascular non-inflammatory molecule 1 (VNN1) was selected for further study.

VNN1 was shown to be upregulated in CRC liver metastases across transcriptomic profiles from all aforementioned models. VNN1 encodes for a membrane-bound vanin-1 enzyme that hydrolyzes its substrate, pantetheine, into endogenous pantothenic acid and cysteamine (Maras et al., 1999; Pitari et al., 2000). Downstream of the pantothenate pathway is responsible for the physiological regulation of fatty acid metabolism, energy production, and oxidative stress response. Pantothenic acid is a structural precursor of coenzyme A (CoA), thus restoration of CoA pools in the tricarboxylic acid (TCA) cycle permits maintenance of oxidative phosphorylation (OXPHOS) and enhance mitochondrial activity (Giessner et al., 2018). Cysteamine inhibits conversion of glutathione (GSH) antioxidant, resulting in higher oxidative stress (Ferreira et al., 2015). High VNN1 levels have been shown to be indicative of poor prognosis in various malignancies, and shown to drive CRC progression. However, the role of VNN1 in cancer metastasis, particularly CRC liver metastasis remains undetermined. In this study, we used the loss-of-function and gain-of-function strategy to manipulate VNN1 expression in American Type Culture Collection (ATCC) human CRC cell lines: HT29, HCT116, and Caco-2 through various molecular tools such as RNA interference by small interfering RNA (siRNA) and short hairpin RNA (shRNA), clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated 9 (Cas9)-mediated knockout, and overexpression. Generation of the cell lines with stable VNN1 knockdown utilizing two shRNAs, as well as stable VNN1 overexpression were achieved. Knockdown and overexpression efficiencies were subjected to validation by quantitative polymerase chain reaction (qPCR) and western blot. shVNN1-6 and shVNN1-7 showed significant knockdown in all cell lines at mRNA level, but not validated at protein level. On the contrary, cell lines transduced with a lentiviral vector encoding VNN1 had significant overexpression was seen in qPCR, and overexpressed glycosylated vanin-1 bands were observed when western blot was performed using cell lysate

harvested from VNN1-overexpressing cell lines. Concurrently, vanin-1 signals in immunofluorescence staining were distinctly higher in overexpression cell lines compared to control cell lines, but not in Caco-2. The overexpression of VNN1 was also confirmed through sandwich enzyme-linked immunosorbent assay (ELISA) by which elevation of secreted vanin-1 levels were quantified. Subsequently, *in vitro* functional assays were conducted on validated overexpression cell lines, including proliferation assay, colony formation assay, and wound healing. Results show that proliferation rate, colony formation, and migratory abilities of VNN1 overexpression cell lines remain unchanged as compared to its control *in vitro*. This may be as a result of the different metabolite levels present in the *in vitro* context compared to physiological *in vivo* setting, with inadequate resemblance of the physiological condition especially in regards to the available metabolites involved in the pantothenate pathway.

Delineation of genetic aberrations that drives metabolic adaptations in CRC liver metastases is crucial to elicit novel therapeutic targets. This study serves as a preliminary yet important step to investigate the role of VNN1 in CRC hepatic metastatic colonization.

1.2 Research objectives

Following the identification of VNN1 as a differentially expressed gene in colorectal liver metastases through unbiased transcriptomic profiling approaches, this study aims to attain the following research objectives: 1) determine VNN1 expression in colorectal cancer liver metastases samples derived from publicly available clinical datasets as validation of preliminary transcriptomics findings, 2) generate stable VNN1 loss-of-function and gain-of-function models in ATCC human CRC cell lines, and 3) characterize phenotypic changes altered with VNN1 overexpression through *in vitro* functional assays.