Summary of dissertation

General Introduction

Tumor cells possess distinct metabolic features compared with normal cells, most notably the Warburg effect, or aerobic glycolysis, where tumor cells preferentially produce lactate even in the presence of oxygen. High lactate concentrations in the tumor microenvironment are associated with poor prognosis and high malignancy in part because lactate influences various immune cells and suppresses anti-tumor immune responses. Furthermore, lactate is taken up by tumor-associated fibroblasts and tumor endothelial cells, promoting their proliferation and creating a favorable environment for tumor growth.

Epigenetic modifications, including DNA methylation, histone methylation, and histone acetylation, regulate gene expression without altering the DNA sequences and influence numerous biological processes such as embryonic development, metabolism, and diseases including cancer. Because epigenetic modifications utilize metabolic intermediates such as acetyl-CoA and lactate as substrates, epigenetic regulation and cellular metabolism are closely interconnected, suggesting that metabolic states may influence epigenetic landscapes. Therefore, integrated investigation of these two systems could provide further insight into cancer pathogenesis and contribute to the identification of potential therapeutic strategies.

Hemangiosarcoma (HSA) is a malignant tumor of vascular endothelial cells that, in dogs, accounts for 1.3–2.8% of all canine tumors and often arises in the spleen, liver, and right atrium of the heart. Given that HSA is highly invasive, rapid metastasis and tumor rupture occur frequently and contribute to a poor prognosis, with median survival times of only about five to six months and a one-year survival rate of less than 16% under standard care. One contributing factor is the incomplete understanding of the molecular pathogenesis of HSA, which prevents researchers from identifying effective therapeutic targets. HSA shares similarities with human angiosarcoma (AS) in morphological features, genetic mutations, and aggressive clinical behavior, and due to the rarity of AS in humans, canine HSA is recognized as a valuable spontaneous model for studying angiosarcoma biology and evaluating novel therapeutics.

Despite increasing recognition of epigenetic regulation in cancer biology, the epigenetic landscape of HSA remains poorly characterized, particularly regarding how modifications interact with metabolic alterations. In this study, I investigated the molecular pathogenesis of HSA focusing on the epigenetic regulation and tumor metabolism. Chapter 1 investigated the role of histone acetylation in HSA using two histone deacetylase (HDAC) inhibitors (SAHA and VPA) and one bromodomain and extraterminal (BET) inhibitor (JQ1), a small molecule that blocks acetylated histone recognition. Chapter 2 analyzed the implication of lysine lactylation under glucose-limited conditions, in which tumor cells experience metabolic

stress to survive. Chapter 3 investigated the roles of lactate and lactate dehydrogenase (LDH) in HSA.

Chapter 1: Manipulating histone acetylation leads to antitumor effects in hemangiosarcoma cells

Histone acetylation regulates transcription and is involved in various biological processes. Acetylated histones can promote transcription by loosening chromatin structure and by recruiting BET proteins, which function as epigenetic readers. Dysregulation of histone acetylation has been increasingly recognized as a key contributor to cancer pathogenesis, making histone acetylation–related regulators attractive therapeutic targets. In this study, I aimed to investigate the role of histone acetylation and the effects of histone deacetylase inhibitors (HDACi) and BET inhibitor (BETi) on HSA.

Global acetylation levels of histones H2B, H3, and H4 were higher in all HSA cell lines compared with normal canine aortic endothelial cells. In clinical HSA cases, global acetylated histone H3 levels were heterogeneous and associated with histopathological patterns, where the ratio of tumor cells classified as negative for H3 acetylation was highest in the solid pattern, followed by the capillary pattern, and lowest in the cavernous pattern. These results suggest that histone H3 acetylation levels are associated with the differentiation status of tumor cells, as solid patterns are considered less differentiated. To examine the role of histone acetylation, HDACi such as SAHA and VPA, and the BETi JQ1 were used. SAHA and JQ1 treatment significantly decreased cell proliferation and induced apoptosis in HSA cell lines, evidenced by increased cleaved caspase 3 expression and annexin V positivity. Furthermore, survival proteins such as AKT and ERK were downregulated by SAHA and JQ1 but not by VPA. Gene set enrichment analysis (GSEA) indicated that inflammatory-related gene expression was positively correlated with SAHA and VPA treatment, with genes such as *Il6*, *Cxcl1*, *Ccl2*, *Ccl7*, and *Oas1a* being upregulated in murine cells, and *OAS1* and other inflammatory genes upregulated in canine cells. Migration assays using RAW264 macrophages showed that SAHA and VPA treatment in HSA cell lines attracted macrophages, suggesting a potential modulation of macrophage-related immune responses. JQ1 treatment activated the initiation of autophagy and impeded the cell cycle, leading to cell cycle retardation and senescence as major effects in murine cells. *In vivo* study revealed that only JQ1 slowed tumor growth and extended survival times in ISOS-1 tumor-bearing mice and suppressed JuB2 tumor growth in immunodeficient mice.

These results suggest that JQ1 has suppressive effects on HSA tumor growth *in vivo* and *in vitro*, and that BET inhibitors represent a promising potential therapeutic strategy for HSA, warranting further clinical evaluation. The limited *in vivo* efficacy of SAHA and VPA

may stem from rapid plasma clearance or suboptimal modulation of the anti-tumor immune response under the conditions tested. The precise mechanisms by which histone acetylation influences tumor progression and immune modulation *in vivo* remain to be fully clarified. Overall, the study suggests that dysregulation of histone acetylation is likely involved in HSA malignancy and that BET inhibitors can be used for HSA treatment.

Chapter 2: Lysine lactylation regulates ATF4-mediated stress responses under glucose starvation in canine hemangiosarcoma

Lactate serves as a substrate for histone lactylation, which has been implicated in promoting immunosuppression and tumor progression in various cancers. While most studies have been conducted under high-lactate conditions, the roles of histone lactylation under glucose-limited metabolic stress remain largely unknown. To better model HSA biology, this study established novel canine HSA cell lines and patient-derived xenograft (PDX) models that preserve histological features and genetic identity.

Glucose deprivation significantly decreased global histone H3 and H4 lactylation levels without altering global histone acetylation levels. Extracellular flux analysis demonstrated that glucose deprivation induced a metabolic shift toward oxidative phosphorylation (OXPHOS), making cells increasingly dependent on fatty acid and glutamine oxidation.

Cleavage Under Targets and Tagmentation (CUT&Tag) revealed that while global levels fell, lysine lactylation (Kla) peaks were redistributed and enriched at the transcription start sites (TSSs) of ATF4-regulated stress-response genes and immune-related genes. TSSs of these stress-response genes were co-occupied by RNA polymerase II phosphorylated at serine 5 (RNAPII), and their expression was upregulated, suggesting that Kla marks were associated with active transcription under nutrient-poor conditions. Representative stress genes like *ASNS*, *DDIT3*, and *SESN2* confirmed concurrent enrichment of Kla and RNAPII and upregulation following glucose withdrawal. The induction of these stress responses was found to be ATF4-dependent and required acute, substantial glucose removal rather than gradual glucose limitation, such as inhibition of the glucose transporter GLUT1. Advanced sequencing results further supported these observations *in vivo*. Single-cell RNA-seq of PDX tumors identified a specific tumor-cell population characterized by stress-response markers. Spatial transcriptomics on a patient tumor mapped these stress-responsive cells to a distinct cluster-like region, suggesting that their distribution may be shaped by the local microenvironment.

Isotope-tracing metabolomics using [U-^{13}C]glutamine confirmed that HSA cells synthesize asparagine from glutamine via the tricarboxylic acid cycle when glucose is scarce. Asparagine supplementation modestly supported cell proliferation under glucose-deprived

conditions, suggesting that glutamine-derived asparagine produced through the ATF4-ASNS axis contributes to cell survival. Immunohistochemistry for H3K18la in clinical HSA tissues indicated that HSA tumor cells showed higher H3K18la levels than normal endothelial cells, and exhibited heterogeneous signal patterns. Dual immunohistochemistry for H3K18la and immune markers revealed a statistically significant negative correlation between average H3K18la signals and the infiltration of Iba-1-positive or CD204-positive M2-like macrophages. Co-culture and conditioned medium assays indicated that glucose-starved HSA cells attract macrophages and polarize them toward an M2-like phenotype.

These findings suggest that lysine lactylation is associated with transcriptional activation that supports tumor cell survival and may contribute to the formation of a pro-tumor microenvironment. Nevertheless, further studies are required to elucidate the detailed mechanisms by which histone lactylation, including its redistribution under nutrient-deprived conditions, regulates gene expression, and how these processes operate *in vivo*, particularly in the context of metabolic stress such as glucose limitation.

Chapter 3: Lactate dehydrogenase is associated with cholesterol/lipid metabolism, and fluvastatin plus dipyridamole suppresses canine hemangiosarcoma growth in patient-derived xenograft models

LDH catalyzes the interconversion of pyruvate and lactate, and its dysregulation is associated with poor prognosis in various cancers. Beyond its central role in glycolytic metabolism, LDH contributes to metabolic symbiosis within tumors by supporting lactate production and utilization across heterogeneous microenvironments. Accumulating evidence has identified LDH as a potential therapeutic target, and pharmacological LDH inhibition has demonstrated antiproliferative effects in multiple cancer types. In this chapter, I therefore aimed to investigate how lactate metabolism and LDH contribute to HSA pathogenesis.

The dual LDHA/LDHB inhibitor (R)-GNE-140 significantly suppressed HSA cell proliferation *in vitro* and reduced global Kla levels. mRNA-sequencing of cells treated with (R)-GNE-140 revealed the downregulation of cholesterol and lipid metabolism-related gene sets. Genetic knockout of LDHA was consistently associated with the downregulation of cholesterol-related genes and decreased the protein expression of SREBP2, a master regulator of cholesterol metabolism, as well as reduced lipid droplet accumulation. In contrast, LDHB knockout results were not consistent among clones. Although LDHA inhibitors like sodium oxamate and statins like fluvastatin showed anti-proliferative effects *in vitro*, they failed to suppress HSA tumor growth *in vivo* as monotherapies. This discrepancy may be due to the tumor microenvironment or the activation of restorative SREBP2-mediated feedback loops that limit statin efficacy. Dipyridamole has been identified as a pharmacological inhibitor of SREBP2 maturation.

Combination therapy using fluvastatin and dipyridamole resulted in significant suppression of tumor volume in HSA PDX models compared to monotherapies, without affecting the body weight of the mice.

These findings suggest that LDH is linked to cholesterol metabolism in HSA cells and that cholesterol pathway inhibition, specifically through the combination of statins and dipyridamole, could offer a therapeutic approach. The precise mechanism linking LDHA loss to the repression of cholesterol-regulated genes remains unclear, although it may be mediated by the reduction of histone lactylation at promoters or potentially through noncanonical nuclear roles of LDHA. Future studies are needed to evaluate LDH expression patterns in clinical HSA tissues and determine if they correlate with therapeutic response. Additionally, further investigation is required to clarify the molecular mechanisms by which LDHA regulates cholesterol metabolic pathways in HSA cells. Collectively, the results suggest that LDH could be associated with cholesterol and lipid metabolism in HSA, and that targeting cholesterol homeostasis is a promising therapeutic approach.

Conclusions

This dissertation examined the molecular pathogenesis of canine HSA with a particular focus on epigenetic regulation and tumor metabolism. By analyzing histone acetylation, lysine lactylation under metabolic stress, and LDH-associated cholesterol metabolism, this work sought to clarify how these processes contribute to tumor progression and potential therapeutic vulnerability. Chapter 1 demonstrated that elevated histone acetylation and its recognition by BET proteins are involved in HSA malignancy, identifying BET inhibition as a potential therapeutic strategy. Chapter 2 revealed that lysine lactylation persists under glucose-deprived conditions and redistributes to TSSs, which might be associated with the activation of adaptive stress-response pathways, such as *de novo* asparagine synthesis that partially supports tumor cell survival, as well as immune-related genes. Chapter 3 established a link between LDH activity and cholesterol metabolism, demonstrating that combination therapy targeting cholesterol synthesis and its regulatory feedback loop could enhance antitumor effects in PDX models.

In conclusion, this dissertation provides evidence that epigenetic and metabolic alterations may contribute to the aggressive phenotype of canine hemangiosarcoma. Although further investigation is required, the findings presented here offer a framework for understanding HSA pathogenesis from an integrated metabolic and epigenetic perspective and may inform future research directions in this disease.