

Role of CXCL7 in Colon Cancer Proliferation

by

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Abstract

Colorectal cancer (CRC) is the third commonly diagnosed cancer worldwide and the second cause of cancer-related mortality in the United States. It is a complex disease, and despite the improvements in CRC screening and treatments approximately half of the patients discovered their cancers at late and aggressive stage of the disease. Thus, there is a need to increase public awareness for the importance of early diagnosis that can interrupt CRC progression, especially among populations who are at higher risk for CRC. Furthermore, understanding the predisposition factors and molecular mechanisms can help to improve preventative strategies and develop an effective-targeted therapy for CRC.

The tumor microenvironment (TME) is heterogeneous, consisting of blood vessels, lymph vessels, secreted proteins, extracellular matrix (ECM), stromal cells, immune/inflammatory cells, and other components. The continuous and dynamic interaction between cancer cells and the TME can promote cancer progression. One of the important players in the TME is the chemokines family, a class of pro-inflammatory cytokines with small molecular weights (~8–14 kDa) that are capable of chemotactic cell-directed movement. Recently, the role of chemokines in the TME has received great attention, and a vast number of studies indicate their role in cancer progression, especially in CRC.

Chemokine (C-X-C motif) ligand 7 (CXCL7)/ NAP2 is an immediate mediator for neutrophils recruitment released from platelets at sites of inflammation, and it has been suggested as a potential biomarker for CRC diagnosis. Literature has reported that CXCL7 drives tumor progression and metastasis by binding to its receptor (CXCR2) in various cancer types

such as cholangiocarcinoma, breast cancer, and CRC. Furthermore, several pieces of evidence indicate that the encoding gene for *CXCL7* (*PPBP*) and *CXCR2* receptors are highly expressed in patients with CRC. Thus, *CXCL7* has been a potential biomarker for diagnosis of many cancers including early lung and CRC. *CXCL7* also has been shown to stimulate glucose transporters and glycolysis in non-cancer cells. Additionally, recent evidence reports a correlation between *CXCL7* and the key glycolytic enzyme; lactate dehydrogenase (LDH) and it is an independent risk factor for CRC prognosis. However, the role of the *CXCL7*-*CXCR2* axis in CRC progression has not been fully understood. Hereby, we carried out this study to examine the role of the *CXCL7*-*CXCR2* axis in mediating colon cancer proliferation. This study was the first to demonstrate that *CXCL7* stimulates proliferation, lactate production, and glycolytic function in human colon cancer cells, suggesting that *CXCL7* may stimulate colon cancer proliferation by enhancing aerobic glycolysis.

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List of Abbreviations

CRC Colorectal cancer

AMPK Adenosine Monophosphate-Activated Protein Kinase

cAMP Cyclic Adenosine Monophosphate

CRY Cryptochrome

CVD Cardiovascular Disease

CXCL7 C-X-C Motif Ligand 7

CXCR2 CXC motif chemokine receptor 2

dsDNA double-stranded DNA

2-DG6P 2-deoxy-d-glucose-6-phosphate

ECAR extracellular acidification rate

ECM extracellular matrix

GLUTs glucose-specific transporters

G6P Glucose 6-Phosphate

GFP Green Fluorescent Protein

GLUT2 Facilitated Transporter Type 2

GPCRs G-protein coupled receptors

HIF1 hypoxia-inducible factor-1

HREs hypoxia response elements

IFN-g Interferon-Gamma

IL-1b Interleukin-1 Beta
IL-6 Interleukin-6
IRS Insulin Receptor Substrate
KO Knockout
MAPK Mitogen-Activated Protein Kinase
MCP-1 Monocyte Chemoattractant Protein-1.
MYC myelocytomatosis oncogen
NAP2 Neutrophil activating protein-2 human
NAD⁺ Nicotinamide Adenine Dinucleotide
NADPH Nicotinamide Adenine Dinucleotide phosphate
NF-kB Nuclear Factor-Kappa B
NMDA N-Methyl-D-Aspartate
OXPHOS Oxidative phosphorylation
OCR oxygen consumption rate
PI3K phosphatidylinositol 3-kinase
PFK Phosphofructokinase
SREBP Sterol regulatory element-binding protein
T2DM Type-II Diabetes Mellitus
TCA Tricarboxylic Acid
TLR4 Toll-Like Receptor 4
TNF-a Tumor Necrosis Factor Alpha
TME tumor microenvironment

Chapter 1: Introduction

Colorectal cancer (CRC) is a heterogeneous disease associated with various etiologies and outcomes. It ranks as the second leading cause of cancer-related deaths in the United States, likely due to the Westernized diet, lifestyle, and other risk factors [1-3]. In the past, CRC was predominantly associated with older age and supported by an elevated incidence rate of CRC in patients over 50 years old [1, 2]. However, recent epidemiological data show rising trends in the incidence and mortality rate in those who are younger than 50 years old, which refers as of early-onset CRC [3]. In the United States, the incidence rates for CRC have augmented by 45% in adults (ages 20–49 years), from 8.6 per 100,000 in 1992 to 13.1 per 100,000 in 2016 [3]. 70% to 90% of CRC patients have a 5-year survival rate and an optimistic clinical prognosis when it is diagnosed before it has metastasized [4]. However, if the tumor has disseminated, patients often have poor prognosis with a 5-year survival rate of less than 20%, even after surgery and comprehensive postoperative clinical care [4]. Hence, it is critical to detect CRC before it metastasizes, enabling a timely and appropriate intervention to inhibit further cancer progression. Given the growing burden of CRC in the adult and elderly populations, understanding the molecular pathways and genetic features for CRC onset and progression is essential to help with early diagnosis and intervention that enhance patients' survival.

Tumors are defined as a collection of mutant cells that have defects in their genome leading to their clonal proliferation in a self-sustaining manner [5]. Mutations give rise to a new genotype, directly shaping the unique cellular phenotype of tumors and significantly diverging from that of normal cells [5]. These variations are observed during metabolic reprogramming at

the basal metabolism level as it regulates the release of oncometabolites [6]. Metabolic reprogramming is used to describe conventional metabolic pathways whose activities are enhanced or suppressed in tumor cells, relative to benign, and oncometabolite refers to metabolites whose abundance increases markedly in tumors [6]. In normal cells, glycolysis starts with glucose uptake by glucose-specific transporters (GLUTs), then the glucose is further metabolized through the glycolysis pathway, converting one molecule of glucose into two molecules of pyruvate, along with the reduction of NAD^+ (Nicotinamide adenine dinucleotide, oxidized form). Under sufficient oxygen supply, pyruvate undergoes oxidation by transforming into an acetyl group attached to a carrier molecule of coenzyme A, which delivered from the cytoplasm to the mitochondria to continue to the citric acid cycle. The need for NAD^+ , which is essential for glycolysis, is compensated by the action of NADH shuttles (Nicotinamide adenine dinucleotide, reduced form), which translocates electrons from the cytosol to a mitochondrial pool of reducing equivalents and the electron transport chain [5]. Noteworthy, glycolysis is a physiological response to hypoxia in normal tissues while cancer cells are constitutively take up glucose and produce lactate regardless of oxygen availability. This phenomenon was first observed by Otto Warburg in the 1920s and is now known as the Warburg effect or aerobic glycolysis [5, 7, 8]. It is a well-known fact that cancer cells increase glycolytic flux and have a greater predisposition for lactic acid formation even under adequate oxygen supply [5, 7]. This, in turn secures suitable levels of biosynthetic precursors (glycolytic intermediates), NADPH, NAD^+ , and rapid ATP production to meet the increased demands of intensively proliferating tumor cells. The end-product of tumor “aerobic glycolysis” is lactate (an oncometabolite), which may support neighboring cancer cells with fuel and facilitate migration and immunosuppression, together enabling cancer progression [5, 6, 9]. Aerobic glycolysis has been correlated with

advanced tumor progression, treatment resistance, and poor prognosis in patients in various cancers, including CRC [10, 11]. Moreover, growth factors in benign cells are important to activate phosphatidylinositol 3-kinase (PI3K), along with its downstream pathways AKT and mammalian target of rapamycin (mTOR). All are important to promote a robust anabolic program involving increased glycolytic flux and fatty acid synthesis through activation of hypoxia-inducible factor-1 (HIF-1) and sterol regulatory element-binding protein (SREBP), respectively [12]. However, tumor cells very frequently contain mutations that allow the proliferative signaling such as PI3K-AKT-mTOR cascade to be constantly active with minimal dependence on extrinsic stimulation such as growth factors [12]. Many of the well-known oncogenes and tumor suppressors reside in the PI3K-AKT-mTOR network, so controlling the activation of this pathway is among the most frequent alterations seen in various sets of cancers [5]. Another commonly deregulated pathway in cancer is the gain of function of MYC (myelocytomatosis oncogene), resulted from gene amplification, chromosomal translocations, or single-nucleotide polymorphisms. MYC increases the expression of many genes that support anabolic growth, including transporters and enzymes involved in glycolysis, glutaminolysis, fatty acid synthesis, serine metabolism, and mitochondrial metabolism [13]. Oncogenes like Kras, which is mutated in one-third of all cancers, have similar physiological effects of PI3K and MYC pathways to promote oncogenesis, maintenance, and subsequent cancer evolution [6, 14, 15]. In addition to oncogenes, tumor suppressors such as the p53 transcription factor can also regulate metabolism [16]. The p53 protein-encoding gene *TP53* (tumor protein p53) is mutated or deleted in 50% of all human cancers, and tumor growth can be inhibited by p53-mediated cell-cycle arrest, cellular senescence, and apoptosis [17]. However, recent evidence indicates that p53 tumor-suppressive roles may also be mediated via regulating metabolism and oxidative stress

[17]. For instance, the loss of function of p53 increases glycolytic flux to speed up anabolism and redox balance, two key processes that induce tumorigenesis [6]. All these signaling pathways, in concert with the transcription factors HIF-1, p53, and c-Myc, modulate the activity and expression of key glycolytic regulatory enzymes, including pyruvate kinase M2 (PKM2), and pyruvate dehydrogenase kinase 1 (PDK1) to tune in the most optimal metabolic setting for the cancer cell [5]. Further discoveries and research into the reprogramming of tumor activity may provide opportunities to predict tumor behavior and prevent tumor progression by blocking essential pathways.

The tumor microenvironment (TME) is very heterogeneous, and it is composed of a diverse cellular and acellular milieu such as immune infiltrated cells, tumor associated fibroblasts, blood vessels, lymph vessels, secreted proteins, extracellular matrix (ECM), stromal cells, immune/inflammatory cells, RNAs, and others. The TME provides a niche for cancer cells to grow and survival. Tumor niche plays a strong role in tumor initiation, progression, metastasis, recurrence, and drug resistance [1]. Cell migration is critical for tumor development and is aided by the function of chemokines.

Chemokines are chemoattractant proinflammatory proteins capable for various functions, including innate and adaptive immunity, organogenesis, and tissue repair through interaction with their seven transmembrane G-protein coupled receptors (GPCRs) that are differentially expressed on all leukocytes [18]. Chemokines are considered the largest family of cytokines, with approximately 50 endogenous chemokine ligands in humans and mice [18, 19]. There are around 20 signaling chemokine receptors and 5 non-signaling chemokine receptors, and they are divided into two groups: G protein-coupled chemokine receptors and atypical chemokine receptors. GPCRs mediate their ligands functions by stimulating pertussis toxin PTX-sensitive

Gi-type G proteins while atypical chemokine receptors modulate chemokine bioavailability by transporting chemokines to intracellular degradative compartments through activation of β -arrestin-dependent pathways in a G protein-independent manner [18-20]. Chemokines and their receptors have a multifaceted role in tumor biology and are implicated in nearly all aspects of cancer growth, survival, and dissemination. Therefore, targeting of chemokines and their cell surface receptor is a promising area for the development of new cancer therapies. Based on the presence or absence of the ELR motif (sequence Glu-Leu-Arg), CXCL chemokines are divided into ELR⁻ sequences and ELR⁺ sequences (glutamic acid-leucine-arginine). Chemokines ELR⁻ motif show anti-angiogenesis properties while chemokines ELR⁺ motif is linked to promote growth and invasion in part due to their angiogenic properties [18-20]. The ELR⁺ motif has been considered a molecular signature for CXCR1/2-targeting chemokines and any change to the ELR motif in these chemokines eliminates their signaling through these receptors [21]. One member of the ELR⁺ CXCL family of chemokines is chemokine ligand 7(CXCL7), known as a platelet-derived growth factor [22, 23]. *PPBP* is the gene encodes for CXCL7 that has several known molecular variants of including platelet basic protein (PBP), connective tissue-activating peptide III (CTAP-III), β -thromboglobulin (β -TG), and neutrophil-activating peptide-2 (NAP-2) that are proteolytically derived from a precursor molecule (pre-platelet basic protein [pre-PBP]) [24]. Each isoform has distinct biological functions, and NAP-2 is the only CXCL7 variant that displays chemotactic activity, especially for neutrophils[24]. Recently, CXCL7 has been observed to be upregulated in various human cancers, which seem to play a role in tumor growth [25-28]. In patients with metastatic colon cancer, elevated transcript levels of CXCR2, the CXCL7 receptor, show a correlation with a reduced disease-free state and worse overall outcomes [25].

Furthermore, several studies have pointed out that serum CXCL7 could be used as non-invasive diagnostic biomarker for CRC [4, 29, 30]. However, the exact mechanism of CXCL7 in promoting colon cancer proliferation has not been studied yet. In particular, the role of glycolytic function of CXCL7 in colon cancer progression. Understanding the link between CXCL7 expression and colon cancer cell proliferation could lead to establish preventative measures to control the CRC incidence and mortality rate. Accordingly, this study aims to examine the role of platelet-derived chemokines (CXCL7) in colon cancer cell proliferation [31, 32].

Chapter 2. Literature Review

2.1. Cancer statistics, epidemiology, and definition

National cancer survivor prevalence, as of January 1, 2022, estimated that more than 18 million Americans with a history of cancer are alive. The top 3 common cancers in 2022 are breast, uterine corpus, and thyroid among women, while prostate, melanoma of the skin, and colon and rectum are the most prevalent among men [33]. The number of cancer survivors is growing in the United States as a result of the combined effects of a growing population as well as continuous improvement of early cancer detection and intervention methods [33].

Nevertheless, many cancer survivors must cope with dramatic functional and cognitive impairments along with other serious psychological and economic sequelae resulting from cancer alone. Recent epidemiologic data garnered from the official databases of the World Health Organization (WHO) and American Cancer Society (ACS) show cancer poses the highest clinical, economic, and social burdens among all other human diseases based on the analytical tool, cause-specific Disability-Adjusted Life Years (DALYs) [34]. DALYs is a comprehensive

assessment tool for major diseases and injuries established by the Global Burden of Disease Study (GBD), and it measures premature mortality (years of life lost, YLL) and spent time in states of reduced health (years lived with disability, YLD) [35].

As expected, the largest number of DALYs is found in the elderly population, after the age of 60 years (124.2 million DALYs; 50.8%). Also, the most frequent malignancies in this group are lung (21%), colorectal (9%), stomach (9%), and liver cancers (9%) [34]. Leukemias (37%), followed by brain and nervous system cancers (16%) and lymphomas (13%) are the top prevalent malignant diseases in the young population, aged 14 years or younger. In the age range of 15-49 years, breast cancer (13%) is the most common malignancy, followed by liver (12%) and lung (9%) cancer [34]. In the population with the age range 50-59 years, lung cancer is the most frequent malignant disease (18%), followed by liver (11%) and breast (9%) cancers. The overall 0-74 years risk of developing cancer is 20.2% (18.2% in women and 22.4% in men, respectively [34]. Thus, there is an urgent need for establishing or reinforcing the current strategies for managing cancer and developing more sensitive screening and diagnostic tools. The term ‘tumor’, or ‘neoplasm’, defines a group of lesions characterized by abnormal and uncontrolled tissue proliferations of the genetically mutant cells, proliferations that exceed the dimensions and speed of all processes occurring in the case of inflammation, regeneration, and repair [15, 36]. Back in 1974, Robins defined neoplastic transformation as a hereditary deviation in cells manifesting through evading from control, enhanced proliferation, alterations in cell membranes, karyotype anomalies, the appearance of new surface antigens, morphological and biochemical changes, as well as other attributes that confer the cells capacity to grow, metastasize, and kill [15]. The term ‘tumor’ is a Latin term, means pathological swelling, whether of inflammatory, cystic, edematous, or other nature [15]. In oncology, the expressions

‘tumor disease’, ‘cancer disease’, and ‘neoplasm’ are interchangeably used. The term ‘cancer’ has been used since ancient times, originating from the Latin ‘cancer’. The term neoplasm is composed of the prefix neo-, which originates from the Greek neos (new) and plasis (formation) [15, 37]. Carcinogenesis is defined as the initiation of a tumor, and oncogenesis refers to the maintenance and subsequent evolution of the tumor [15]. It is now widely accepted that the etiology of cancer could be a result of the gradual accumulation of driver gene mutations that dramatically increase cell proliferation [5, 38, 39]. Tumorigenesis, or cancer development, is a slow and stepwise process, typically taking multiple years to several decades, and it can be grouped into three phases: initiation, promotion, and progression [38, 40, 41]. Initiation happens after exposure to mutagens and involves genomic alterations within the “cancer cell”, such as gene deletion and amplification, point mutations, and chromosomal rearrangements leading to permanent cellular abnormalities [38, 40, 41]. Tumor development depends on the survival and clonal expansion of these “initiated” cells [38, 40]. As the initiated clone expands and divides, random cells within it acquire additional mutations, forming unique subclones, and this step in the process is called promotion [40]. While initiation results in little or no observable changes in the cellular or tissue morphology, tumor promotion requires non-mutagenic tissue disruption by wounding or inflammation leading to the formation of a non-malignant tumor which may progress without further stimulus [41]. Progression is manifested by substantial growth in tumor size and malignant transformation requires some additional tissue disruption, though much of cellular evolution in this phase seems to require no external stimulus [36, 38, 41]. However, acquiring of new mutations accelerates as cellular mutation repair machinery itself becomes dysfunctional and mutated [40]. The rate of growth is also increased due to mutations in cell division signaling systems [41]. However, mutation is not the only factor shaping the cancer

genome: evolutionary selection also plays a significant role. Selection is defined as the situation where one group of cells within the TME is evolutionarily ‘favored’ over others. These favored cells have more offspring than the non-favored population, making the cell adapt to a new phenotypic trait that gives it an advantage in the current TME [42]. For instance, a cell with a low metabolic demand might proliferate faster than a cell with a high metabolic need when both cells are together in a nutrient-poor microenvironment [42]. Consequently, selection plays a central role in shaping the frequency distribution of mutations within a tumor. Since any mutation in the dominant clone (favored) becomes more common in the TME, minor clones (not favored) become relatively less frequent [42]. Therefore, understanding tumorigenesis requires understanding both mutation and selection process and how these two phenomena together control the pattern and frequency of mutations in the cancer’s genome [43].

Back to cancer development stages, during the early phase (initiation) cancer remains invisible, and mutation is undetectable unless tissues or cells are examined microscopically [40]. Initiation and promotion phases are mediated by tissue-generated proliferation constraints through promotion mutations in oncogenes, and tumor suppressor genes that lead to induce senescence pathways [41]. In contrast, the last stage or progression is largely primed by microenvironmental factors such as blood flow and substrate diffusion that control the concentrations of oxygen, glucose, and extracellular pH in the TME [41]. Cancer progression is restricted by concentrations of oxygen, glucose, or H^+ , and this is facilitated through adaptations such as the upregulation of aerobic glycolysis and resistance to acid-mediated toxicity that lead to the formation of invasive cancer [41]. As cancer proliferates, it inherits mutation-driven changes in both form and function. Carcinogenesis progresses when cancer cells change in form and manifest themselves as deviations from benign cells in size, shape, and orientation [40]. The early changes in cell

morphology are known as dysplasia. The aberration becomes aggressively more visible, from mild to severe dysplasia, until they have morphological characteristics of a malignant neoplasm, the most common trait of which is cell-to-cell heterogeneity [40]. At this stage, the neoplasm looks well-circumscribed with smooth borders as it is confined to its anatomical niche of origin in the epithelium and is known as carcinoma in situ [40, 44]. With further progression, the neoplasm acquires new characteristics and abilities that allow it to invade and destroy normal cells [40]. The neoplasm overtakes the tissue beneath the epithelium of origin, migrates into lymphatic and/or blood vessels, and survives in either the high-pressure blood circulatory system, the low-pressure lymphatic, or both [40]. These circulatory systems carry the malignant cells to other sites in the body, either to the regional draining lymph nodes via the lymphatics or to distant organs and tissues via the blood. The capability of neoplasm cells to spread from the primary site, in situ, to the secondary location within the body is referred to as metastasis [45]. The 'seed and soil theory' proposed by Stephen Paget laid out an early insight into metastasis [46]. Paget hypothesized crosstalk between selected cancer cells (the 'seeds') and specific organ/metastatic niches (the 'soil') [46, 47]. Paget was struck by the correlation between the relative blood supply and the frequent incidence of metastases in some organs [47]. He examined more than 900 autopsy records of patients with different primary tumors. His investigations documented a non-random pattern of metastasis to bones and visceral organs, and he concluded that the outcome of metastasis was not due to chance (the prevailing viewpoint of that time), but that certain tumor cells, 'seeds', have a specific affinity for invading and growing in certain organs. In other words, metastasis was established only when the seed and soil were compatible [47]. When metastatic cancer cells exit the vessels and establish new colonies of tumor, they invade and destroy the normal tissue in the metastatic site [40]. By the time the neoplasm has

succeeded in migration to distant body sites, the cancer has higher treatment resistance and is much more likely to be lethal to the host [40, 41]. Conventional oncology treatments face numerous challenges when targeting metastatic disease. Despite significant improvements in early diagnosis, surgical procedures, general cancer patient care, and local and systemic adjuvant therapies, most deaths from cancer results from metastases that are resistant to current cancer therapies [47].

Many epidemiological studies have long been discussing the main cause of tumor initiation such as mutation, including the role of environmental factors in cancer development, to find fundamental implications for primary prevention [39, 48]. Carcinogenesis can be diagnosed in several aspects, and one way to describe this process is to illustrate the essential features of both cancer cells and tumors that collectively refers as “hallmarks” of cancer [49]. Cancer development or hallmarks of cancer requires the acquisition of six fundamental properties: self-sufficient proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, unlimited replicative potential, and the maintenance of vascularization [36, 49]. Literature has been addressing these six biological features associated with the multistep development of new plastic diseases [49]. These hallmarks established an organized principle for understanding the complexities of tumorigenesis. Figure 1. illustrates the six hallmarks of cancer, including constitutive proliferative signaling, inhibition of growth suppressors, resistance to apoptosis, replicative immortality, speeding angiogenesis, and activating migration and metastasis[49]. Genomic instability, along with genetic diversity and inflammation, serves as the primary initiator of cancer development, collectively mediating cancer hallmarks. Genomic instability associates with auto constitutive inflammatory signaling from tumor cells, which has recently been accounted as a driving force for metastasis [45].

The current perspective considers tumors more than just an insular masse of proliferating cancer cells that show distinct traits. Instead, they are collective tissues composed of many different cell types that participate in heterotypic interactions with one another. Solid tumors possess a unique environment that is known as the tumor microenvironment (TME) and is composed of two distinct compartments, the parenchyma, and the stroma, just like normal tissues [50]. The parenchyma typically represents the cancer cells, while all non-malignant cells and the other connective tissue elements are considered as the stromal compartment [50]. The stroma is composed of very heterogeneous molecules, including various cell types, soluble proteins, and adhesion molecules, both contributing to the functional activity and structural support of the TME itself [51]. Infiltration of Immune cells in the tumor microenvironment is necessary for a successful and prompt suppression of the primary tumor. Each cell type herein supports the maintenance of the microenvironment itself both functionally and biochemically through orchestrating cell-cell interactions and secreting a massive number of chemokines and cytokines[22].This dynamic interactions determine the timing and degree of the immune response, as well as facilitate appropriate modulation and maintenance of angiogenesis. The net effect of this strictly related phenomenon may either be the stimulation of a tumor-specific immune response or tumor escape [51]. In addition to the recruitment of immune cells, tumor-associated stromal cells are active participants in tumorigenesis and contribute to the development and expression of certain hallmark capabilities in TME.

Clearly, the development of this supportive niche is critical to the continued uncontrolled growth of the tumor. During the ensuing decade, this notion has been solidified and extended, revealing that the biology of tumors can no longer be understood simply by enumerating the

traits of the cancer cells but instead must encompass the contributions of the TME to tumorigenesis [49].

In summary, with the high incidence of cancer globally, cancer has become a major public health and economic issue. Hence, thorough understanding for the causes, stages, and hallmarks of tumorigenesis is important to establish preventative measurements and affective treatment for cancer.

2.1.1. Cancer is an over-healing wound: platelets and cancer cells interactions

Similarities between tumorigenesis and the inflammatory response associated with wound healing have been discussed since the 19th century when Rudolph Virchow proposed his hypothesis on cancer development as the product of unresolved inflammation [49]. Virchow established the notion of tumor-associated inflammation and discussed how tumors acquire a specialized microenvironment by invoking an inflammatory cascade and attracting immune cells that are needed for survival and growth [45, 49, 52]. Wound healing is a dynamic process that is composed of an inflammatory stage, followed by epithelial cell proliferation and finally, tissue remodeling. These three phases are regulated similarly during tumor metastasis [45, 53]. As with damage to any tissue, the wound healing process begins with inflammation to clear cellular debris and resist infection [45]. This inflammatory phase that occurs during normal wound healing has been linked to the pathological state of many cancers [53]. Tissue injury induces immediate recruitment of and infiltrating of leukocytes, starting with neutrophil recruitment, which is later replaced by lymphocytes and macrophages [52, 53]. Leukocyte infiltration induces the secretion of growth factors, inflammatory cytokines, and chemokines that stimulate the proliferation of progenitor cells and the recruitment of endothelial cells and keratinocytes during

the proliferative phase of wound healing [54]. At this stage, granulation tissue is generated, angiogenesis is accelerated, and a new extracellular matrix (ECM) is produced [53]. Epithelial cells undertake epithelial-mesenchymal transition (EMT) and travel to the edges of the wound to perform re-epithelialization of the damaged cells [53]. In the final phase of wound healing, the maturation/tissue remodeling stage, fibroblasts differentiate into myofibroblasts, forming scar tissue [45]. Most importantly, the inflammatory phase only lasts 3–14 days, and failure to exit the inflammatory phase leads to abnormal tissue remodeling that is associated with impaired wound healing in many disorders, including diabetes mellitus, vasculitis, and cancer [55]. Figure 2 summarizes the wound healing stages [45]. There is growing evidence that inflammation is the root cause of many malignancies [55], and genomic instability, a result of ongoing errors during mitosis, could be a driver for inflammation [45]. In fact, chromosomal instability leads to the formation of rupture-prone micronuclei – an intrinsic source of chronic inflammatory signaling in cancer cells themselves. Consequently, genomic instability can drive tumor progression forward and yield aggressive and metastatic cancers that are resisted treatments [45]. Genomic instability promotes the engagement of wound healing processes related to migration, regeneration, proliferation, and remodeling by inducing inflammatory signaling through sensing of double-stranded DNA (dsDNA) in the cytosol [45].

Other possible etiologies for inflammation-associated malignancy are chemical and physical agents, autoimmune and inflammatory reactions, and most importantly, obesity. Obesity results from Western diet intake that is rich in sugar and hydrogenated/saturated fat which creates low-grade unresolved inflammatory status in the body [56]. Epidemiological evidence shows that being obese can increase the risk for tumor-associated inflammation, and obesity itself has been linked to several malignancies such as endometrial cancer, postmenopausal breast

cancer, and colon cancer, [56, 57]. However, Cancer associated with chronic inflammation is manifested by continuously recruiting platelets to the TME [58, 59]. In fact, platelets represent the link between wound healing and inflammatory response with a putative role in tumorigenesis [59-61].

Platelets arise from megakaryocytes, a bone marrow stem cell lineage. These anucleate cells can influence inflammation and immune regulation [59]. Platelets serve as the “first responders” during normal wounding, and when blood vessels are injured, they release subendothelial elements that initiate platelets’ first response through receptor-based recognition of the ECM [59]. This recognition motivates platelet activation and aggregation within a matter of seconds by changing the platelets’ shape, inducing degranulation, and releasing proteins, bioactive lipids, and growth factors. These factors attract additional platelets and immune cells along with initiating thrombogenesis and clotting to fix any exposed gaps [59]. Accordingly, platelets, as first responder, are essential in maintaining homeostasis, threat recognition, immune function, and even cancer progression [24, 58, 59, 62, 63]. Besides their vital role in coagulation and maintaining hemostasis, platelets express abundance receptors on their surfaces and store many bioactive molecules in their granules that can contribute to mediate inflammation, cancer progression, and metastasis [64]. Based on the biophysical view, platelets are optimized to do all these functions due to their tiny size and discoid morphology that facilitate their spreading around vessel walls to maintain vascular integrity and react quickly to vascular lesions. Functionally, platelets normally trigger inflammation and enhance rapid clotting and vasoconstriction in wounds that help to sterilize and repair injured tissues [59]. In addition, platelets can facilitate tumor escape/extravasation by decorating them, and sense phagocytized pathogens in the bloodstream [63]. Thus, platelets are considered the first responders during

chronic inflammation and pathogen invasion [59]. When platelets are activated, they release a plethora of inflammatory cytokines, chemokines, platelet-derived chemokines, and growth factors that establish TME, and mediate two major cancer hallmarks: proliferation and metastasis [58, 62, 63]. In terms of proliferation, platelets secrete several cancer growth and angiogenesis factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and Angiopoietin-1(ANG-1) [65]. Platelet microparticles (PMPs) are extracellular vesicles (EVs) derived from activated platelet storage units and are used for different active proteins in platelets, mainly chemokine CX ligand 7 [66]. Michael *et al.* found that PMPs infiltrated TME and transferred platelet RNA to cancer cells *in vivo* and *in vitro* [66, 67]. Multiple of platelets' secretion can exert proliferation properties. A study showed platelets' derived growth (CXCL7) increased the phosphorylation of mTORC1, S6K1, and 4EBP1 in glomerular endothelial cells (GEnCs) in a dose-dependent manner. Additionally, by introducing the CXCL7 receptor blocker (CXCR2 antagonist), the PMP-induced mTORC1 activation is reduced [66]. mTORC1 is a rapamycin-sensitive protein kinase complex that regulates cell growth, proliferation, metabolism, and autophagy by phosphorylating p70 S6 kinase 1 (S6K1) and 4EBP1 [66, 68]. Hyperactivation of mTOR signaling is commonly observed in various types of cancers that promotes cell proliferation and contributes to tumor initiation and progression while negatively regulating autophagy [68]. Similarly, platelet-derived lysophosphatidic acid (LPA) increases tumor cell proliferation by activation of the LPA receptor on cancer cells such as breast (MDA-BO2) and ovarian (CHO) cancer cells [65]. LPA also contributes to bone metastasis by stimulating the release of the bone osteoclast resorption stimulators interleukin IL-6 and IL-8 [65, 67]. When tumor cells begin to disseminate from the primary tumor and access the blood, platelets are the first host cells they encounter [64]. The interaction of tumor cells with

platelets is essential for successful hematogenous metastasis. When cancer cells arrive in the blood, they immediately activate platelets to create a permissive microenvironment [64]. Sequestration of circulating tumor cells (CTCs) by platelets protects them from shear forces and immune surveillance, such as assault of NK cells by encapsulating tumor cells in a thrombus or protecting them from cytolysis [64, 65, 69]. Platelets secrete several factors that help in metastasis, particularly PDGF that confers a mesenchymal-like phenotype to tumor cells and opens the capillary endothelium to support cancer cells extravasation in distant organs [70]. Platelets also release high concentrations of TGF β , which plays a role in platelet-colon cancer cell crosstalk, leading to cancer metastasis to the lung and increasing the expression of genes involved in epithelial-mesenchymal-like transition (EMT) [60, 63]. A recent vivo study found that P-selectin (adhesion molecule stored on platelets α -granules) deficiency inhibits the direct interaction between platelets and colon cancer cells leading to reduced tumor growth by modulating the expression of genes involved in cell cycle progression, such as CyclinD1, Ccne1, and PCNA [60]. Thus, cancer is considered a non-healing wound associated with chronic inflammation, and it can be progressed by platelet mitogenic properties. To maintain stable adhesion between cancer cells and platelets, cancer cells activate platelets through several mechanisms, which are also the reason for hypercoagulation and increased incidences of thrombosis in cancer patients [64, 71]. Cancer-associated thrombosis can result from the direct pressure of tumor mass on blood vessels or low physical activity of most cancer patients, especially after invasive treatment or resection surgery [71]. In addition, malignant tumors normally secrete heparanase, the only mammalian enzyme that degrades heparan sulfate residues. Heparan sulfate is an anticoagulant and an important component of the extracellular matrix [46, 71, 72]. Tumor cells also release soluble platelet mediators like thromboxane A₂

(TXA2), or high-mobility group box 1 (HMGB1), which interacts with toll-like receptor 4 (TLR4) to prime a local platelet activation to accelerate the thrombogenesis/clotting process [59, 64]. Recently, Ward et al. showed that cancer cell-expressed adhesion GPCR CD97 induced platelet activation, which leads to lysophosphatidic acid (LPA) release from platelets. LPA, in turn increases vascular permeability to promote tumor cell invasion and to induce trans endothelial migration [64]. Most cancer cells have constitutively active tissue factor (TF) on their cell membranes, which initiates the plasmatic coagulation cascade and finally generates thrombin, which in turn induces platelet activation [64, 65, 73]. Thus, malignancy creates a “vicious cycle” in which greater tumor burden correlates with greater thrombin and platelet-tumor interaction [65]. Besides the activation of the coagulation cascade and platelets, thrombin is critical for almost every step of the metastatic process [64]. Thrombin contributes to cancer cell proliferation and tumor growth in several ways, such as by activation of its G-coupled seven transmembrane protease-activated receptor PAR-1 and fibrinogen [65]. In TME, thrombin-stimulated fibroblasts and macrophages secrete monocyte chemotactic proteins, which favor pro-tumorigenic myeloid cell invasion [64]. Thrombin also modulates endothelial cells to support angiogenesis, such as by potentiation of the mitogenic activity of VEGF on endothelial cells [74]. Thrombin-stimulated endothelial cells have rounded morphology and loss of adherent junctions, which permits tumor cell transendothelial migration [64]. Moreover, thrombin acts as an anti-apoptosis factor and increases the proliferation and differentiation of vascular progenitor cells [64]. In particular, the thrombin-fibrinogen axis is observed to play an important role in CRC. Previous study indicates a six-fold increased likelihood of developing CRC in people who have homozygous carriers of the prothrombotic factor V Leiden mutation compared to non-carriers, suggesting that increased thrombin level is positively correlated to tumorigenesis in

CRC [70, 75]. In contrast, patients with atrial fibrillation who were on long-term anticoagulating agents were shown to have a lower incidence of colon cancer [75].

In conclusion, the wound healing process can be corrupted and co-opt cancer progression since the mechanisms that regulate tissue repairing are like the mechanisms that promote the growth of tumor. Today, studies are focusing more on exploring the contribution of tumor inflammatory microenvironment and platelets activation on tumor metastasis and progression.

2.1.2. Metabolic reprogramming: a hallmark of malignancy

It is hard to discuss cancer's metabolism without first mentioning Otto Warburg, a pioneer in respiration research. In 1920, Warburg observed that cancer cells rely on aerobic glycolysis to meet the heightened anabolic needs for cancer cell proliferation and reduce the use of tricarboxylic acid cycle (TCA) irrespective of oxygen availability and the presence of completely functional mitochondria [8, 76-78]. In contrast, normal or differentiated cells mainly rely on mitochondrial oxidative phosphorylation to fully oxidize the glucose and generate high energy. Warburg was awarded a Nobel Prize in 1931 for his observations on metabolic reprogramming in cancer. Since that time, literature has recognized constitutive aerobic glycolysis as a hallmark of cancer and the main criterium for tumorigenesis [79]. Observation of the Warburg effect has been documented for over 90 years and extensively studied over the past 10 years, with thousands of papers reporting the complexities of neoplasticity [6, 80]. However, compared to mitochondrial oxidative phosphorylation (OXPHOS), the Warburg effect is less efficient in terms of ATP production, with nearly 16-fold lower efficiency of energy production by glycolysis compared to OXPHOS [81]. Under oxygen availability, one molecule of glucose is first converted to two pyruvates via glycolysis in the cytosol, followed by CO₂ production via the

TCA cycle in the mitochondria [81]. A total of 30 or 32 ATP molecules are formed during this process. In contrast, under anaerobic conditions, glycolysis is dominant, and less pyruvate is shifted to the oxygen-consuming mitochondria. Thus, only two ATP molecules from one glucose molecule are generated through glycolysis under anaerobic conditions [81]. Many studies have been done to discuss why most tumor cells rely on a less efficient system like aerobic glycolysis to meet their energy demands. It should be noted that, to a great extent, the current understanding of cellular metabolism primarily relies on the observations of nonproliferating cells within differentiated tissues [82]. These cells mainly metabolize glucose to CO₂ through the complete mitochondrial oxidation of pyruvate after glycolysis is complete, followed by a large amount of ATP molecules being released [81]. For proliferative organelles like cancer cells, in addition to energy requirements, they must meet their needs for a huge number of building blocks for macromolecular synthesis, including lipids, nucleotides, and amino acids [81]. Thus, as nutrient flux increases into cancer cells, they will preferentially utilize the fastest proliferating processes rather than the maximization of ATP production. Interestingly, many unicellular organisms preferentially rely on fermentation, converting pyruvate to lactate, similar to aerobic glycolysis in tumor cells to maintain sufficient supply for cell division even in the presence of oxygen [81]. One explanation for this phenomenon is the glycolytic switch in rapidly proliferative cells allows rapid flux of glycolytic intermediates to many biosynthetic pathways, which in turn support the synthesis of the biomacromolecules and other materials required for rapidly dividing cells and producing daughter cells [81]. Similarly, tumor cells need to replenish all cellular components, and meet their large need for nucleotides, lipids, and amino acids [81]. If glucose is used to generate ATP through mitochondrial OXPHOS, the carbon resources for supplying biomacromolecule synthesis would be very limited. Thus, it is becoming increasingly evident

that metabolizing glucose to CO₂ through mitochondrial OXPHOS to release ATP is unfavorable pathway for the rapidly growing tissues such as cancer cells.

Noteworthy, the increase in glycolytic dependency, accelerated glucose uptake, and fermentation lead to accumulation of lactate in TME. In fact, lactate produced from tumors can reach a concentration 20-fold higher than normal tissues, leading to further diminish the efficiency of the TCA cycle and OXPHOS in cancer cells [80, 83, 84]. Since lactic acid is a weak hydrophilic acid, it requires specific transporters, monocarboxylate transporters (MCTs) such as MCT1 and MCT4, that are strongly associated with the hyperglycolytic phenotype of cancer cells and upregulated in them [78]. MCTs permit the shuttle of lactate from cancer cells to neighboring cells such as tumor stroma, and tumor-associated endothelial cells, and induce metabolic rewiring. Stromal cells in TME can recycle the accumulated lactate excreted by glycolytic cancer cells to pyruvate, which in turn can be extruded to supply cancer cells [85]. A cooperative micro-metabolic system normally is created between anaerobic tumor cells and aerobic stromal cells, similar to the Cori cycle between liver and skeletal muscle cells [85]. Similarly, there is a symbiotic relationship between well-oxygenated cancer cells (oxidative tumor) and poorly oxygenated cancer cell (glycolytic tumor) populations. Oxidative tumors can import and utilize lactate from glycolytic tumors, which depends on the expression of MCT1, while glycolytic tumors should express MCT4 to export lactate [77, 78]. Targeting MCTs has been proven to suppress tumor growth in cancer cells, for instance, MCT1 and MCT4 inhibitors impair leukemia cell proliferation and enhance their sensitivity toward chemotherapy [86, 87]. However, cancer cells do not exclusively rely on glycolysis for ATP supply and carbon sources, but they consume vast amounts of glutamine to sustain many biosynthetic processes, such as the production of nonessential amino acids, lipids, and DNA bases [81]. Finally, the intermediates

and byproducts of both glycolysis and glutaminolysis are utilized for generating biomass to meet the needs of a rapidly growing tumor [81].

Because the Warburg effect generates less ATP than that of oxidative phosphorylation, cancer cells compensate for this disadvantage by upregulating the uptake of glucose and accelerating the rate of glycolysis [88]. An accelerated rate of glycolysis is usually parallel with the increased expression of many glycolytic enzymes. Based on this, it is unsurprising that glycolytic enzymes are exponentially upregulated 2-500-fold in many tumors [89].

Overexpression of glucose transporters and certain glycolytic enzymes, such as hexokinase 2 (HK2), in many kinds of cancers is linked with poor prognosis after radio- or chemotherapy [88].

Current research in cancer therapy focuses on targeting glycolytic mediators such as hexokinase (HK), PKM2, glucose-6-phosphate dehydrogenase (G6PD), lactate dehydrogenase-A (LDHA), and glucose transporters (GLUTs), to enhance treatment sensitivity and patient prognosis [89].

Pyruvate is the cytosolic end-product of glycolysis, and it acts as a junction between glycolysis and the TCA cycle in mitochondria [90]. It is well known that the metabolic fate of pyruvate reflects the extent to which cells are involved in aerobic glycolysis [91]. To facilitate the entry of pyruvate carbons into the TCA cycle, pyruvate needs to be oxidized by the pyruvate

dehydrogenase complex (PDHC) [90]. PDHC is negatively regulated by PDK, and inhibition of the PDK would suppress aerobic glycolysis by promoting the oxidation of glucose carbons in the TCA cycle at the expense of fermentation. It has been shown that genetic suppression of PDKs could slow cancer cell growth in vitro and in vivo [20, 90]. Hexokinase is the first enzyme that catalyzes the glycolytic pathway and converts glucose to glucose 6 phosphate. Hexokinase has four isoforms (HK1-4) with different cellular distributions and glucose affinity [77]. HK1 and HK2 are located on the outer mitochondrial membrane and are associated with the AKT

signaling pathway involved in cell proliferation and survival [92]. HK2 overexpression is solely observed in cancer cells, and studies show HK2 is markedly upregulated in advanced lung cancer stages, recurrence of breast cancer, and poor prognosis cancer patients in general [93, 94]. Targeting HK2 diminishes cell proliferation and shifts cancer metabolism from glycolytic to oxidative phosphorylation [94]. An example for most commonly reported HK2 inhibitor is 2-Deoxy glucose (2-DG), a glucose analog that triggers the intracellular accumulation of 2-deoxy-d-glucose-6-phosphate (2-DG6P) and inhibit glycolysis [95]. Many clinical trials show that administering 2-DG to the tumor can improve the outcome of chemotherapy and radiotherapy by sensitizing cancer cells and inhibiting glycolysis [95, 96]. Recently, a study found administering 2-DG could sensitize glioblastoma cells to chloroethyl nitrosourea by adjusting glycolysis, intracellular reactive oxygen species (ROS) formation, and endoplasmic reticulum stress induction [97]. Curcumin is another HK inhibitor with a proven anti-tumor effect that can inhibit HK expression by targeting SLUG, a key transcriptional regulator for cancer development. Treating prostate cancer patients with the combination of curcumin and docetaxel improves their sensitivity to treatment with low toxicity, and improves patients tolerance in general [98]. Another glycolytic enzyme that could mediate the Warburg effect is lactate dehydrogenase (LDH), an NADPH-dependent enzyme. In fact, the Warburg effect phenomenon could be blocked by targeting LDH and enforce cancer cells to revert to OXPHOS to reoxidize NADH and produce ATP [89]. LDH is a key glycolytic enzyme that upregulated in aggressive cancers and is essential for tumor progression and maintaining conversion of pyruvate to lactate, even under aerobic conditions [77]. Recent pre-clinical and clinical studies have supported the combined use of LDH inhibitors, e.g. pyruvate analog and oxamate (OXM), with concurrent treatments as a promising strategy for cancer therapy [77, 99-101]. Studies have found

administering OXM triggers tumor reduction in the brain by reducing glycolysis and ATP levels, inducing apoptosis, and increasing ROS production [101]. Combined therapy of LDH inhibitor (OXM) with other chemotherapeutic agents, including mTORC1 inhibitor, provided synergistic inhibitory effects on colon cancer cells by significantly suppressing colon cancer cells glucose uptake and lactate production [100]. 3-Bromopyruvate acid (3BrPA) is another classic LDH inhibitor that can also target many other enzymes in the glycolytic pathway, including HK2, and is a potent inhibitor of cancer cell growth [77, 81].

Another glycolytic mediator that is upregulated in cancers is glucose transporters (GLUTs) family, a critical mediator to initiate glucose metabolism. Literature indicates inhibition of GLUTs can potentiate the therapeutic efficacy for many cancers' drugs and enhance patients outcomes. [102]. According to a meta-analysis study, increased expression of GLUT1 is associated with poorly differentiated tumors, larger tumor size, and positive lymph node metastasis [103]. In addition, GLUT1 inhibition significantly decreases the self-renewing capacity and tumor-initiating potential of cancer stem cells. It also substantially declines the AKT/MTOR signaling cascade, a key proliferative pathway in cancer [103]. The serine/threonine kinase mTOR is important in the PI3K signaling cascade, which mediates tumor growth, metabolism, metastasis, and invasion via inducing lipid synthesis and metabolism [100]. Using inhibitors of GLUT1 to downregulate glycolysis, combined with routine cancer therapies, has been proven to potentiate the effects of cancer therapy in a synergistic and additive fashion in pre-clinical studies for various cancers [103, 104].

Overall, altered energy metabolism of aerobic glycolysis is considered a hallmark of cancer cells that has been linked with tumor aggressiveness and resistance to cancer therapy.

However, the precise mechanism underlying the role of aerobic glycolysis mediators in tumor progression and therapies resistance is still unclear. Further studies should be done to target cancer cell metabolism, which may enable tumor cells to be sensitized to chemotherapy and enhance the therapeutic efficacy.

2.1.2.1 Oncogenic shapes Warburg effect

Oncogenes play a critical role in triggering the unique glycolytic phenotype for cancer cells. Metabolic reprogramming in cancer cells is associated with genetics and proteomic alterations that impact various cellular and physiological functions in cancer cells including energy metabolism, vascularization, invasion, senescence, stem cell maintenance, genetic instability, and drug resistance [105]. Genetic alterations have resulted from high expression of specific transcription factors or oncogenic tumor pathways that can sustain the Warburg effect [86]. In addition, the Warburg effect reflects a pseudo-hypoxic state that activates many transcription factors associated with tumorigenesis. Principal pathways that are activated in malignant cells are hypoxia-inducible factor-1alpha (HIF-1 α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), phosphatidylinositol-3-OH kinase (PI3K), and myelocytomatosis oncogene (MYC) [86]. Current evidence indicates that the upregulation of these transcription factors, in particular HIF-1 and c-Myc, is essential to increase the activities of the glycolytic enzymes and activate cancer's proliferative pathway PI3K/Akt and Ras [91, 106]. HIF1 α promotes the expression of various glycolytic enzymes, including HK2, aldolase, LDHA, and glycolytic transporters such as GLUT1 and MCT4, and increases the intracellular level of glycolysis [106]. In cancer cells, the upregulation of HIF-1 and c-Myc is usually accompanied by inhibiting tumor suppressor (*p53*), mitochondria dysfunction, upregulating the expression of

various glycolytic enzyme, mainly LDH, leading to the metabolic switch from OXPHOS to fermentation in the tumor niche [89, 91]. Accumulative evidence has shown that *MYC* performs an essential role in the regulation of metabolic reprogramming, permitting rapid generation of bioenergetic substrates and building blocks to support the uncontrolled growth of cancer cells [107]. Thus, the net result from the dysregulation of oncogenes and drivers will alter cancer metabolism and confer adaptive, proliferative, and survival advantages for cancer cells.

Hypoxia is a core hallmark of TME resulting from an imbalance between increased oxygen consumption and limited oxygen supply due to a rapid cellular growth that outgrows the surrounding vasculature [108]. Cancer cells adapt to hypoxia by altering their metabolism and upregulating the transcriptional factor HIF-1, which orchestrates the cellular adaptive mechanisms triggered in response to a low-oxygen environment [108, 109]. In normal cells under hypoxic conditions, cell proliferation declines to prevent further need for oxygen consumption by the cells. However, in cancer cell genetic mutations that alter tumor-suppressing genes and increase high capacity for metabolic reprogramming, permit cell proliferation, disregarding low-oxygen conditions. In fact, mitochondria, the primary organelles responsible for cellular respiration, are severely damaged by decreased oxygen availability in tumor. In addition, loss of function for *p53* in cancer cells blocks expression of the synthesis of cytochrome c oxidase (*SCO2*) gene, and inhibits mitochondrial respiration [110].

However, mitochondria adjust their metabolism in response to hypoxia in different ways, including exchanging or modifying subunits of the respiratory chain, lowering TCA cycle intermediates, decreasing OXPHOS, altering ROS production, and decreasing β -oxidation [108, 111]. HIF-1 also can modulate mitochondria dysfunction and bind to most oxygen-regulated genes if they contain specific responsive elements (hypoxia response elements, HREs) leading to

an adaptive response that support tumor progression [108]. HIF-1 α also upregulates the expression of key glycolytic mediators that convert glucose into lactate such as GLUTs, HK2, LDHA, and the lactate-extruding monocarboxylate transporter 4 (MCT4) [112-114]. Interestingly, HIF-1 α blocks the entry of pyruvate into the TCA cycle by overexpression of PDK1, driving tumor cell energy towards glycolysis [86]. The major oncogene *Ras*, when mutated, can also increase glycolysis through the activation of the mammalian target of rapamycin complex I (mTORC1) [78]. Also, in tumors Akt -PI3K is activated resulting in increasing glucose uptake and glycolytic flux, while HK2 targets the mitochondria, and the transcription factor MYC induces glutaminolysis and upregulates MCT1 expression [78]. In this context, it could be assumed that the Warburg effect in some cases is driven by genetic alterations/oncogene activation that could be mediated by the current needs of the TME.

Clearly, instead of one event inducing the Warburg effect, numerous factors and genes participate in determining the fate of glucose in cancer cells. The active symbiosis relationship between oncogenes and cancer cells is crucial in promoting tumor growth and survival. Prevention of metabolic rewiring via targeting oncogenes may represent a promising approach for the treatment of cancers.

2.1.2.2. Warburg Effect: Beyond Aerobic Glycolysis

The Warburg effect phenomenon has been proposed to be an adaptive mechanism to support high energy needs and the biosynthetic requirements for uncontrolled growth in cancer cells [8, 76, 83]. However, altered tumor metabolism predicts not only glycolytic capacity but also other malignant biological behaviors such as survival and cancer phenotypes [79].

Because cancerous cells perform constitutive aerobic glycolysis, their ability for glucose uptake outgrows the surrounding cells. Since TME has a limited amount of glucose, cancer cells will compete with other cells for the glucose, and this will decrease glucose availability for many other organelles, such as immune cells, which suppress the immune response in TME and promote cancer cell survival[115]. To explain, the availability of glucose appears to be a result of direct competition between the tumor and tumor-infiltrating lymphocytes (TIL). The high rates of glycolysis for tumor growth will decrease the availability of glucose to TILs. This is important since TILs need sufficient glucose for their effector functions, so less glucose leads to a diminished immune response [83]. Therefore, targeting aerobic glycolysis in the tumor has the added benefit of enabling the supply of glucose to TILs, thus promoting their main function which is to eradicate the tumor cells [83]. Recent evidence suggests that in cancer cells, changes to the cellular environment were altered greatly to increase ATP demand, which is associated with changing the demand for ATP-dependent membrane pumps. This leads to inducing aerobic glycolysis while oxidative phosphorylation remains constant [83]. This provides further explanation for the impact of the Warburg Effect on rapidly generating ATP to support the fast cancer demand for ATP [83]. Furthermore, the increase in glucose metabolism provides a carbon source for anabolic processes that support rapid cellular proliferation in the cancer [116]. These excess carbons are utilized in anabolic processes such as the de novo synthesis of nucleotides, lipids, and proteins to meet the demand for increasing cellular proliferation[83]. Thus, the new insight in cancer research argues that the glycolytic pathway allows cancer cells to meet their urgent and rapid needs not only for the ATP but also for the cells building blocks and specific carriers. For instance, the production of electron carriers is required as a cofactor for redox reactions in cells, utilizing reducing equivalents in the form of NADPH [8, 76, 83, 89, 91].

Increased glucose uptake permits the greater synthesis of these reducing equivalents in the oxidative branch of the pentose phosphate pathway (PPP) in cancer [5, 83]. The fermentation step is the conversion from pyruvate to lactate via LDH and requires NADH to regenerate NAD⁺ to avoid the excessive accumulation of NADH [83, 91]. Also, the PPP induces the oxidative decarboxylation of G6P via the oxidative branch, resulting in ribulose 5-phosphate (Ru 5P). The Ru 5P is isomerized into ribose 5-phosphate (R 5P), which serves as a nucleotide precursor and can be metabolized through the non-oxidative branch of the PPP to yield glycolysis mediators such as fructose 6-phosphate (F6P) and glyceraldehyde-3-phosphate (G-3-P), via multiple enzymatic reactions [21]. At the same time, this process allows cancer cells to transform NADP⁺ into NADPH, which is an essential coenzyme in lipid metabolism [8, 76, 83, 89]. Despite high glycolytic flux and the depletion of NAD, the end metabolite product of Warburg effect pathway, lactate, can recycle/regenerate NADPH from NAD to keep glycolysis continuous [89]. Further, NADPH participates in scavenging for ROS, maintaining proper redox control, and protecting the tumor from oxidative damage [116], and as a result of the decrease in ROS production in the TME, tumor proliferation will be enhanced. The increase in cell growth and proliferation will raise the demand to synthesize more nucleic acids and proteins through PPP that diminishes fully oxidizing glucose to CO₂ and H₂O to sustain the proper intermediates for membrane growth [116]. This is accomplished partially by slowing the entry of pyruvate into mitochondria, decreasing the conversion to acetyl-CoA, and slowing the rate of the TCA cycle [83, 116]. Since pyruvate will accumulate from aerobic glycolysis, it will be converted to lactate and secreted from the pool to keep glycolysis active [116]. The buildup of lactic acid increases acidity in TME, which is highly correlated with cancer aggressiveness, resistance, and poor survival [88, 89]. Lactate can also exaggerate tumor hypoxic conditions in a paracrine dependent

manner. Accumulation of lactate increases acidosis that often precedes angiogenesis and stimulates HIF expression independently of hypoxia [117]. Data from studies in different cancer lines showed that lactate could activate HIF-1, the master transcription factor involved in cancer cell glycolysis [89]. In addition, lactate is considered an oncometabolite with signaling properties that regulate multiple hallmarks of cancer by supporting cell proliferation and promoting immune suppression, angiogenesis, and metastasis [78, 84, 89]. Lactate can also serve as an intracellular signaling mediator, serving a role in hypoxia adaptation. This adaptation is facilitated by lactate in HIF-1 α -dependent [direct HIF-1 α stabilization by prolyl hydroxylase (PHD) 2 inhibition] and independent [activating N-Myc downstream-regulated (NDRG3) protein, preventing association with PHD2] manners [78]. Lactate promotes HIF-1 α -mediated vascular permeability factor (VEGF) expression in the cancerous cell to stimulate the formation of new blood vessels [118, 119]. Further, enhanced glycolysis in cancer cells relies on LDH-mediated production of NAD⁺ from NADH, reducing NADH: NAD⁺ ratio that inhibits the function of tumor-suppressor genes (*p53*) and promotes cell survival [120]. High glucose metabolism and lactate accumulation lead to inefficient vascular clearing and elevated pH in the TME which causes extracellular matrix remodeling. This is also associated with enabling blood vessel invasion in response to angiogenic factors produced by the tumor [90, 116]. Moreover, lactate stimulates macrophages to secrete pro-angiogenesis factors. Tumor-associated macrophages (TAMs) can uptake lactate through their MCTs leading to lactate-induced activation of HIF- α , thus enhancing transcription of vascular endothelial growth factor VEGF [106]. Interestingly, administering LDH inhibitors in colon cancer markedly decreases the extracellular level of lactate which leads to an inhibition of TAM-derived VEGF and tube formation [106]. In addition, lactate can also enhance cancerous cell survival by avoiding

surveillance and destruction of the immune system [52]. In particular, CD8⁺ T cells are important anti-tumor factors and are often negatively affected by lactate and tumor metabolic microenvironment, leading to impaired cell proliferation, activation, and survival [121]. Tumor-derived lactate inhibits CD8⁺T cell cytotoxicity by inducing a switch of pyruvate utilization from pyruvate carboxylase to pyruvate dehydrogenase. Pyruvate dehydrogenase inhibition permits pyruvate carboxylase activity and T-cell cytotoxicity by enhancing the TCA cycle and increasing succinate secretion, in turn leading to the potentiation of tumor therapy with immunotherapy [121]. Many studies report that lactate induces key tumor pathways by interfering with immune signaling that leads to IFN- γ secretion by cytotoxic T cells, activates the IL-23/IL-17 proinflammatory pathway, and promotes polarization of macrophages toward an M2-like phenotype [78]. In addition, LDH-A, glycolytic enzyme primes converting pyruvate to lactate, overexpression induces AKT phosphorylation and PI3K, which induces cyclin D1 and c-Myc expression in cancer cells [122, 123]. LDH upregulation is also correlated with epithelial-mesenchymal transition (EMT)-related genes, SNAIL and SLUG, and is thus involved in regulating the invasion and metastasis of cancer cells [77]. Therefore, LDH-A levels could predict patient prognosis and be used as a diagnostic biomarker for cancer [124].

In addition to lactate excretion, the increase of glucose metabolism in the cancer niche is accompanied by high H⁺ formation and excretion [83]. This, combined with poor perfusion, will further increase the acidity of the extracellular matrix in malignant tumors (pH 6.5–6.9) compared with normal surrounding tissues under physiologic conditions (pH 7.2–7.4) [125]. Acidosis of the tumor microenvironment is a typical criterion of a malignant phenotype, and many *in vivo* and *in vitro* studies showed that the low pH in the microenvironment of a tumor provides a niche with an engineering strategy that facilitates local invasion and subsequent

growth of the tumor [126]. The acid-mediated invasion has been considered a consequence of lactate build-up and the subsequent increased flow of H^+ ions along concentration gradients from the tumor into adjacent normal tissue. This phenomenon induces tissue remodeling at the tumor-stroma interface [126]. While the tumor's acidic environment is toxic to normal cells, it has a significant role in tumor formation and migration. Tumor microenvironment acidity promotes degradation of the ECM by proteinases, increasing angiogenesis through the release of VEGF, and suppresses the immune response to tumor antigens [89, 126]. Thus, suppressing the monocarboxylate family of transporters, which includes lactate transporters, is being considered as a potential therapeutic target for cancer treatment [52]. Figure. 3 summarizes the proposed functions of the Warburg Effect beyond enhancing aerobic glycolysis. Overall, cancer metabolism fuels and drives cancer development. The Warburg effect is the earliest metabolic feature that is found in tumors and contributes to other hallmarks of cancer. Thus, targeting Warburg metabolism mediators could be a promising strategy to innovative targeted cancer therapy.

2.2. Colon Cancer

A recent study estimated that more than 1.4 million people living in the United States have a history of CRC and 151,030 new cases will be diagnosed with the disease in 2022 [33]. In fact, CRC is one of the most common cancers worldwide and the second most common cause of cancer deaths in the United States, most likely due to the Westernized diet, which is generally defined by overeating, frequent snacking, and a prolonged postprandial state [2, 56, 127-129]. CRC is a heterogeneous disease that is associated with various etiologies and outcomes. Previously, CRC was common in older age, with an elevated incidence in patients over the age

of 50 [1, 2, 32]. However, recent epidemiological data shows rising trends in the incidence and mortality of early-onset CRC in those who are younger than 50 years old [2, 3]. Data shows that 73% of CRC survivors are aged 65 years or above, and 5% of them are younger than 50 years old [33]. Interestingly, The median age at diagnosis for colorectal cancer has rapidly shifted to younger than 65 years old for males and 68 years old for females since the early 2000s due to the rising incidence of CRC in younger adults (< 50 years), while numbers decreased in older age groups [33]. The reasons for increased CRC incidences in young adults remain unclear but may be related to changes in lifestyle, diet, physical inactivity, and the obesity epidemic [33].

Typically, colon tumors develop through multistep stages (ranging from 0 to IV) containing a series of histological, morphological, and genetic abnormalities that accumulate over time [130]. The earliest stage of colorectal cancer is called stage 0 when the cancer is in situ, carcinoma, or intramucosal carcinoma (Tis), which has not grown beyond the inner layer (mucosa) of the colon or rectum [131]. As a rule of thumb, the lower the stage number, the less likely the cancer has metastasized, and high patient prognosis. In contrast, highest stage (IV) means the cancer has metastasized to other areas of the body associated with worse patients prognosis. Typically, cancers with similar stages express a similar properties, and are often treated with similar therapies [131]. The staging system used for colorectal cancer is the American Joint Committee on Cancer (AJCC) TNM system, which is based on 3 classified categories of information. Firstly, the extent (size) of the tumor (T), which describes how far the cancer has grown into the wall of the colon or rectum. Secondly, the spread to nearby lymph nodes (N), including the degree of cancer spread. Lastly, the metastasis of the tumor to distant sites (M), which represents cancer metastatic activity whether it has spread to distant lymph nodes or organs such as the liver or lungs [129, 131]. Most importantly, the 5-year

survival rates for those with early-stage, localized disease (Stages I and II) can reach up to 90%, whereas the 5-year survival rate for those diagnosed with late-stage CRC, is 13.1%. Late-stage patients mostly receive palliative care during a time when treatment-related financial burdens are the greatest [130]. Similarly, the majority of CRC patients with stage I and II undergo a colectomy without chemotherapy (84%), while approximately two-thirds of patients with stage III colon cancer receive adjuvant chemotherapy to lower the risk of recurrence [33]. In addition, CRC survivors may suffer from neurotoxicity, bladder dysfunction, sexual dysfunction, infertility, and negative body image. Studies indicate that survivors of CRC have a higher prevalence of sexual dysfunction than survivors of other cancers, especially among patients with a permanent ostomy. Patients within their reproductive years who are treated with pelvic radiation could suffer from serious side effects and impairments in the ovaries or testes because of exposure [33, 132]. Given the rising burden of CRC in the adult and elderly populations, understanding the molecular and genetic features for CRC onset and progression is essential to help with early diagnosis and intervention that could increase patients' survival.

A new trend in CRC research has led to specific CRC classification to be based on bulk transcriptomics [2, 132]. Researchers were successful in subdividing CRC by comprehensive gene expression profiling, which resulted in the identification of four primary consensus molecular subtypes (CMS), and one unclassified subtype. Gene set enrichment analyses (GSEA) have segregated each of the four groups in the CMS classification into unique biological clusters [56]. CMS1 is characterized by high immune infiltration, microsatellite high (MSI), and an upregulation of immune checkpoints. CMS2 is recognized as the canonical subtype of CRC which is characterized as the classical MYC and WNT upregulation of downstream targets. Since CMS3 expresses widespread metabolic dysregulation at the transcriptome level, it is

defined as the metabolic subtype of the CMSs [2]. Lastly, CMS4 or mesenchymal subtype has been termed due to the upregulation of epithelial-mesenchymal transition (EMT) genes [2]. Patients diagnosed with early-stage CMS4 are highly susceptible to increased risk of distant relapse and death [2]. CMS4 is characterized by an upregulation of wound-like responses involving platelet activation and complement activation [5]. Interestingly, obesity has been classified as low-grade and unresolved inflammation that could trigger cancer, especially if it is associated with the high consumption of Western diet rich in hydrogenated and saturated fat [56]. A recent study showed that complement activation was due to an obesity-enriched gene set in CMS4 tumors and that the platelet marker *PPBP* appeared to be an obesity-linked hub gene in CMS4 tumors [2]. Although research has made many breakthroughs in CRC classification, diagnosis, and treatment over the past few decades, CRC-related mortality remains high [133].

1.2.1. Colon cancer risk factors

Despite massive improvements in screening tools for cancer, approximately half of CRC patients discovered their cancer at a later stage of the disease [134]. Hence, oncologists emphasize the importance of prevention and early detection strategies that can interrupt CRC progression, especially among populations who are at higher risk for CRC. Although anyone can develop CRC, several factors are associated with an increased risk for the disease, such as obesity, lifestyle, age, genetics, and environmental factors. These factors can be classified as non-modifiable and modifiable risk factors [135]. Medical history (sex, age, race, the history of adenomatous polyps, inflammatory bowel disease (IBD) history) and family history are referred to as non-modifiable risk factors that cannot be controlled by individuals. The modifiable factors are related to individual habits or lifestyles that can be altered to reduce the risk for CRC [136].

It is noteworthy that 50% of CRC cases are attributed to modifiable factors especially unbalanced diet. For instance, epidemiological data show that sedentary people who are highly consume red and processed meat and alcohol while their intake of vegetables is low are vulnerable to developing CRC [128]. A cohort study done on more than 4,000 patients indicated that frequent meat intake was linked to an elevated incidence of proximal colon cancer in males and rectal cancer in women [137]. Those consuming an average of 76 g/day of red and processed meat had a 20% higher risk of CRC than those who consume 21 g/day, according to a prospective study that was done in the UK [138]. Interestingly, consuming white meat such as fish and poultry is safe and not linked with CRC risk [139]. Also, a high-fiber diet, which is rich in vegetables, fruits, whole grains, and cereals is linked with a low incidence of CRC [139]. The potential underlying mechanisms between excessive intake of red and processed meat and CRC risk include carcinogenic substances produced during the cooking process and lead to increased exposure to N-nitroso compounds (NOCs), heterocyclic amines (HCAs), and polycyclic aromatic hydrocarbons (PAHs) [48]. Heme iron molecules in red meat can liberate carcinogens while acting as DNA mutagens themselves. Precautions against this include avoiding the exposure of meat surfaces to flames, wrapping meat with safe materials before oven roasting, and microwave cooking, which can all help to decrease the formation of some carcinogens such as HCAs [140]. Similarly, polyunsaturated fatty acids (PUFAs) such as non-human sialic acid, bile acids, and infectious pathogens are all potential mechanistic triggers of carcinogenesis [36, 39]. A diet rich in fiber is recommended to minimize the effects of carcinogenic influences. An unhealthy diet, smoking, obesity, and lack of physical activity are well-known preventable risk factors [2]. Both aging and genetic factors are non-modifiable factors that determine the risk of developing CRC. For example, the risk of developing CRC in patients with long-standing ulcerative colitis and

Crohn's disease increases with age [141]. A larger sect of the US adult population has a moderately elevated risk of CRC due to a family history of CRC, likely due to a combination of shared polygenic and environmental risks [141]. Hereditary pathological conditions such as familial adenomatous polyposis (FAP) and Lynch syndrome are associated with an extremely high lifetime risk of CRC while accounting for a minority of all CRCs [136, 141]. A family history of CRC in a first-degree relative (FHCRC), and a previous history of colon or rectum adenomatous polyps, increase the risk for colon cancer [18]. The lifetime risk of developing CRC is raised by 100% in individuals with FHCRC, and the magnitude of increasing risk is dependent on the number of relatives, the degree of relation, and the age of the relative at diagnosis [142] [143]. In addition, chronic inflammation plays a role in CRC pathogenesis [33]. A history of IBD, such as ulcerative colitis or Crohn's disease, is a predisposed factor for CRC [18]. A meta-analysis of 13 trials, including 45,000 patients with IBD, showed the risk of CRC to be approximately a triplicate greater in people with IBD (RR 2.93, 95% CI 1.79–4.81) compared to the control-people with no IBD [33]. Inflammatory disease creates chronic mucosal inflammation, increased cell turnover, and increased rates of sporadic mutations [36]. Systemic inflammation also has wide-ranging effects on CRC initiation and progression. Systemic inflammation can support primary tumor growth by many means, such as promoting tumor cell proliferation, enhancing angiogenesis through providing the availability of pro-angiogenic molecules, suppressing anti-tumor immunity by recruiting anti-inflammatory cell types, and creating pre-metastatic niches to facilitate subsequent metastasis [33]. Thus, systemic inflammatory biomarkers, such as acute phase proteins, cytokines, exosomes, and leukocytes, may help to diagnose and classify CRC patients into useful prognostic categories. However,

additional large-scale studies are needed to determine optimal marker combinations for selecting patients to receive specific treatments [128].

2.2.2. Obesity linked CRC

Considerable evidence has linked an obesogenic diet and excess body fat with cancer in general and CRC progression in particular [2, 56, 129, 135, 139, 144]. Previous epidemiologic studies have reported that 14% and 20% of all cancer mortality in men and women, respectively, are due to overweight and obesity, and there is a significant association between increased body mass index (BMI) and several major cancers, including CRC [135, 145, 146]. In terms of colon cancer, there is strong evidence supporting an association between body size and the risk of CRC, and it has been estimated that more than 10% of cancers of the colon can be attributed to excess weight [145]. Recent evidence indicates that obesity impacts the CRC tumor transcriptome in a CMS-specific manner [2]. Interestingly, body mass index (BMI, in kg/m^2) is positively associated with CRC risk in males, while females show weaker correlations. Also, abdominal obesity, which is assessed by waist circumference (WC, in cm), is closely correlated with CRC risk in both genders [129]. This disparity could be attributed to males and females having distinct body compositions, with fat constituting a higher percentage of body mass in women (30%) than in men (20%) [129]. A further explanation might be that abdominal obesity mediates metabolic abnormalities, leading to chronic diseases, including cancer [129]. It has been hypothesized that obesity, diabetes, insulin resistance, inflammation, and oxidative stress might elevate the risk of cancer [143]. However, the association between these risk factors and cancer has not been fully explored [143]. Adipose tissues have an ultimate role in insulin sensitivity especially abdominal adipose tissues that are composed of heterogeneous cell types

and function as an active endocrine and metabolic organ can have effects on the physiology of other organs [56]. In response to endocrine and metabolic signals from other organs, adipose tissue reacts by either elevating or decreasing the release of free fatty acids — an energy-providing fuel for skeletal muscle and other tissues. Adipocytes release peptide hormones such as adiponectin, leptin, resistin (in rodents), and tumor necrosis factor- α (TNF α) which are also important in the regulation of energy balance, lipid metabolism, and insulin sensitivity[147]. Obesity-associated imbalance energy intake is coupled with increased release of free fatty acids, resistin, and TNF α by adipose tissue and reduced release of adiponectin that triggers insulin resistance state, a metabolic state characterized by the reduced metabolic response of tissues, such as muscle, liver, adipose, to insulin and to compensatory hyperinsulinemia [139]. Previous reports have indicated the role of adipocyte secretions in increasing the proliferation of cancer cells in vitro: culturing colon cancer cell lines, such as HT-29, with adipocytes or in adipocyte-conditioned media stimulated cancer cell proliferation [56]. The pathophysiological abnormalities associated with obesity, such as an accumulation of visceral fat and hepatic dysfunction, lead to systemic insulin resistance and inflammation. Studies suggested the obesity-cancer link could be justified by these mechanisms involving hypercellular proliferation due to hyperinsulinemia, cell growth from growth hormones, and decline of apoptosis [2, 143]. Not only colon cancer but also several types of cancer, such as pancreatic cancer, endometrial cancer, and breast cancer could be affected by chronic hyperinsulinemia is associated with progression and metastasis [137, 142, 143, 145]. To explain, insulin invokes the production and activity of insulin-like growth factor 1 (IGF1), leading to enhanced cellular proliferation and decreased apoptosis in an over-nutrition state [137]. Although IGF1 is not relatively increased as BMI increases, researchers assume that free IGF1 can cause cell proliferation [139]. In an

experimental animal study, tumor growth was diminished when an IGF1 receptor was knocked out or when the IGF1 concentration was reduced [138]. Furthermore, hyperinsulinemia can affect the production of sex hormones such as androgen and estrogen, which could potentiate cancer growth [143]. Chronic inflammation and oxidative stress associated with obesity and insulin resistance can cause massive dysregulation of adipokines, pro-inflammatory cytokine, acute-phase protein synthesis, and dysbiosis (microbial imbalance) that lead to a systemic inflammatory microenvironment [2, 148]. Studies show insulin-resistant mediators and visceral adipose tissue participates in crosstalk with CRC and facilitates a favorable tumor microenvironment by secreting metabolites, growth factors, and proinflammatory cytokines such, as tumor necrosis factor α (TNF- α) and interleukin 6 (IL6) [2, 56, 136, 149]. Studies involving animal models showed obesity and IR induced pathophysiological state inflammation stimulates CRC tumor growth [2]. Elevated levels of pro-inflammatory cytokines associated with obesity induced IR are linked with an increased risk of CRC, specifically the circulating inflammatory signature (high miR-21, IL-6, and IL-8) that are clinical indicators for the prognosis and overall survival of patients with metastatic CRC [2]. Proinflammatory proteins, such as IL-6, are highly correlated with late tumor stages and poor prognosis in CRC patients. Additionally, the literature indicates IL6's role in stimulating the migration/motility of CRC cells by promoting epithelial-mesenchymal transition [149]. Interestingly, the use of anti-inflammatory drugs like aspirin reduces the incidence of cancers [150]. Overall, obesity-associated insulin resistance causes metabolic disturbances and creates low-grade chronic inflammation that could trigger cancer. The characteristic of a chronic low-grade inflammatory state associated with obesity has been indicated as a risk factor for tumor initiation and progression. In this regard, the current research has been focusing on the role of distinct pro-

inflammatory factors, chemokines, cytokines, immune cells, and other mediators in the promotion and progression of CRC [2, 128, 129, 135, 141].

2.3. Chemokines: old stars and new players in tumor microenvironment.

Recently, the role of chemokines in the TME has received great attention. Chemokines are a class of pro-inflammatory cytokines with small molecular weights (~8–14 kDa). These chemokines are secreted proteins, and as their name implies, are capable of chemotactic cell-directed movement. Chemokines usually have 60-100 amino acids at maturity and they mediate all immune cell migratory patterns ranging from necessary migration for immune cell development and homeostasis to what is required for the stimulation of primary and amnestic cellular and humoral immunity, to the pathologic recruitment of immune cells in inflammation and disease [1, [151]. In vivo, chemokines participate in innate and adaptive immunity (e.g. leukocyte migration), embryogenesis, angiogenic activity, tissue repair, and coordinate tumor microenvironment [18, 50, 152]. In addition, chemokines also mediate lymphoid organ development and T-cell differentiation and have recently been shown to participate in the nervous system as neuromodulators [32, 153]. Thus, abnormal chemokines' secretions are usually associated with an abnormality in the biological processes, leading to the development of relevant pathological conditions, such as inflammatory disorders, carcinomas, and metabolic diseases [154]. Consistent with their various functions, chemokines are produced from various tissues such as endothelial cells, immune cells (e.g. monocytes), platelets, inflammatory cells, fibroblasts, and cancer cells [155-157]. Chemokines can be classified into two broad categories based on their biological functions. Homeostatic chemokines are mostly involved in the basal leukocyte movement and normal immune surveillance, resulting in screening of any potential

pathogens at all times [151]. Inflammatory cytokines, on the other hand, find their functionality mostly in times of inflammation caused by many different pathological conditions [151].

In these conditions, the role of inflammatory chemokines is to actively recruit and direct immune cells at the site of inflammation. Another way to classify chemokines is based on the number and spacing of two conserved NH₂-terminal cysteine residues in the protein structure. To explain, chemokines can be divided into four distinct subfamilies: CXC (called α -chemokines), CC (called β -chemokines), C that have only two cysteines (called γ chemokines), and CX₃C which has three amino acids between the two cysteines (called d-chemokines) [34, 158]. The CXCL family is one of the main chemokine families that is composed of small molecular weight proteins secreted by cancer cells, leukocytes, fibroblasts, endothelial cells, and epithelial cells [154]. As a significant member of the chemokine family, the influence of CXCL family expression in chemokine-linked diseases cannot be ignored. The CXC chemokines subfamily is further classified according to the presence or absence of a three amino acid sequence, glutamic acid-leucine-arginine (Glu-Leu-Arg) (the “ELR” motif), immediately proximal to the CXC sequence. ELR+ CXC chemokines include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, and CXCL17 while ELR– CXC chemokines include CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CXCL16 [34, 154, 159-161]. While the ELR-positive CXC chemokines are involved in angiogenesis, the ELR-negative CXC chemokines are known as potent inhibitors of angiogenesis [21, 116]. CXCL chemokines are involved in tumor progression by activating various proliferative signal transductions such as MAPK, PI3K/AKT, STAT3, NF- κ B, Ras, TGF- β and β -catenin ERK (extracellular signal-regulated kinase)- and JNK (c-Jun N-terminal kinase) [154, 162]. Angiogenic CXCLs can specifically expand the growth of local tumor cells by modifying the tumor microenvironment to

support the introduction of cancer cells to the circulatory system through the invasion of the ECM and modulation of the vascular basement membrane leading to metastasis to distant organs [154, 163]. Convincing data reports that CXCL chemokines regulate these aspects of tumor hallmarks (i.e. angiogenesis, proliferation, and migration) in an autocrine manner or by facilitating crosstalk between tumor cells and their microenvironments leading to induce survival, cell cycle progression, and tumor growth [164]. All these chemokines' effects and functions can be mediated through activating G protein-coupled receptors (GPCRs) with seven transmembrane structural domains that are divided into four types (CXCR, CX3CR, XCR, and CCR) [18, 50, 152]. These receptors are selectively located on the surface of their target cells, and they are classified according to the ligands they bind, denoted by an R (representing receptor) and a number corresponding to the order of discovery. Among these, CXC chemokine receptors are the largest family and play vital roles in several physiological and pathological processes [152]. The CXCR family contains seven members: CXCR1–CXCR7. The ELR+ CXCLs are the ligands for CXCR1 and/or CXCR2, while the ELR- CXCLs are primarily the ligands for CXCR3, CXCR4, CXCR5, CXCR6 or CXCR7. In general, ELR+ CXCLs/CXCR1/2 signaling contributes to tumor progression, while ELR- CXCLs/CXCR3-7 signaling has anti-tumor effects [165]. As shown in Figure 5, CXC chemokine ligands are known as GRO- α (CXCL1), GRO- β (CXCL2), GRO- γ (CXCL3), ENA78 (CXCL5), GCP-2 (CXCL6), NAP-2 (CXCL7), IL-8 (CXCL8), MIG (CXCL9), IP-10 (CXCL10), I-TAC(CXCL11), SDF-1(CXCL12), BCA-1(CXCL13), and SR-PSOX(CXCL16) [152]. To date, up to 50 chemokines are produced from various cells in humans and mice, and more than 20 of their receptors have been recognized [166]. With twice the number of ligands as receptors, it is not surprising that most receptors activate multiple chemokines. Recent evidence indicated that some CXCR2

ligands exhibit organ-specific expression [167]. For example, in the spleen, only CXCL1, CXCL6, and *PPBP* are expressed, while in the appendix and gallbladder, all CXCR2 ligands show high expression levels, except for *PPBP* [167]. The urinary bladder displays high expression of all CXCR2 ligand genes, excluding CXCL2 and *PPBP*. In the liver, only CXCL2, CXCL3, and CXCL8 are expressed. The bone marrow tissues express all CXCR2 ligand genes, except for CXCL5 and CXCL6 [167]. CXCL5 demonstrates tissue-specific expression in the lymph node, while CXCL3 and CXCL5 exhibit specific expression in the stomach. CXCL8 shows specific expression in the esophagus, CXCL3 in the colon, and CXCL1 in the small intestine. The lung expresses CXCL2, CXCL3, and CXCL5 while bone marrow and spleen exclusively express *PPBP* [167]. Although monogamous pairs do exist, some chemokine ligands bind to more than one receptor. Initially, this could be a consequence of evolutionary pressure to organize the immune system against multiple pathogens and avoid immune resistance. However, research on chemokine signaling shows unique functions for each chemokine-receptor pair [168]. The literature describes the chemokine interaction network as selective promiscuous, neither fully specific nor fully promiscuous, where overlapping high-affinity chemokine-receptor pairs exist as a subset of all possible combinations [168]. ELR+CXCL specifically binds to their GPCR receptors CXCR-1 and CXCR-2, leading to the activation of different downstream signals related to proliferation and antiapoptotic [85-90]. These GPCR receptors are 77% similar in their amino acid sequence, but the differences at the end of the sequences result in differences in ligand binding [167, 169]. Interestingly, a single chemokine can interact with different chemokine receptors. Their interactions form a complex regulatory network that facilitates cell migration and angiogenesis and are closely related to the development of inflammation and tumorigenesis. The strongest agonist for CXCR2 is CXCL3,

whose half-maximal effective concentration (EC_{50}) is 1 nM, while the weakest agonist is CXCL5, whose EC_{50} is 10.6 nM [169, 170]. In contrast, chemokines CXCL1, CXCL2, CXCL6, CXCL7, and CXCL8 activate CXCR2 in the EC_{50} range of 3 nM–7 nM [56]. Another way for classifying CXC chemokines that are ligands for CXCR2 is by those that activate it at similar concentrations to CXCR1 activation (CXCL6 and CXCL8) and those at considerably higher concentrations (CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7) [169]. [171]. Certain chemokines share the same location and function but do not express similar structures or bind to the same receptor. For example, CXCL1, CXCL4, CXCL7 and CXCL8. These chemokines co-exist in platelet granules, and they release upon platelet activation orchestrating neutrophils to the injury site. While CXCL7, CXCL1, and CXCL8, characterized by the conserved N-terminal “ELR” motif, direct neutrophil trafficking by activating the CXCR2 receptor, CXCL4 is not a CXCR2 ligand as it lacks the ELR motif. However, CXCL4 also plays a key role in promoting neutrophil adhesion [4]. Evidence from functional studies has shown that chemokines work in “synergy,” so the presence of multiple chemokines results in improved or altered activity [4]. Synergy is thought to play a significant role at the onset of inflammatory signaling and has been attributed to altered receptor signaling and/or heterodimer formation [4]. As an example, the functional potential of chemokine synergy was demonstrated in an animal model using peptides that block the CXCL4/CCL5 heterodimer in hyperlipidemic mice with atherosclerosis [61]. Despite the ELR+CXCL/CXCR1/CXCR2 axis being a key component of tumor development, the significance of co-expression in the same cell of CXCR and CXCL is controversial since this autocrine pathway can drive both senescence and tumor development [172]. Figure 4 shows the classification of CXCR2 ligands based on their functions [173, 174]. To wrap up, chemokines are soluble proteins that orchestrate the migration of many cell types through interactions with

their G protein-coupled receptors (GPCRs). Studies have shown that CXC chemokines are dysregulated in multiple types of cancer and are closely correlated with tumor progression by activating their receptor (CXCR2).

2.3.1. Role of CXCLs-CXCR2 axis in inducing cancer

The chemokine receptor CXCR2 is predominantly expressed in various cell types, mainly neutrophils, and to a lesser extent in macrophages, eosinophils, oligodendrocytes, endothelial cells, neurons, neural crest-derived cells, and some tumor cells such as colon cancer cells [153, 175]. Since CXCR2 is expressed in various tissues, it has a significant role in mediating many biological processes from combating bacterial and viral infections, to have a role in development, blood vessel narrowing, leukocytes trafficking, diseases of many organs, including the heart, lung, liver, and brain, as well as tumor growth and metastasis [153, 176]. Recently, the impact of CXCR2 on metabolism and tumorigenesis has drawn researchers' attention. Many studies block the CXCLs-CXCR2 axis or perform tissue-specific knockout (KO) of *Cxcr2*, to understand the role of CXCR2 in shaping metabolism [153]. Initial reports have indicated that *Cxcr2*^{-/-} mice are protected against obesity-induced insulin resistance, and blocking the CXCL5-CXCR2 axis with neutralizing antibody or antagonist enhanced insulin sensitivity in mice models of insulin resistance [177]. Another *vivo* study shows CXCR2 KO female mice have thinner skin and less subcutaneous adipose layer compared to WT mice [178]. The authors justified their observations by attributing them to the reduced expression of adipogenesis-related genes associated with adipocyte-specific CXCR2 signaling in *Cxcr2*^{-/-} mice [178]. Adipocytes themselves express CXCR2, and the expression level is higher than in pre-adipocytes [153, 178]. It has been observed that upregulation and a promoting effect for CXCL3 during adipocyte

differentiation through activating ERK (extracellular signal-regulated kinase)- and JNK (c-Jun N-terminal kinase)-mediated activation of C/EBP β and δ [153]. Since CXCR2 is important to recruit and activate neutrophils, many studies discussed using CXCR2 blockade as a possible treatment of several neutrophil-driven pathologies such as obesity-driven insulin resistance [179]. Researchers use selective CXCR2 antagonists to permit exploration of the effect of neutrophil-selective impairment independently of macrophages on insulin sensitivity and liver pathology under high-fat diet conditions [162]. They found that used selective CXCR2 antagonist (AZD5069) in high-fat fed mice enhanced insulin-induced suppression of hepatic glucose synthesis, decreased hepatic lipid storage, and prevented progression towards liver pathology reminiscent of non-alcoholic fatty acid disease (NAFLD) [162]. In the context of tumor biology, CXCR2 blockades reduce neutrophil infiltration in tumors and inhibit tumor growth [180]. Deficiency or inhibition of CXCR2 reduces inflammation-driven tumorigenesis and spontaneous intestinal adenocarcinoma in mouse models of intestinal tumorigenesis [181]. Furthermore, CXCR2 transcript levels were correlated with a decline in disease-free and overall survival in patients with metastatic colon cancer [182]. Hence, the literature discussed the potential implication of CXCR2-dependent signaling pathways in tumor relapse. CXCLs-CXCR2 axis can contribute to tumor progression by activating multiple proliferative and survival signaling pathways including phosphatidylinositol-3 kinase (PI3K)/Akt pathway, the phospholipase C (PLC)/protein kinase C (PKC) pathway, and the Ras/Raf/extracellular signal-related kinases (ERK1/2) pathway, and each of these pathways exerts a distinct biological impact on tumor progression [33, 153, 171, 183]. For instance, overexpression of CXCL3 in cervical cancer induced cancer proliferation through activating the ERK/proliferation signal cascade associated with enhancing the BCL2/Bax ratio [184]. Furthermore, certain chemokines, such as

CXCL5, exert anti-apoptotic effects while they activate the PI3K/AKT/mTOR pathway that is chemotherapy-resistant in gastric cancer cells [185]. Proliferative signaling PI3K/AKT is also the major downstream signal cascade for CXCR2/CXCL8, which plays a critical role in mediating cancer cell survival, angiogenesis, and migration [186, 187]. Xu et al. (2018) showed that the phosphorylated AKT and cyclooxygenase-2 (COX2) might be two major mediators in the CXCR2 signaling to promote breast progression, metastasis, anti-apoptosis, and anti-senescence [188]. Generally, CXCR2 can mediate angiogenesis and migration through Akt phosphorylation, and its activation is one of the most frequent changes in malignant tumors [174]. Figure 6 visualizes all signal transductions that are mediated by CXCR2-ligand interactions [189]. Recent research scrutinizes the expression of the CXCR2 ligands CXCL1, CXCL2, CXCL3, CXCL5/CXCL6, CXCL7, and CXCL8 in 590 gastric tumors. They found that the expression on cancer cells was frequent for CXCL1 (46.2%), followed by CXCL7 (36.4%), CXCL2 (20.7%), and CXCL3 (17.1%) [27]. Detailed information about the classification, signaling pathways, and physiological and pathological effects of the CXCLs-CXCRs axis are summarized in Figure 7 [165]. Hence, the literature indicates that targeting CXCRs may have immense potential for cancer therapy. Knockout CXCR2 decreased the recruitment of immune cells to tumor nest and extended the survival in a mouse model of pancreatic ductal adenocarcinoma mice through reduced cell invasion/migration and a shift of immune-inflammatory microenvironment [190]. In a murine model of lung cancer, using anti-CXCR2 antibodies dramatically suppressed lung metastases. In addition to neutralizing antibodies, multiple antagonists of CXCR2, such as SB225002, AZD5069, and danirixin were observed to suppress tumor growth [165]. Although further investigations are necessary to elucidate the detailed mechanisms of the CXCLs–CXCR2

axis dependency of tumor progression, inhibiting this axis could be a promising therapeutic strategy for cancer by blocking the crosstalk between tumor and stromal cells.

2.3.2. Chemokine 7

Chemokine ligand 7 (CXCL7) is one member of the ELR⁺ CXCL family of chemokines that promotes tumor growth and invasion due to their angiogenic potency through the G-protein-coupled receptors CXCR1 and CXCR2 [23]. CXCL7 is a cleavage product of the platelet α -granule component platelet basic protein (PBP) and connective tissue-activating peptide III (CTAP-III) [191]. It is also known as neutrophil-activating peptide-2 (NAP-2). After platelet activation, the expression of CXCL7 is significantly upregulated [176]. CXCL7 is translated as a propeptide, then cleaved into many smaller isomers that are each reported to have unique functions. The longest form, called PPBP (pro-platelet basic protein) or LDGF (leukocyte-derived growth factor), is reported to be a fibroblast mitogen [192]. The shortest form called NAP-2 (neutrophil-activating peptide-2), which is produced by megakaryocytes and stored in platelets, is a potent chemotactic agent for neutrophils. Interestingly, NAP2 has been reported to inhibit megakaryocytopoiesis in an autocrine fashion to inhibit thrombopoiesis and therefore may contribute to a negative feedback mechanism for regulating platelet counts [172]. In the past, *PPBP* gene expression was thought to be restricted to cells within the megakaryocytic lineage. However, recent consecutive studies have identified other cell types that may produce the protein product (chemokine), specifically stromal-stimulated monocytes and lymphocytes [32, 157, 172, 191]. CXCL7 has been reported to participate in different cellular processes such as glycolysis, DNA synthesis, mitosis, intracellular cAMP accumulation, prostaglandin E2 secretion, and the synthesis of hyaluronic acid and plasminogen activator [30, 193]. In addition,

it is an antimicrobial protein with bactericidal and antifungal properties [193]. Other less-defined functions include heparinase activity of PBP and CTAP-III, which is important for the modulation of the matrix proteins. Thus, pro platelet protein is a protein derived from a single gene but exerts various biological activities depending on its cleavage. This appears to be directly or indirectly dependent on signals from other cells [172]. Meanwhile, several clinical studies have identified CXCL7 as a novel biomarker for early diagnosis of various cancers including colon cancer, lung cancer, and renal cell carcinoma [134, 194]. Subsequently, CXCL7 can help in the prediction of treatment efficacy. Since CXCL7 belongs to the subfamily of ELR⁺ CXC chemokines, it is considered a potent regulator of angiogenesis [191]. Multiple functions and modes of action have been advocated for CXCL7 and its receptor CXCR2 in the progression of primary and secondary tumors. NAP-2 can play its physiological role by binding CXCR2 and activation of several proliferative and anti-apoptotic signaling cascades such as Ras/Raf/mitogen-activated protein kinase (MAPK) and PI3K/AKT/mTOR signaling pathways [32, 66, 195, 196].

CTAPIII/CXCL7 may play a role in the progression, diagnosis, and prognosis of many malignancies, including pancreatic disease, breast cancer, clear cell renal cell carcinoma (ccRCC), lung cancer, gastric cancer, and colon cancer [30, 182, 197]. In breast cancer, MCF10AT cells showed metastatic behavior after they were transfected with CXCL7 due to induced expression of lymph angiogenesis factors VEGF-C/D and heparinase activity [26]. Another study showed that co-culturing breast cancer cells with human monocyte THP-1 cells induced secretion of CXCL7 from monocytes, which in turn acted on cancer cells to promote FAK activation, MMP13 expression, migration, and invasion [157]. Interestingly, in this study, authors treated a xenograft mouse model with CXCL7 neutralizing antibodies, and they observed

markedly declined in the tumor size, the abundance of M2 macrophages, and the metastatic behavior of the tumor [157, 179]. A similar finding was observed in a study including tumors established from Lewis lung carcinoma (LLC) cells. The LLC cells were overexpressing CXCL7, and showed increased infiltration of M2 macrophages at the early stages of tumorigenesis [32]. These CXCL7-overexpressing LLC tumors also grew faster than control tumors, suggesting that CXCL7 traffics macrophages, especially at the tumor site, and may accelerate lung tumor development in the early stages [32].

CXCL7 has been reported to enhance breast cancer progression through its receptor CXCR2, and many studies showed increased expression of CXCR2 in human breast cancer samples [26, 157, 171]. CXCL7 can also accelerate ccRCC development through binding CXCR1/CXCR2 and activating survival signal transduction/ ERK pathway [195]. In terms of CRC, several studies reported that CXCR2 and its ligand CXCL7 also are highly expressed in CRC [30, 182]. Furthermore, CXCL7 expression is correlated with the strongest driver of angiogenesis (VEGF), clinicopathological features in CRC patients, poor prognosis, with shorter free and overall survival [30, 196]. Thus, serum CXCL7 has been identified as a potential diagnostic and prognostic marker for CRC, especially in OCRC patients [196]. Desurmont *et al.* reported that overexpression of CXCL7 and CXCR2 in liver metastases from colon cancer was associated with shorter disease-free and overall survival [182]. CXCL7 may also play a promoting role in the malignant progression of cholangiocarcinoma. Literature shows CXCL7 expressed in cholangiocarcinoma tissues compared to adjacent non-tumor tissues. Moreover, increased expression of CXCL7 was significantly associated with poor differentiation, vascular invasion, lymph node metastasis, advanced clinical stage, as well as shorter overall survival time [193]. Furthermore, the expression of CXCL7 was significantly associated with the protein

expression of proliferative marker Ki67 [193]. Transfection of SiRNA-induced inhibition of CXCL7 significantly reduced the proliferation and invasion of QBC939 cells while overexpression of CXCL7 markedly promoted these malignant phenotypes of QBC939 cells [193]. Increased proliferation rate in QBC939 overexpressing CXCL7 cells was associated with increasing AKT activity, suggesting the proliferative role of CXCL7 [193]. In the early stage of lung cancer, CXCL7 can be used as a plasma diagnostic biomarker with high sensitivity and specificity [181, 198]. It is reported that the elevation can be detected 29 months before the diagnosis of lung cancer [198]. Furthermore, Higher gene expression of CXCL7 and CXCL8 has been associated with worse overall survival in patients with lung adenocarcinoma [181]. Studies report relapsed GC patients had higher CXCL7 levels in tumor drainage blood and peripheral blood, and increases in CXCL7 expression were correlated with lymph node metastasis [27]. In contrast, there is a significant decrease in plasma CXCL7 levels in patients with pancreatic cancer [199]. However, the functional mechanism of CXCL7 chemokine is not well established, which is crucial for understanding its role in tumorigenesis and designing new therapeutic approaches to control tumor growth and metastasis. Hence, developing a strategy to block the CXCL7-CXCR2 axis may be effective and will allow for effective targeted therapy for different malignancies.

Figure 1.

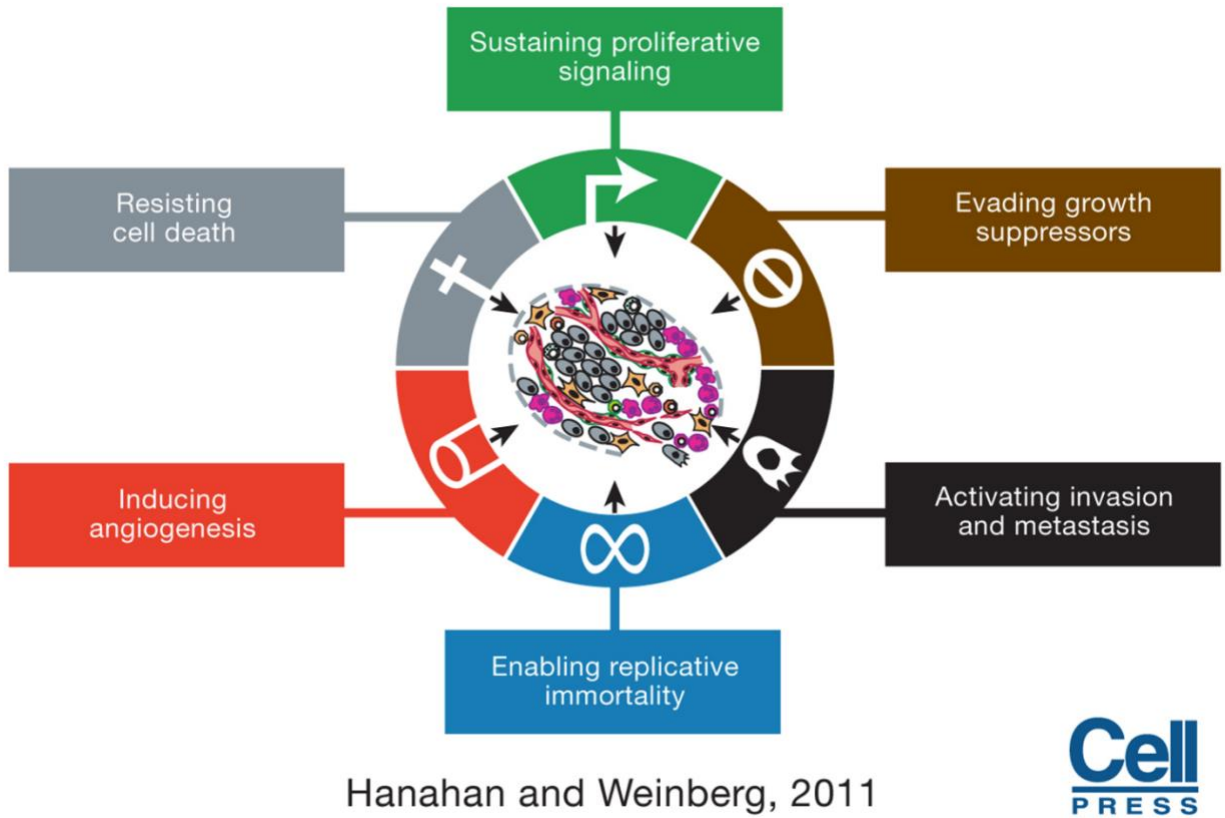


Figure 1. The Hallmarks of Carcinogenesis. Retrieved from: Hanahan & Weinberg, 2011[49].

Figure 2.

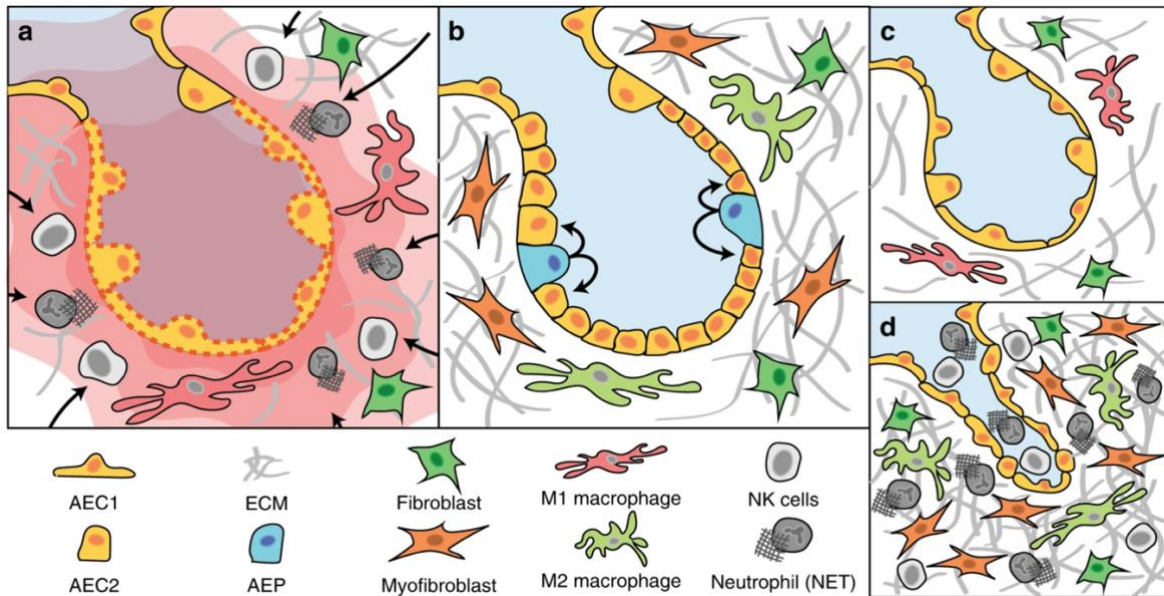


Figure. 2: Stages for wound healing. The wound healing process comprises three stages: inflammation, regeneration, and remodeling. During inflammation (**a**), damage to epithelial cells (AEC1/AEC2) releases inflammatory signals (red waves). Regeneration (**b**) of the wound begins when immune cells such as macrophages transition from a pro-inflammatory (M1) to an anti-inflammatory (M2) phenotype. This triggers the release of growth factors that engage the stem/progenitor functions of epithelial cells to replace cells. Concurrently, the surrounding extracellular matrix (ECM) is restored by macrophages, activated fibroblasts, and myofibroblasts. **C** Remodeling completes the wound-healing pathway by removing excess ECM and cells. When remodeling fails (**d**), unchecked proliferation of immune cells, collagen, and myofibroblasts causes them to persist in the wound microenvironment, leading to scarring and fibrosis. Retrieved from Delley et al., 2021[45].

Figure 3.

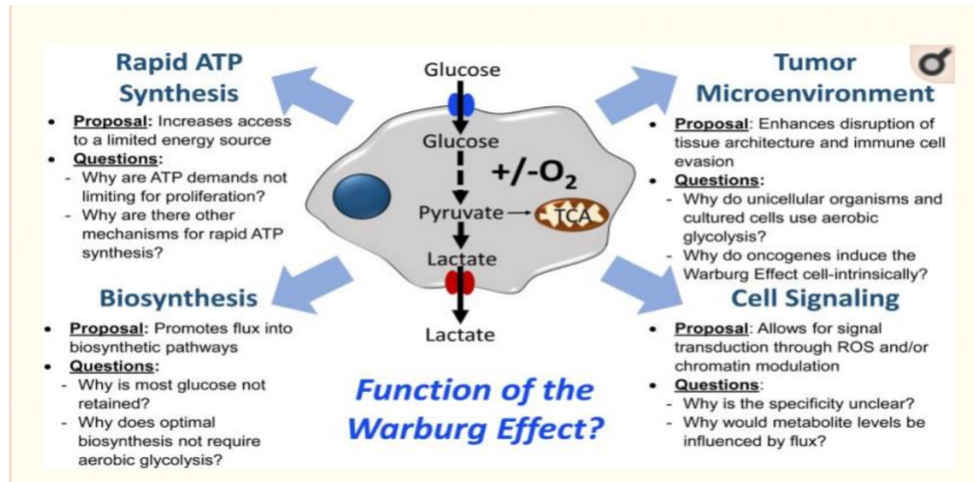


Figure 3. Summary of the proposed functions of the Warburg Effect.

Retrieved from; Liberti & Locasale (2016)[83].

Figure 4.

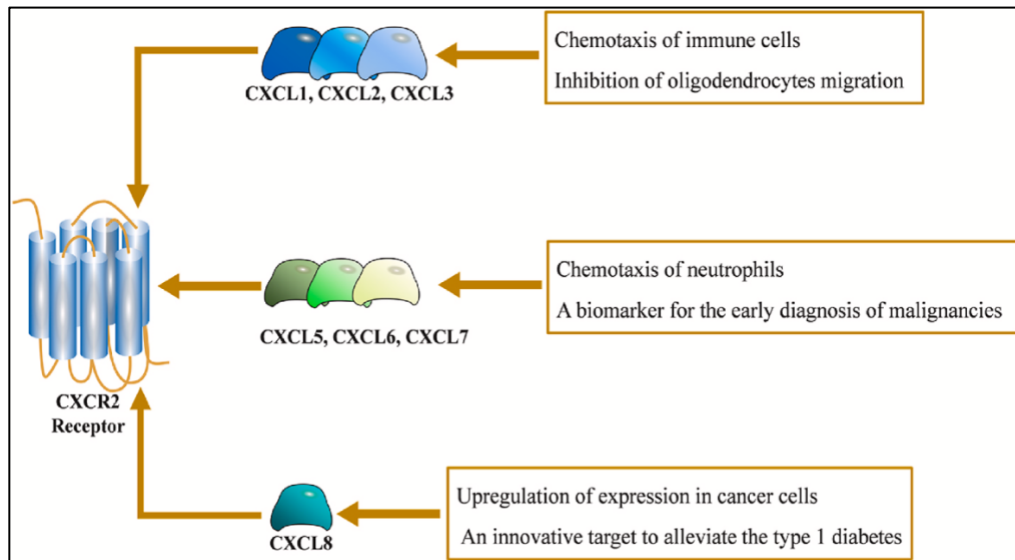


Figure 4. Overview of ELR⁺ CXCL chemokines ligands for CXCR2 and their regulatory functions. Retrieved from Xie et al., 2023[189].

Figure 5.

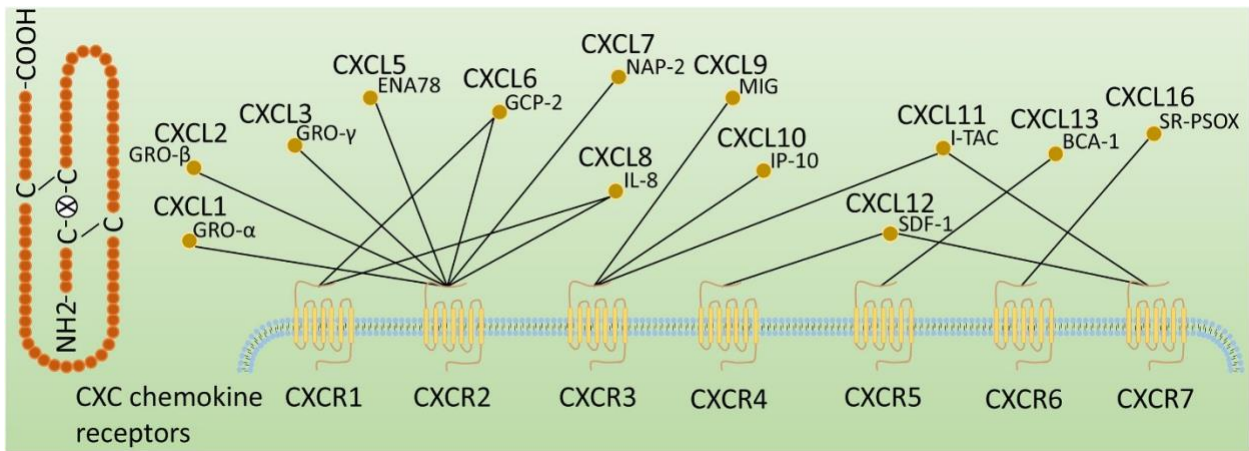


Figure 5. Classification of CXC chemokines based on their receptors. Retrieved from Wang et al., 2021[152].

Figure 6.

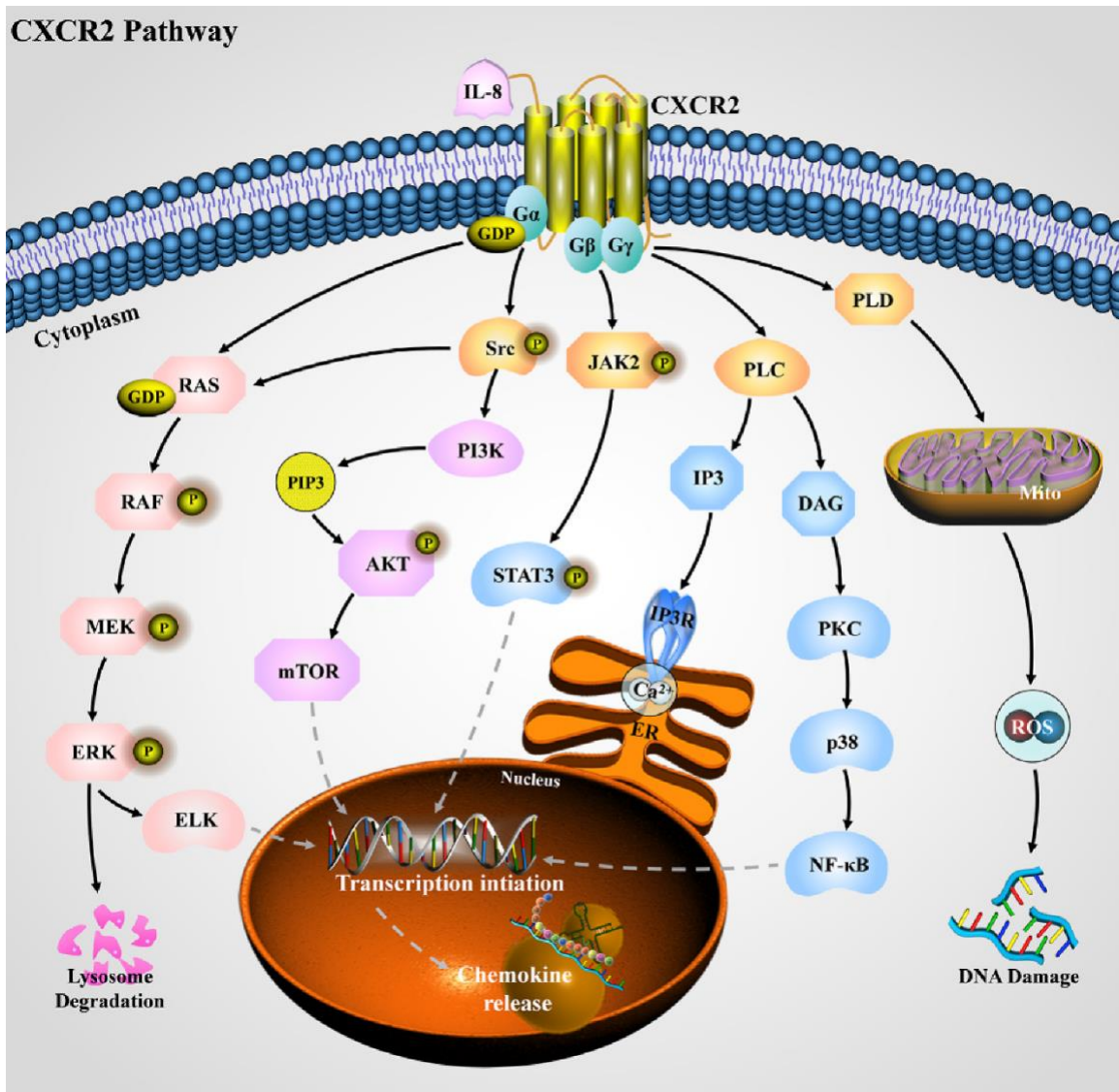


Figure 6. Signaling pathways of CXCLs/CXCR2 axis. Retrieved from Xie et al., 2023[189].

Figure 7.

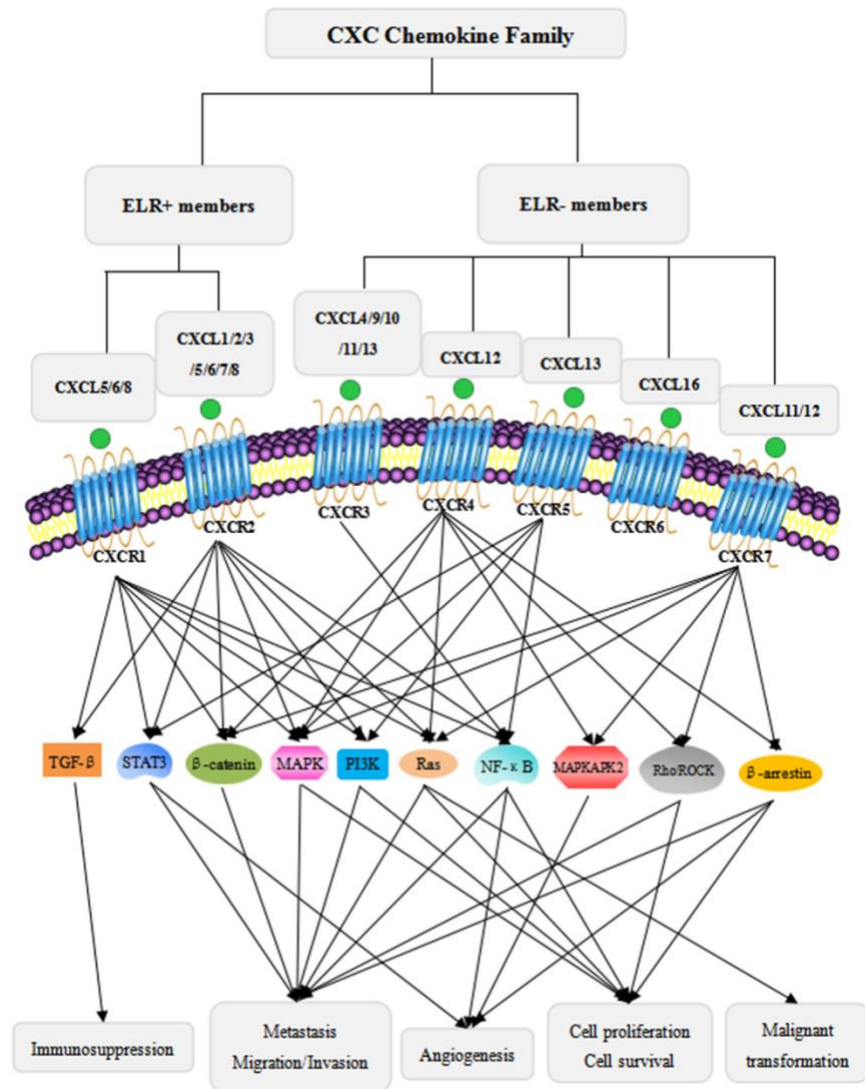


Figure 7. CXC chemokine family and their physiological affects. Retrieved from Wu, T., et al. 2022 [165].

Chapter 3: Role of CXCL7 in Colon Cancer Proliferation

3.1 Abstract

Chemokine (C-X-C motif) ligand 7 (CXCL7) – a protein product of the *pro-platelet basic protein (PPBP)* gene – is a potential biomarker for colorectal cancer (CRC) diagnosis. However, role of CXCL7 in CRC progression is not known yet. Hence, we examined the impact of CXCL7 in colon cancer cell proliferation and aerobic glycolysis. We first examined the effect of CXCL7 on cellular proliferation and glycolytic flux using human colon cancer (HT-29) cells transfected with CXCL7 or an empty vector as a control. Stable CXCL7- and vector-expressing cells were selected using hygromycin B. In addition, we treated HT-29 cells with CXCL7 obtained from the conditioned media (CM) from CXCL7-transfected HEK-293T cells or CM from vector-transfected (control) cells. CXCL7 expression was confirmed by analyzing *PPBP* expression using RT-qPCR, and the CXCL7 level in the conditioned media was quantified by ELISA. The specificity of CXCL7 actions was examined using a CXCL7 neutralizing antibody and an antagonist (SB225002) to C-X-C motif chemokine receptor 2 (CXCR2). Cancer cell proliferation was assessed by measuring DNA synthesis (EdU incorporation) in the colon cancer cells. Glycolytic flux was monitored by measuring glucose uptake using a fluorescent glucose analog 2-deoxyglucose (2-NBDG), Seahorse glycolysis stress test analysis, measuring lactate accumulation in the cultured cell's media, and using Western blotting for analyzing glycolytic enzyme protein expression. To further investigate CXCL7-mediated proliferation, we examined EdU incorporation and colony size growth in 3D engineered tissues and tumor xenograft growth

in nude mice injected with CXCL7- and vector-expressing HT-29 cells. The proliferation rate in CXCL7-expressing HT-29 cells and HT-29 cells treated with the CXCL7-CM were significantly higher (1.2-fold and 1.8-fold, respectively) compared to controls ($p < 0.01$). Consistently, the proliferation rate in CXCL7-expressing HT-29 3D-engineered tissues was significantly enhanced compared to vector-expressing tissues ($p < 0.05$). Importantly, blocking the CXCL7/CXCR2 axis using a CXCL7 neutralizing antibody and a CXCR2 inhibitor abrogated CXCL7-stimulated proliferation. Lactate levels were two-fold higher in the culture media from both CXCL7-expressing HT-29 cells and HT-29 cells treated with CXCL7-CM compared to the controls ($p < 0.05$). Glucose uptake was also greater in CXCL7-overexpressing cells and CXCL7-CM treated cells compared to vector controls. Data from the Seahorse assay and Western blots confirmed that CXCL7-expressing HT-29 cells and HT-29 cells treated with CXCL7-CM have higher glycolytic capacity and increased glycolytic enzymes' protein expression compared to the controls. *In vivo*, CXCL7-expressing HT-29 xenograft tumor weight and PCNA protein levels were 100% and 60% greater, respectively, than vector-expressing tumors ($p < 0.05$). Our study showed that, for the first time, CXCL7 stimulates colon cancer cell proliferation and enhances aerobic glycolysis.

3.2 Introduction

Colorectal cancer (CRC) is the third most common cause of cancer in the world and the second most cancer-related cause of mortality in the US [200, 201]. Previously, the risk of developing CRC was linked with increasing age, especially after 50 years. Now the incidence trend has been rising among the younger population, specifically those less than 55 years old [202]. In fact, the proportion of cases among those younger than 55 years increased from 11% in

1995 to 20% in 2019 [200]. CRC patients have a favorable clinical prognosis if the disease is diagnosed in an early stage with a 5-year survival rate reaching 90%, but this ratio dramatically drops to <20% if the disease is diagnosed in late stage after metastasis, even with surgery and comprehensive postoperative treatment [203]. Therefore, revealing the underlying mechanism of CRC development and progression may improve the development of effective therapeutic approaches.

Recently, the landscape of tumor microenvironment (TME), especially tumor-infiltrating immune cells and chemokines, has been recognized as a key factor in affecting cancer progression[157]. Chemokines are small, secreted proteins that have low molecular weight ranging from 7 to 15kDa and are best known for their roles in trafficking immune cells and lymphoid tissue development via activating their receptors [204, 205]. Chemokines are the largest subfamily of cytokines and can be classified into four main classes based on the location of the first two cysteines residues in their protein sequence: the CC-chemokines, the CXC-chemokines, C-chemokines and CX₃C-chemokines [20, 22, 157, 205-207]. CXC chemokines are further divided into ELR⁺ CXC and ELR⁻ CXC, according to the presence of the amino-terminal ELR motif [157]. In the TME, chemokines can be secreted by various immune cells and cancer cells, and in response to specific chemokines, many immune cells move into the TME and mediate tumor immune responses in a spatiotemporal fashion [22]. In addition, chemokines can directly perform their functions in various cells in the TME, including cancer cells and vascular endothelial by mediating cancer cell stemness, proliferation, invasion, and metastasis [22, 160, 208].

Cancer cells uncouple glycolysis and oxidative phosphorylation (OXPHOS), a phenomenon known as the Warburg Effect after the German Scientist Otto Warburg, who first

described it [8]. This metabolic reprogramming is one of the ten hallmarks of cancer and has been linked with tumor progression [209]. Many studies have indicated that chemokines have a role in tumor glucose metabolism and function by mediating various aspects of proliferation, metastasis, and other cancer properties [210]. Therefore, chemokines, directly and indirectly, influence tumor progression by shaping cancer metabolism, TME biological phenotypes, and targeting TME components should be considered for future improvement of CRC therapy.

Chemokine (C–X–C motif) ligand 7 (CXCL7; also known as NAP-2) is a member of the CXC chemokine family and is known as a crucial player in neutrophil recruitment upon vascular injury by binding to the CXCR2 receptor[157]. In cancer cells, the CXCL7-CXCR2 axis exerts the promotion of cancer development and invasion on different types of cancer [134, 157, 193], including lung cancer, breast cancer, and renal cell carcinoma[157, 211]. Noteworthy, knocked down of CXCL7 decreased proliferation in non-cancer cells and cancer cells, except CRC [28]. Furthermore, CXCL7 has also been also reported to have a role in enhancing the expression of glucose transporters, GLUT1, and subsequently glucose uptake in connective tissue cells [212]. However, a limited number of studies were done to explore the role of CXCL7 in colon cancer cells. In addition, the few studies that were performed to analyze CXCL7 expression in CRC patients have been limited to the Asian population. Nevertheless, the underlying mechanism for CXCL7 in colon cancer proliferation remains unclear. Hereby, we performed this study to examine the role and mechanism of the CXCL7-CXCR2 axis in colon cancer cells proliferation.

3.3 Materials and Method

3.3.1. 2D Cell culture

Colon cancer cell lines: HT-29, CL40, and SW480 and HEK-293T were cultured in Dulbecco's Modified Eagle's Medium Hi Glucose DMEM (GIBCO®, Life Technologies, Carlsbad, CA) medium containing 10% Fetal Bovine Serum (FBS) (catalog no. S11550, R&D Systems, Atlanta, GA) and 1% Penicillin/Streptomycin (P/S). For lactate assay only we used Dialyzed FBS one shot (catalog no. A3382001, Thermo Fisher Scientific) to supplement the cell culture media. CL-40 cells were purchased from Leibniz-Institut DSMZ-Deutsche, Germany. The rest of the cell lines were purchased from the American Type Culture Collection (ATCC, USA). Cell lines were tested for pathogens, including Mycoplasma. All cells were cultured at 37 °C and 5% CO₂ with humidity.

3.3.2. Transfection of plasmid

HT-29 cells and HEK-293T were transfected with the Human CXCL7/PPBP Expression-Ready ORF Clone (pCMV3-Untagged) plasmid with the hygromycin resistance gene (Catalog no. LS-N18861-1, LSBio, Seattle, WA, USA) or control empty vector, with only hygromycin resistance gene (Catalog no. SC1822, GenScript, Piscataway, NJ, USA) by using Lipofectamine™ 3000 Transfection Reagent (catalog no. L3000001, Invitrogen, San Diego, CA). Briefly, HT-29 cells and HEK-293T cells were split in a 6-well plate and they were 80% to 90% confluent on the day of transfection. Stable CXCL7 expressing HT-29 cells and HEK-293T cells were selected by using 500 µg/ml and 200 µg/ml Hygromycin B, respectively, 24 hours after transfection. Transfection efficiency, as confirmed by green fluorescence protein expression was >70% at 48 hours. The CXCL7 expression was confirmed by Q-PCR using *PPBP* primers, measuring secreted CXCL7 in culture media by ELISA, and Western blotting using anti-human CXCL7 antibodies. After confirming the *PPBP* expression, the Hygromycin B dose was reduced

to 200 µg/ml and 100 µg/ml for HT-29 and 293T transfected cells, respectively, to maintain pressure on selected cells while continuing culturing of transfected cells.

3.3.3. Drugs and Reagents

CXCR2 inhibitors that were used; SB 225002 (Catalog no. 559405, Millipore sigma) and AZD-5069 (Catalog no. ab285434, abcam). Recombinant human CXCL7/NAP2 (Catalog no. 393-NP), monoclonal mouse IgG isotype was used as control (Catalog no. MAB002) for human CXCL7/NAP2 antibody treatment (Catalog no. MAB393), and all were purchased from R&D Systems (Minneapolis, MN, USA) and diluted in phosphate-buffered saline (PBS; GIBCO). Other reagents for specific assay will be explained later.

3.3.4. L-lactate assessments.

Lactate analysis was performed on lactate secreted in the culture media using the Lactate-Glo, a luminescence-based assay (catalog no. J502, Promega). Briefly, cells were seeded at 50K density per well in a 24-well plate in hygromycin-free experimental media. We used DMEM with glucose [2.5 g/L], fortified with 3% dialyzed FBS (catalog no. A3382001, Thermo Fisher Scientific, Waltham, MA) to prepare experimental media for culturing HT-29 transfected cells while Wildtype HT-29 cells treated with conditioned media (CM) were growing in serum-free media. For HT-29 transfected cells, samples from culture media were collected after 24 hours and diluted at (5:100) with PBS. For HT-29 cells that were treated with CM, samples from culture media were collected after 8 hours of incubation and diluted at 1:100 in PBS. Lactate assay was performed in 96-well plate following the kit's protocol and luminescence values were recorded by using a SpectraMax M2 plate reader.

3.3.5. Cell proliferation assay

Proliferation was quantified by measuring the incorporation of fluorescence-conjugated EdU [10 μ M] (Click-iT EdU, catalog no.C10499, Invitrogen) in cells that were seeded at 7K and allowed to adhere and incubated with EdU for 24 hours at 37°C before performing the assay. We prepared experimental media, DMEM with glucose [2.5 g/L], fortified with 3% FBS for culturing HT-29 transfected cells, while HT-29 cells treated with CM were cultured in experimental serum-free media. Post incubation, EdU was directly detected using AmplexTM Ultra Red, a fluorescent reagent, and the fluorescence values were recorded by using a SpectrMax M2 plate reader.

3.3.6. Glucose uptake assay

Glucose uptake was assessed by administering fluorescence d-glucose analog 2NBDG in cells that were seeded at 5K density and allowed to adhere for 24 hours in 96-well at 37°C before performing the assays. We prepared experimental media, DMEM with glucose [2.5 g/L], fortified with 3% FBS for culturing HT-29 transfected cells, while HT-29 cells treated with CM were cultured in experimental serum-free media. After incubation, all wells were washed twice with PBS and incubated with a fluorescent derivative of 2-deoxyglucose (2-NBDG)/glucose analog (Catalog no. N13195, Invitrogen) [200 μ M] for 30 min at 37°C in a humidified atmosphere of 5% CO₂ prior to measure the fluorescent signal intensity by microplate reader. Nonspecific uptake was determined in the presence of 10 μ M cytochalasin B and was subtracted from all values. The reaction was stopped by adding a twofold volume of ice-cold PBS, and the wells were washed again with ice- cold PBS three times. The fluorescent signal was measured

using the fluorometric spectrophotometer, and nonspecific uptake was determined in the presence of 10 μ M cytochalasin B (catalog no. C6762, Millipore Sigma, WI), and they used as background and subtracted from all values.

3.3.7. Seahorse assay

Glycolysis stress assay was performed using the Seahorse XFp analyzer (Seahorse Bioscience, Agilent, batch# 0006742900) according to the manufacturer's instructions to analyze real-time changes in ECAR (extracellular acidification rate) and OCR (oxygen consumption rate) in cells culture media. Briefly, 5k cells/well were seeded into an 8-well XF cell culture microplate in experiment growth medium 24 h pre-assay. The experimental media was DMEM with glucose [2.5 g/L] fortified with 3% FBS for HT-29 transfected cells, while HT-29 cells treated with CM were cultured in experimental serum-free media. After incubation, the cells were starved in glucose-free media with [2mM] glutamine and incubated in a non-CO₂ incubator for 1 h at 37 °C prior to assay. The extracellular acidification rate (ECAR) was quantified using an XFp analyzer in XF base medium (pH = 7.4) containing [1 mM] glutamine following sequential additions of glucose [10 mM], oligomycin [1.5 μ M] and glucose analogue, 2-Deoxy- d-glucose (2-DG) [50 mM]. Data were analyzed by the Seahorse XF Glycolysis Stress Test Report Generator package.

3.3.8. RNA Extraction and Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR)

RNeasy plus mini kit (Qiagen, Louisville, KY) was used to isolate total RNA from adherent cells following the manufacturer's protocol. RNA quantity and quality were assessed

using a NanoDrop (Thermo Scientific). Reverse transcription was performed on mRNA extracted from the cells by using a Superscript IV Reverse Transcription Kit (Invitrogen, Catalog# 18091050). qPCR was performed using the RT² SYBR Green qPCR Mastermix (Qiagen) and gene-specific primers (IDT) using a MyIQ Real-Time PCR system (Bio-Rad, Hercules, CA). After amplification, melt curve analyses were performed on each reaction to confirm specificity. *gapdh* and *B2M* were used as reference genes to normalize gene expression. The $2^{-\Delta\Delta CT}$ method was used to analyze the qPCR data and measure relative expression.

3.3.9 Enzyme-linked immunosorbent assay

CXCL7 protein concentration in conditioned media (CM) was measured by enzyme-linked immunosorbent assay (ELISA) using human CXCL7/nap 2 DuoSet ELISA Development System (catalog no. DY393; R&D Systems, Minneapolis, Minnesota). The optical density of each well was determined using a plate reader, SpectrMax M2. The reading at 570 nm was subtracted from the reading at 450 nm.

3.3.10. Samples extraction, Western blotting, and antibodies

Adhered cells in 10 cm plate was placed on ice and washed two times with chilled PBS. Then 400 μ l of RIPA (1X) buffer (contained also 2 μ g/mL leupeptin, aprotinin and protease inhibitor cocktail) was added to cells. By using scraper, the cells were extracted, and the cell lysate was collected in microcentrifuge tube on ice and stored in -80°C. After one day, the samples were thawed on ice and centrifuged for 20 min at 20,000 g at 4°C to collect the supernatants. For tumor samples preparation, the tumors were ground with liquid nitrogen, and 60 mg of grinding tumor powder was mixed with 400 μ l of RIPA (1X) buffer and homogenized

by using a Potter-Elvehjem homogenizer followed by centrifugation for 20 min at 20,000 g at 4°C. Protein concentrations of the supernatants from cells and tumors samples were determined by the Pierce BCA Protein Assay Reagent Kit (Thermoscientific, catalog no. 23228). We prepared the desired total amount of protein to be loaded in equal total volumes for each sample, and the sample volumes were adjusted using LDS sample buffer (1X) (mixed with 0.1M DTT) then the samples were boiled in a hot block (95°C for 9 minutes). A total concentration of 40 µg of protein was separated on 10-20% polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were blocked in 5% non-fat milk TBS-T solution at room temperature for one hour and then incubated with the following primary antibodies in 1:1000 ratio at 4°C overnight: Hexokinase I (C35C4) Rabbit mAb, Hexokinase II (C64G5) Rabbit mAb, LDHA (C4B5) Rabbit mAb, PKM2 (D78A4) XP® Rabbit mAb, Pyruvate Dehydrogenase (C54G1) Rabbit mAb, and PKM1/2 (C103A3) Rabbit mAb detects endogenous levels of total PKM (including M1 and M2) protein. (catalog no.8337T, cell signaling), PCNA (Catalog no.13110S, cell signaling), Caspase-3 (catalog no.14220, cell signaling), and in 1:500 ratio at 4°C overnight for CXCL7 (catalog no.500-P03, PeroTech). Equal loading of the protein was verified with β-Actin (catalog no.4967, cell signaling). Immunoblots were then incubated with goat anti-rabbit IgG secondary antibody (IRDye® 680RD Goat anti-Rabbit IgG Secondary Antibody, catalog no. 926-68071, LI-COR) for one hour at room temperature before imaging with Odyssey fluorescence imager (LI-Cor, Lincoln, NE, USA).

3.3.11. Tumor xenograft formation and size evaluation

HT-29 cells transfected with either CXCL7 or control/vector were growing in monolayers using aseptic techniques in the laboratory, harvested by trypsin digestion, washed 3x in sterile

phosphate buffered saline and resuspended in sterile serum-free RPMI-1640 medium. Eight nude male mice (*AthymicNude-Foxn1tm* mice, Jackson Laboratory) were injected subcutaneously on the flank immediately caudal to the axilla with 5×10^6 cells of HT-29 transfected cell resuspended in sterile serum-free RPMI-1640 medium mixed 1:1 with sterile low growth factor Matrigel matrix (Catalog no.356230, Corning). Body weights were collected a minimum of once weekly. Using a digital caliper, tumor size (length x width) was measured at least weekly, and the volume was calculated using the following formula: $V = (L \times W^2)/2$. Tumors were harvested after 28 days, and all animal procedures were IACUC-approved.

3.3.12. Materials and Methodology for 3D experiments

All chemicals were obtained from Sigma-Aldrich, St. Louis, MO, unless mentioned otherwise.

3.3.12.1. In vitro cell culture and maintenance

HT-29 human colorectal cancer cells transfected with CXCL7, or vector. HT-29 cells with these genes were cultured separately in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO® , Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1% (v/v) non-essential amino acids (NEAA) (Lonza, Walkersville, MD), 1% (v/v) penicillin/streptomycin (GIBCO®) and 1% (v/v) Glutamax (GIBCO®). HT29 cells were maintained in McCoy's 5a Modified media (VWR, Radnor, PA) with 10% FBS and 1% (v/v) penicillin/streptomycin.

3.3.12.2. PEG-fibrinogen (PF) synthesis from PEGDA and characterization

Poly (ethylene glycol) diacrylate (PEGDA) was synthesized by reacting poly (ethylene glycol) (PEG) with acryloyl chloride in anhydrous dichloromethane with triethylamine. Bovine fibrinogen was then covalently coupled to PEGDA by dissolving fibrinogen in urea-PBS buffer, adding Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl), adjusting the pH, and slowly adding the clear PEGDA solution. After allowing the reaction to proceed, the solution was diluted, precipitated in acetone, and dialyzed against sterile PBS. The final product, PF, was stored at -80°C . The protein concentration and net solid weight of the dry product were determined using a Pierce™ BCA Protein Assay Kit. This PEG Fibrinogen was used to make tissues both for CXCL7 and vector transfected cells.

3.3.12.3. Fabrication of Hydrogel Precursor solution and cell encapsulation

To prepare hydrogel molds, Poly (dimethyl siloxane) (PDMS) sheets of $600\ \mu\text{m}$ thickness were fabricated between two glass slides with spacers, then punched to obtain circular molds using a biopsy punch (diameter: $4\ \text{mm}$). These PDMS molds were securely attached to the bottom of a 6-well plate to prevent leakage of the hydrogel precursor. The hydrogel precursor was formulated by mixing PF solution in PBS with 1.5% (v/v) triethanolamine (TEOA), $37\ \text{mM}$ 1-vinyl-2-pyrrolidinone (NVP), and $0.1\ \text{mM}$ Eosin Y in PBS as a photoinitiator. A $10\ \mu\text{L}$ volume of precursor was pipetted into each PDMS mold and crosslinked with visible light exposure (Light intensity: $203\ \text{mW}/\text{cm}^2$) for 2 minutes. After crosslinking, the PDMS mold was removed, leaving behind disc-shaped photo-crosslinked hydrogels. These hydrogels were allowed to swell in PBS overnight before further analysis. PDMS sheets of $600\ \mu\text{m}$ thickness were punched to create circular molds and attached to a 6-well plate. A hydrogel precursor, composed of PF solution, TEOA, NVP, and Eosin Y, was pipetted into the molds and crosslinked with visible

light for 2 minutes. After removing the PDMS mold, disc-shaped hydrogels were obtained and allowed to swell in PBS overnight for analysis. For cell encapsulation within the PF-based hydrogels, HT-29 cell lines were trypsinized, counted, and resuspended in the polymer precursor at 20×10^6 cells/ml before photo-crosslinking. HT-29 cells were transfected with CXCL7 and an empty vector for control. The effect of CXCL7 on proliferation, viability, cell colony, and tissue size were examined. Cancer cell proliferation was assessed by measuring EdU incorporation in DNA after 8 days of encapsulation, and viability was observed on days 1, 8, and 15. Cells were cultured inside the 3D tissue for 30 days. Additionally, both HT-29 overexpress CXCL7 transfected, and HT-29 vector transfected cells were treated with modified culture media of 6% FBS, and differences between the two types of tissue were observed over 30 days.

3.3.12.4. Morphological analysis

Quantification of morphological features indicative of cancer cell growth and spreading was performed via phase contrast microscopy and image analysis over a 30-day period in culture. Following encapsulation of cancer cells, tumor constructs maintained in 3D culture were observed using a Nikon Ti inverted microscope equipped with an Andor Luca S camera at intervals of 7 days. Z-stacks with a thickness of 200 μm and individual slices spaced 5 μm apart were acquired and analyzed using ImageJ software. Parameters such as colony area, diameter, circularity, aspect ratio, as well as cellular area, diameter, circularity, aspect ratio, and elongation length were determined. Morphological quantification was carried out by sequentially focusing through individual planes from the top to the bottom of the z-stacks. Morphological features were quantified only at planes where cell or colony edges and boundaries were sharpest and most distinct, disregarding out-of-focus regions. For both HT29 CXCL7 and vector transfected cells,

only colonies fully encapsulated within the hydrogel constructs were considered, excluding those extending beyond the hydrogel edge. Similarly, for vector transfected cells, only fully encapsulated colonies were used for morphological analysis. A minimum of 50 colonies or cells from three independent tumor constructs were analyzed for each time point and experimental condition.

3.3.12.5. Viability assay

The viability assessment of cells within the 3D tissues was conducted using fluorescence microscopy and image analysis. Tumor constructs were cultured over a 15-day period, with images captured on days 1, 8, and 15. Following a PBS wash, the tissues were incubated with Live/Dead cell viability stain (Invitrogen, Carlsbad, CA) for 30 minutes. After the removal of excess stain with PBS, z-stack images of the samples were obtained using an inverted Nikon Ti microscope. The acquired z-stacks, with a thickness of 200 μm and individual slices spaced 5 μm apart, were analyzed using ImageJ software. Cell counting was performed manually in each slice of the z-stack. A minimum of 5 z-stack images from three independent tumor constructs were quantified for each time point and experimental condition.

3.3.13. Statistical analysis

All values were reported as the mean \pm standard error of the mean (SEM). Analysis of variance (ANOVA) combined with Tukey's test was used to analyze multiple comparisons, and a Student's t-test was performed to test differences between two groups with significance determined at $p < 0.05$ (GraphPad Prism San Diego, CA).

3.4. Results

3.4.1. *PPBP* expression is elevated in colon adenocarcinoma and its expression positively correlates with cell proliferation and glycolysis gene sets.

To confirm prior findings that *PPBP* expression is elevated in colon adenocarcinomas[210], the TCGA COAD cohort was queried using UCSC Xena platform. As shown in Figure 8A, *PPBP* expression is significantly elevated ($p < 0.001$) in the primary tumor compared to normal colon tissue. Consistent with this finding, colon adenocarcinoma *PPBP* expression significantly positively correlated (FDR Q value < 0.001) with genes enriched in the MSigDB Grade_Colon_Rectal_Cancer_Up gene set (Fig. 8B). We next examined whether colon adenocarcinoma *PPBP* expression positively correlated with genes enriched in the MSigDB cell proliferation-related and glycolysis-related gene sets. As shown in Figure 8 C and D, a significant positive correlation with genes enriched in the MSigDB GOBP_Cell_Growth (FDR Q value < 0.030) and GOBP_Endothelial_Cell_Proliferation (FDR Q value < 0.001) gene sets. We also observed a significant positive correlation with genes enriched in the MSigDB Hallmark Glycoysis (FDR Q value < 0.034) but not the KEGG Glycolysis_and_Gluconeogenesis (FDR Q value = 0.576) gene sets (Fig. 8 E and F). Analysis of Glycolytic enzyme genes contributing to enrichment in both the Hallmark Glycoysis and KEGG Glycolysis_and_Gluconeogenesis revealed that six genes in the glycolytic pathway are enriched (Fig. 14). Taken together, these findings suggest that *PPBP* expression is elevated in colon adenocarcinoma and that it may play a role in cancer cell proliferation and aerobic glycolysis.

3.4.2 Overexpression *CXCL7* promotes colon cancer cell proliferation while blocking the *CXCL7-CXCR2* axis abrogates the cell growth *in vitro*.

To experimentally examine the proliferative effect of CXCL7, we generated a CRC cell line overexpressing CXCL7. We selected the cell line by assessing the mRNA expression of CXCL7 receptor (CXCR2) in several common CRC cell lines including HT-29, CL40, SW480, and HCT116 as indicated in Supplementary Figure 1. The gene expression of CXCR2 was positively expressed in these CRC cell lines. However, we used HT-29 cells in the following experiments since it is most frequent cell lines that are used in colon cancer studies. HT-29 was transfected with CXCL7 ORF plasmid or blank vector, (HT-29-CXCL7) and (HT-29-Vector) respectively. We validated *PPBP* expression and CXCL7 secretion in HT-29 transfected cells by RT-qPCR and ELISA. First, we found that HT-29-CXCL7 cells markedly express mRNA of *PPBP* compared to the HT29-vector cells ($p < .001$, Suppl. Fig. 1B and C). ELISA data further supported that the CXCL7 levels are 70-fold higher in HT29-CXCL7 when we measured the secreted CXCL7 in the CM [approximately 860 ng/mL/48 hours] than those in control group (HT29-Vector) ($p < 0.01$, Suppl. Fig. 1D). Next, we examined the impact of stably overexpress CXCL7 (HT29-CXCL7) on colon cancer cell proliferative phenotype compared to vector-transfected cells (HT29-Vector) by using a sensitive fluorescence assay that directly detected the newly synthesized DNA in the actively dividing cells. Briefly, the cells were seeded at 7k cells density/ well in 96-well plate with experimental media that mixed with EDU [10 μ M] for 24 hours before performing the proliferation assay. The result showed a 1.2-fold increase in HT29-CXCL7 proliferation rate compared to HT29-Vector ($p < 0.01$, Fig.9A). This result provided a rationale for evaluating the specificity of CXCL7 signaling in inducing the cells' proliferation by using a neutralizing antibody. Blocking CXCL7 by neutralizing antibodies (Ab) [20 μ g/ml] decreased the proliferation level of HT29-CXCL7 to the level comparable to the control (HT29-Vector) treated with IgG or Ab ($p < 0.01$, Fig. 9A). The functionality and the role of CXCL7

receptor (CXCR2) expressed by HT-29 cells on proliferation was determined through administering a competitive inhibitor for CXCR2 (SB225002), which has been used in research involving of inflammatory pathologies and tumor progression [176]. Treating HT29 overexpressing CXCL7 cells with CXCR2 antagonist for 24 hours decreased HT29-CXCL7 proliferation rate in dose-response fashion, and 50% inhibitive concentrations of SB225002 on HT29-CXCL7 cells was observed with SB dose [56.8 μ M] ($p < 0.0001$, Fig. 9B). Similar results were found when we used other CXCR2 antagonists (AZD-5069 and GSK1325756) to treat HT-29-CXCL7 cells. As indicated in Fig. S15E, both CXCR2 inhibitors AZD and GSK significantly decreased proliferation levels for HT29-CXCL7 ($p < 0.0001$). We further examine the effect of SB225002 on CXCL7 naturally producing CRC cell line, CL-40 that shows highest expression of *PPBP* when we analyzed among vast number of CRC cell lines (Suppl. Fig. 1H). We treated CL40 with CXCR2 blocker (SB225002) following a similar previous method and dose for 24h pre-proliferation assay. As expected, blocking CXCL7 receptor by CXCR2 antagonist markedly decreased CL40 cells proliferation, which provides further evidence for the proliferative impact of CXCL7 in colon cancer cells ($p < 0.01$, Fig. 9F).

To further test our hypothesis, we examined the paracrine effect of CXCL7 on colon cancer cell proliferation by treating wild-type HT-29 cells with conditioned media (CM) derived from HEK-293T cells that were transfected with CXCL7 ORF plasmid or blank vector, (CM-CXCL7) and (CM-Vector) respectively. Transfection efficiency was validated by measuring the level of relative mRNA *PPBP* expression via q-PCR and analyzing secreted CXCL7 in the culture media of transfected cells ($p < 0.01$, Suppl. Fig. 1F and G, respectively). As shown in Figure 9 C, treating HT-29 cells with CM from 293T cells overexpressed CXCL7 (CXCL7-CM) increased the proliferation rate in a dose-dependent manner with respect to the vector. However,

blocking the CXCL7/CXCR2 axis by using the CXCL7 neutralizing antibody [20 µg/ml] and CXCR2 inhibitor [56.8 µM] abrogated CXCL7 stimulated proliferation ($p<0.01$, Fig.9 D-E). We further assessed the proliferative effects with using the CM from transfected 293T on other colon cancer cell lines that highly express CXCR2 (SW480) with equivalent results ($p<0.01$, Suppl. Fig. 2 A-B). These findings confirmed that the CXCL7-CXR2 axis may play a significant role in colon cancer cell proliferation in autocrine and paracrine loops.

3.4.3. CXCL7 induces lactate secretion in colon cancer cells in *vitro*.

Our previous observation prompted us to examine if the increased in the proliferation in the colon cancer would result from inducing in aerobic glycolysis. Previously we noticed that enrichment of lactate dehydrogenase gene in colon cancer patients (Fig. 14), so we examined the impact of overexpress CXCL7 in modifying the lactate secretion in *vitro*. Lactate is the final metabolite of aerobic glycolysis that is produced from glucose fermentation, which may therefore be used as an index of glycolysis activity. Literature pointed out that lactate secreted levels is correlated well with cytokine production specifically CXCL7 level [213]. First, we measured the secreted lactate in culture media of HT-29 transfected cells (5000 cells/well in 48-wellplate) by using the Lactate-Glo, a luminescence-based assay (Promega) after 24 hours of incubation. Results showed that the lactate levels were two-fold higher in culture media of CXCL7-expressing cells (HT29-CXCL7) compared to vector transfected cells (HT29-Vector) ($p<0.05$, Fig.10A). However, introducing CXCL7 neutralizing antibody decreased the concentration of secreted lactate in HT29-CXCL7 culture media to the level comparable to HT29-Vector ($p<0.001$, Fig. 10A). Similarly, HT29 cells that treated with CXCL7-CM secreted higher level of lactate compared to the control; HT-29 treated with Vector-CM after 8 hours of

incubation ($p < 0.05$, Fig. 10B), but treating cells with CXCL7 neutralizing antibody abrogated the impact of CXCL7 in inducing lactate secretion from HT-29 cells treated with CXCL7-CM ($p < 0.05$, Fig. 10B). Moreover, blocking the CXCL7/CXCR2 axis by using the CXCR2 antagonist (SB225002) dramatically changed the concentration of lactate in culture media for both HT29-CXCL7 cells and HT-29 treated with CM-CXCL7 to level comparable to HT29-vector and HT-29 cells treated with CM-Vector ($p < .001$, Fig. 10C and D). Hence, we analyzed the protein expression for lactate dehydrogenase (LDH), the enzyme that responsible for converting pyruvate to lactate in aerobic glycolysis, in HT29-Vector treated with DMSO (Vector-DM) or CXCR2 inhibitor (SB225002) (Vector-SB) and HT29-CXCL7 treated with DMSO (CXCL7-DM) or CXCR2 inhibitor SB225002 (CXCL7-SB). Results from Western blotting showed that LDH protein expression was upregulated in HT-29 cells overexpress CXCL7 (CXCL7-DM) compared to the control (Vector-DM) but administering CXCR2 antagonist (SB) downregulated LDH to the level comparable to the control (Vector-DM) ($p < .01$, Fig. 10E). This supports our assumption for the stimulatory impact of CXCL7 on LDH activity leading to increase the lactate secretion and aerobic glycolysis consequently in colon cancer cells. In conclusion, CXCL7 can enhance colon cancer cells glycolytic activity as evidenced by increasing the lactate secretion and the activity of LDH in HT29- CXCL7 and HT-29 cells that are treated with conditioned media from 293T cells overexpress of CXCL7 compared to control. This could explain the ability of CXCL7 to drive growth in colon cancer cells.

3.4.4 CXCL7 stimulates glycolytic flux and capacity in colon cancer cells.

To support our finding regarding role of CXCL7 in inducing proliferation and lactate secretion, we further investigated the effect of CXCL7 expression on CRC glycolytic function.

First, we measured the glucose uptake directly by incubating colon cancer cells with a fluorescent derivative of 2-deoxyglucose (2-NBDG)/glucose analog. As shown in Fig. 11A, HT29-CXCL7 cells treated with vehicle (IgG) showed significantly greater uptake for glucose analog (2-NBDG) compared to HT29-CXCL7 cells treated with CXCL7 neutralize antibody [20ug/ml] and vector transfected cells ($p<0.01$). Similarly, introducing CXCR2 blockers (SB225002) [56.8uM] dramatically inhibited the uptake of 2-NBDG in both HT29-CXCL7 and HT29-vector transfected cells compared to HT29-CXCL7 cells that were treated with vehicle (DMSO) ($p<0.01$) (Fig.11B). Nevertheless, the level of glucose uptake for HT29-CXCL7 cells was also higher than HT29-vector cells when both treated with vehicle as shown in Fig.11B. Hence, we examined if there is difference on the activity for significant glycolytic enzymes between HT29-vector and HT29-CXCL7 that could explain the difference in glucose uptake between the two groups. As expected, Western blotting results revealed the protein expression for hexokinase 2 (Hexo2) and pyruvate dehydrogenase (PDH) were markedly upregulated in HT29-CXCL7 treated with vehicle (DM) compared to HT29-Vector treated with (DM) or when both HT29-CXCL7 and HT29-Vector were treated with CXCR2 antagonist, ($P<0.05$) (Fig.11C-D). Next, to confirm our observations, we repeated the glucose uptake assay in HT-29 cells treated with CM. Results showed that treating HT-29 cells with CM from 293T transfected with CXCL7 (CXCL7-CM) enhanced glucose uptake more than two fold when compared to control, while co-treatment with CXCL7 neutralizing antibody markedly reversed the induction of glycolytic activity resulted from CXCL7 in HT-29 cells (Fig. 11E). In addition, blocking CXCR2 with (SB) antagonist significantly decrease the glycolytic activity for HT-29 cells treated with CXCL7-CM cells to the level comparable to HT29 cells treated with vector-CM ($p<0.001$). However, glucose uptake activity for HT-29 cells treated with CXCL7-CM and SB inhibitor

remained similar to HT-29 cells treated with Vector-CM and SB or vehicle ($p>0.05$) (Fig. 11F). Measuring the protein expression for key glycolytic enzymes showed that HT-29 cells treated with CXCL7-CM have higher protein expression for PKM1/2 and hexo2 compared to HT-29 cells treated with Vector-CM. However, again blocking CXCL7 receptor with SB downregulate the protein expression for these glycolytic enzymes in HT-29 cells despite treating them with CXCL7-CM ($p<0.05$, Fig. 11G-H). Hence, we performed glycolysis stress test as an advanced tool that can critically monitor the glycolytic activity for the cancer cells. As we expected, HT29-CXCL7 that incubated in experimental media for 24 h prior to exposure to 10 mM glucose, 1 μ M oligomycin and 50 mM 2-DG at various time points, as demonstrated in Fig. 11I exhibited higher glycolytic function and unique metabolic phenotype, compared to the control (HT29-Vector). Moreover, using CXCL7-CM to treat HT-29 cells for 24 h prior to perform the glycolysis stress test significantly induced the glycolytic function comparing to incubate HT-29 cells with Vector-CM (Fig. 11J). Similar results were obtained when treated another colon cancer cell line (SW480) with CXCL7-CM, and the cells showed enhanced in their glycolytic capacity compared to control (Suppl. Fig. 2 C-D). Apparently, CXCL7 can exert differences in metabolic phenotype and fuel utilization capability in colon cancer cells that may influence tumor proliferation and progression. Altogether, the results suggest that CXCL7-CXCR2 axis plays a vital role in colon cancer cells proliferation through enhancing the glycolysis efficiency and capacity.

3.4.5 CXCL7 role in 3D engineered model.

To assess the impact CXCL7 on cancer cells, we imaged PF-based tumor constructs via phase-contrast microscopy and analyzed different morphological features like cancer cell

proliferation and viability. Fig. 12A shows the individual tissues we made with CXCL7 and vector chemokine, which grows over 29 days. CXCL7 and vector transfected cells encapsulated within 3D constructs appeared as individual cells on day 0, which form distinct colonies through the whole duration of culture. Fig. 12B represents the proliferation of cells inside the 3d construct, which we studied with EdU incorporation assay on day 8 of cell encapsulation. It shows proliferation for CXCL7 transfected cells are higher than that of the vector (*p < 0.05). From Fig. 12C, individual colonies created inside of the CXCL7 transfected tissue are significantly increases over the time till day 15 for the 3D hydrogel construct (*p < 0.05) and Fig. 12D represents the progression for three different batches of data where no significant difference was observed in colony area or diameter among the vector transfected hydrogel groups, suggesting that vector cells may be less responsive to changes in matrix characteristics compared to CXCL7 cells. Notably, vector cells formed smaller colonies in the tumor constructs compared to CXCL7 cells for all the hydrogel compositions. Notably, standard deviations of measured parameters were considerably higher for CXCL7 cells compared to the vectors.

In evaluating the viability of cancer cells encapsulated in 3D culture, we did Live/Dead staining on the tumor constructs and assessed cell viability using fluorescence imaging. Initially, on day 1 post-encapsulation (day 0), all cell lines exhibited high viability (>90%), regardless of cell type. There was no apparent effect on viability across any cell line, and viable cells appeared evenly distributed within the tumor constructs, with no spatial variations. From Fig. 12E, by the final time point on day 15 of culture, some instances showed a decrease in cell viability compared to day 1 values for both cell types. Additionally, Fig. 12F shows the graphical representation of the viability difference on day 15 (*p < 0.05). Notably, the viability of CXCL7 transfected tissues exceeded that of vector-transfected tissues. Initially, on day 1, the viability

difference between CXCL7 (90%) and the vector (85%) was modest. However, by day 8, the viability of CXCL7-transfected tissues increased to 93%, while vector-transfected tissues lagged significantly at 75%. This trend persisted through day 15, where the viability of vector-transfected tissues further declined to 53%, while CXCL7 maintained robust viability at 92%.

3.4.6 Overexpression of CXCL7 accelerates the growth of HT-29 xenograft tumors *in vivo*

We examined CXCL7-mediated tumor growth *in vivo* conditions in nude mice implanted with HT-29 colon cancer cells transfected with an empty vector or CXCL7. While in the vector group the tumors were detected on day 14 post-injection, CXCL7 tumors appeared as early as day 8 after injection as shown in the schematic diagram for the *in vivo* study's method (Fig. 13A). At day 30 post-injection tumor growth reached excessively large (tumor >17 mm x 17 mm) for some animals (mainly in CXCL7 group), so the mice were euthanized and sacrificed, and the tumors were collected for further analysis. Results indicated that CXCL7 tumors average weight was more than two-fold higher than vector tumors average weight (Fig. 13B, $p < 0.0001$). Increased proliferating capabilities for CXCL7 tumors were confirmed by analyzing the protein expression of the proliferative cell nuclear antigen (PCNA) in vector and CXCL7 tumors that PCNA was significantly upregulated in CXCL7 tumors with respect to control ($p < 0.05$) (Fig. 13C). In contrast, when we analyzed the protein expression for the apoptotic marker Caspase3, we found Caspase3 downregulated in CXCL7 tumors compared to control ($*p < 0.05$) (Fig. 13D). Thus, we concluded that CXCL7 enhances tumor growth *in vivo* possibly through enhancing the cancer cell proliferation and survival.

3.5. Discussion, conclusion, and limitations

Colorectal cancer is the second-leading cause of cancer-related mortality in the United States [131]. Thus, gaining a better understanding of the CRC and its molecular mechanism can help to develop targeted therapy for cancer patients and increase their prognosis [131].

Chemokines play a pivotal role in the development of the tumor microenvironment. Consecutive studies have indicated that high expression of ELR⁺CXC chemokines *in vivo* and *in vitro* is correlated with a patient's poor prognosis [116, 214, 215]. Recent studies have pointed out the importance of neutrophil-activating peptide-2/CXCL7 in driving cancer progression and suggest using CXCL7 level as a diagnostic biomarker [4, 25, 30, 216-218]. However, few studies have examined the impact of CXCL7 on CRC, and most of these studies lack of showing the specific mechanism for CXCL7 in driving CRC progression. This is the first study that analyzes the effects and mechanism of CXCL7 in colon cancer cell proliferation.

Altered expression of CXCL7 has been observed in several human cancers, generally playing an oncogenic role [30, 193, 211]. In the present study, consistent with the literature, we noticed that *PPBP*, gene encodes CXCL7, is highly expressed in colon adenocarcinomas human samples compared to normal colon tissues [30, 210]. Furthermore, we find novel evidence shows *PPBP* expression is significantly and positively correlated with gene enrichment sets for cell growth, proliferation, and glycolysis based on RNA sequencing data generated by using the colon adenocarcinoma TCGA cohort.

In fact, our *in vitro* data indicates that the CXCL7/CXCR2 axis contributes to colon cancer progression, consistent with previous studies that were done on the role of CXCL7 in various types of cancers [25, 157, 175, 193]. Most importantly, we show, for the first time, that CXCL7 exerts proliferative effects on colon cancer cells through inducing aerobic glycolysis, as evidenced by increasing glucose uptake, glycolytic capacity, and lactate secretion. It is well known that tumor

growth and metastasis require glucose metabolism to be reprogrammed to glycolysis [30]. Traditionally, CXCL7 involves in mediating glycolysis in non-cancer cells by inducing GLUT1 transporters [219], besides it shows proliferative properties in cancer cells (except CRC) and non-cancer cells [28]. In this study, we observe that CXCL7 improves cell proliferation through inducing aerobic glycolysis *in vitro* while it accelerates tumor growth *in vivo*. Interestingly, we observed that CXCL7 can enhance DNA synthesis by significantly increasing the proliferation rate in human colon cancer cells via autocrine and paracrine-dependent loops. This observation was consistent with previous work done by Guo et al. (2017), who noticed that the knockdown of CXCL7 caused a significant decrease in the proliferation and invasion of cholangiocarcinoma (QBC939) cells. Similarly, the authors found that treating QBC939 cells with recombinant CXCL7 [50ng/ml], or CXCL7-derived from conditioned media, promoted the malignant phenotypes of cholangiocarcinoma cells in a paracrine-dependent manner [193]. This study concluded that the increase of proliferation in CXCL7 overexpressed cells was attributed to enhance the activity of the proliferative pathway/AKT [193]. Similarly, Grépin et al. (2014) found that activating the CXCL7/CXCR2 axis enhanced the activity of the PI3K/AKT/mTOR and ERK cell proliferative signaling pathways in ccRCC cells lead to increase the proliferation in the cancer cells [28]. In fact, the interaction between the PI3K/AKT/mTOR signaling cascade and aerobic glycolysis in cancer cells has been reported in the literature [220]. However, in our study, we speculate the specificity for glycolytic and proliferative effects of CXCL7 in colon cancer cells by introducing CXCL7 neutralizing antibody and CXCR2 antagonist (SB225002) to HT-29-CXCL7 and colon cancer cells treated with CXCL7-CM. As expected, blocking the CXCL7-CXCR2 axis abrogates the proliferative effect of CXCL7 on colon cancer cells. This observation is strongly matched with previous studies that had observed blocking CXCR2

signaling with a CXCR2 antagonist inhibits the proliferation and migration of colon carcinoma cells in *in vitro* settings and the growth of tumor xenografts in immunodeficient mice [25]. This result provides further insights for the CXCR2 role in tumor proliferation and progression that has been reported in the literature [22, 221]. Despite many studies show that CXCL7 mediates its proliferative effects via AKT kinase activation, literature shows mixed results about the effect of AKT itself on glycolysis activity and proliferation of cancer cells. To explain, vast evidence in the literature has pointed out that CXCL7 can enhance proliferation via enhancing the activity of AKT, while knockdown of CXCL7 is associated with down-regulation of AKT activity and, consequently proliferation [134, 193]. In contrast, the literature also indicated that inhibiting AKT can directly mediate glycolysis by enhancing glucose uptake and increasing the expression of the key glycolytic enzyme, hexokinase [222]. Thus, it is possible that CXCL7 directly enhances cancer cell aerobic glycolysis and, consequently, the proliferation without relying on AKT signaling cascade. However, further study should be designed to elaborate on the nuclear transcription factor or receptor that mediates CXCL7 glycolytic effect.

Nevertheless, in this current study, we examine the impact of overexpression CXCL7 in colon cancer cells' glycolytic pathway by using multi-approaches and biochemical assays. Our data revealed that CXCL7 dramatically increased glucose uptake and lactate production in culture media of HT-29 cells transfected with CXCL7, but as anticipated, blocking CXCL7/CXCR2 axis significantly abrogates the glycolytic impact of CXCL7 on colon cancer cells. In addition, results from real-time monitoring for glycolytic rate in HT-29-CXCL7 cells by using the Seahorse glycolysis stress test provided further evidence for glycolytic activity of CXCL7 as manifested by increasing the extracellular acidification rate (ECAR) and glycolytic capacity in HT29-CXCL7 compared to HT29-vector. Since other normal cell types in the TME

could also secrete CXCL7, we studied whether treating colon cancer cells with CXCL7-CM could promote aerobic glycolysis and, consequently proliferation, in a paracrine-dependent manner. The results were equivalent to the previous observations and gave further evidence for the role of the CXCL7-CXCR2 axis in colon cancer proliferation by inducing glycolysis in both autocrine and paracrine loops.

Despite the Warburg effect being inefficient in terms of net ATP generation, it can provide rapid supply of ATP that can be instantly tuned to support the high demand for ATP in the highly divided cancer cells [83]. Also, the increased in glucose consumption support the cells with carbon source for anabolic processes needed to support the increased rate of cell proliferation, so the activation of glycolytic pathways promotes tumor cells to adapt to grow quickly [8]. Several noteworthy studies have revealed a relationship between chemokines and glycolysis [210]. In particular, CXCL7 has been reported to be involved in mediating the expression of glucose transporters and, thereby, glucose uptake in connective tissue cells [219]. However, our study shows novel evidence for the role of CXCL7-CXCR2 axis in boosting proliferation via aerobic glycolysis in colon cancer cells. Literature indicates that glycolysis-associated enzymes and molecules are highly expressed in tumor cells, which also play an important role in supporting aerobic glycolysis [223]. Thus, in this current study, we analyzed the expression of key enzymes in the Warburg effect, and we found them markedly upregulated in both HT-29-CXCL7 and colon cancer cells that were treated with CXCL7-derived CM, compared to control. In particular, we noticed that pyruvate kinase M2 (PKM1/2) was significantly upregulated in colon cancer cells overexpressing CXCL7. Studies have pointed out that PKM2 expression in tumors promotes a shift from a normal respiratory chain toward aerobic glycolysis with increased lactate production [224]. There are pieces of evidence reported that

knocking down PKM2 leads to decreased proliferation and cancer metabolic activity while increasing apoptosis in cancer cells [225]. Similarly, hexokinase 2, the first enzyme that catalyzes the first committed step of glucose metabolism, was also significantly upregulated in both approaches, indicating higher glycolytic efficiency for colon cancer cells under the stimulation of CXCL7. Lactate dehydrogenase (LDH) is a crucial enzyme in aerobic glycolysis, catalyzing the last step of glycolysis, i.e., the conversion of pyruvate to lactate, so it has been considered a biomarker of aerobic glycolysis efficiency [210, 226]. In fact, LDHA knockdown could inhibit cell growth and migration *in vitro* and impair tumorigenesis *in vivo*, so it contributes greatly to cancer proliferation and survival [227]. In our study, we found that both HT-29-CXCL7 and HT-29 cells treated with CXCL7-CM had a statistically significant double-fold change in lactate production and LDH protein expression compared to control. This is consistent with the findings of Liang et al., (2024) that indicated the expression of CXCL7 is positively correlated with the level of LDH and predicts prognosis in patients with colorectal cancer. In addition, novel evidence in the literature reported that the increase in CXCL7 and CCL5 levels in response to inflammatory/infection status is associated with an increase in microfibril-associated protein 3-like MFAP3L expression that promotes lactate production and glucose uptake [213].

Nevertheless, we found that blocking the CXCL7-CXCR2 axis dramatically downregulates these glycolytic enzymes and lactate production in HT-29-CXCL7 cells and colon cancer cells treated with CXCL7-CM, which support the specificity of CXCL7 in inducing glycolysis. These data are supported by the outcomes of analyzing a publicly available dataset of RNA-seq for CRC patients and normal colon samples from TCGA COAD. We found that *PPBP* expression positively correlated with gene expression of glycolytic enzymes enriched in

glycolysis-related gene sets in colon adenocarcinomas comparing to normal human colon samples. In addition, glycolytic enzyme genes that are enriched in both the C1 Hallmarks gene set (Glycolysis) and KEGG gene set (Glycolysis_and_Gluconeogenesis) in the TCGA COAD dataset are hexokinase 2, PKM2, and lactate dehydrogenase. Given these results, we assumed that CXCL7 may promote glycolysis by improving levels of glycolytic enzymes and lactate secretion, thus providing more energy and synthetic raw materials for colon cancer cells to proliferate and progress.

These previous results provided us a rationale for testing the impact of CXCL7 on the development of HT-29-CXCL7 and HT-29-vector xenografted tumors. Consistent with our results from *vitro* study, we found that CXCL7 tumors have a greater weight and markedly express the proliferative marker PCNA compared to control. PCNA is involved in the DNA synthesis of cancer cells [228], survival, and possibly in energy metabolic pathways, such as glycolysis [229]. Thus, many studies have discussed role of this protein in highly proliferating malignancies. A study found that PCNA expression increased with advancing cancer stages and correlated significantly with many chemokines such as IL-6 and prognostic factors, LDH [230]. Studies in the past decades have established that the functions of PCNA are regulated through post-translational modifications, including chromatin-bound PCNA being phosphorylated at Y211 (phospho-Y211) by the EGFR (epidermal growth factor receptor), leading to upregulation during the S phase of the cell cycle, consequently promoting proliferation of cancer cells [231]. However, the regulation of PCNA function in cancer cells is not fully understood. One possible interpretation based on proteomic analysis data has suggested that PCNA is involved in the coordination of glycolysis via direct interactions with certain glycolytic enzymes in the cytoplasm [232]. Similarly, a study reported interaction between endogenous PCNA and LDH

and PKM2 in human leukemia cells, suggesting that these interactions might be mediated by some PCNA post-translational modifications, which might also mediate resistance to chemotherapy [233]. Interestingly, treating human leukemia cells with the PCNA inhibitor decreased the glycolytic mediator (intracellular NAD⁺) concentration, decreased extracellular acidification rate (ECAR) in a dose-dependent manner, and inhibited glycolysis rate in general. This suggests that cytosolic PCNA is a key factor in shaping the energy metabolism that mediates the Warburg effect in cancer [233]. However, whether PCNA expression and glycolytic enzymes activities are regulating each other is not studied yet. Furthermore, how CXCL7 can stimulate PCNA expression, and how this interaction can be related to the increased speed of aerobic glycolysis and proliferation in colon cancer cells, as seen in our study, are needed to answer with further studies. One possible explanation for this mechanism is based on what we know from earlier studies that PCNA is regulated by EGFR, the receptor for many signaling transduction including the PI3K-AKT cascade. This raises an assumption that AKT activity can induce glycolytic enzyme activity, and all are potentially regulated by CXCL7 expression. However, further studies should be done to speculate on this assumption.

To replicate our study in TME mimic conditions, we created 3D tissues from the transfected HT-29 cells. The creation of 3D biomimetic microenvironments within tissue-engineered cancer models is imperative for faithfully replicating the intrinsic tumorigenic characteristics of cancer cells, encompassing cellular mechanobiology, as well as cell-cell and cell-matrix signaling dynamics[234]. PEG-fibrinogen (PF) emerges as a pivotal biomaterial for crafting these 3D tumor models, offering versatility to incorporate additional elements of the complex tumor microenvironment, such as cancer-associated fibroblasts, endothelial cells, and macrophages[235]. In terms of viability, CXCL7 transfected tissues exhibited superior viability

compared to the vector-transfected counterparts throughout the experimental timeline. The disparity in viability, though modest on day 1, became markedly pronounced by day 8 and further accentuated by day 15, underscoring the resilience of CXCL7-transfected tissues against cellular adversity. This is consistent with our in vivo study's outcome when we observed that CXCL7 xenografted tumor expressed lower levels of apoptotic marker, Caspase 3, and higher levels of proliferative marker, PCNA, compared to control. Moreover, colony formation assays revealed a substantial increase in colony area in CXCL7 transfected tissues compared to vector-transfected tissues, which is further confirmed the heightened proliferative capacity associated with CXCL7 expression. Lastly, replicate the proliferation assays in 3D engineered tissues demonstrated a discernible uptick in growth and proliferation rates of cells expressing CXCL7, underscoring the pivotal role of this chemokine ligand in fueling cellular proliferation pathways in cancer cells. Our current observations provide further insights into the potential impact of CXCL7 signaling in promoting colon cancer cell growth and progression. However, there are several potential limitations in this current study. First, since this study has shown glycolytic and proliferative properties of CXCL7 in colon cancer cells, an important question is raised here about the specific transcription factor that mediates CXCL7 effects. This could be answered by doing further vigorous analysis for the available samples in our study or performing transcriptomic analysis to explore any regulatory transcription factors for CXCL7. In addition, it is well known that chemokines in general, act synergistically to perform their function, and here we overexpressed CXCL7 solely in the colon cancer cells to monitor its effects. Thus, we need to use a more physiologically relevant model to examine role of CXCL7 in the TME. For instance, treating 3D engineered cancer tissues (generated from PDX lines that express CXCR2) with IL-6 and IL-1 β , that are known to upregulate *PPBP* gene expression, would show a mechanism for

CXCL7 in inducing CRC progression. Further, we could examine the specificity by using neutralizing CXCL7 antibodies and CXCR2 inhibitors to block CXCL7-CXCR2 axis.

Future directions would be included conducting additional experiments to examine the impact of administering neutralizing CXCL7 antibodies and CXCR2 inhibitors in 3D and *in vivo* models. Our current plan for future study is exploring the impact of knocking down *PPBP* in CL-40, human colon cancer cells that naturally produce high level of CXCL7. Once we validate that CXCL7 is knocked down, we will repeat previous methods to assess the impact of knocking down CXCL7 in CL-40 proliferation and glycolytic properties compared to control.

In conclusion, our study is the first to show that the CXCL7-CXCR2 axis can enhance proliferation in colon cancer cells by inducing aerobic glycolysis in *vivo* and *vitro*. However, further investigations using more cancer models would be useful to fully examine the role of CXCL7 in colon cancer progression.

3.7 Figures and legends

Figure 8

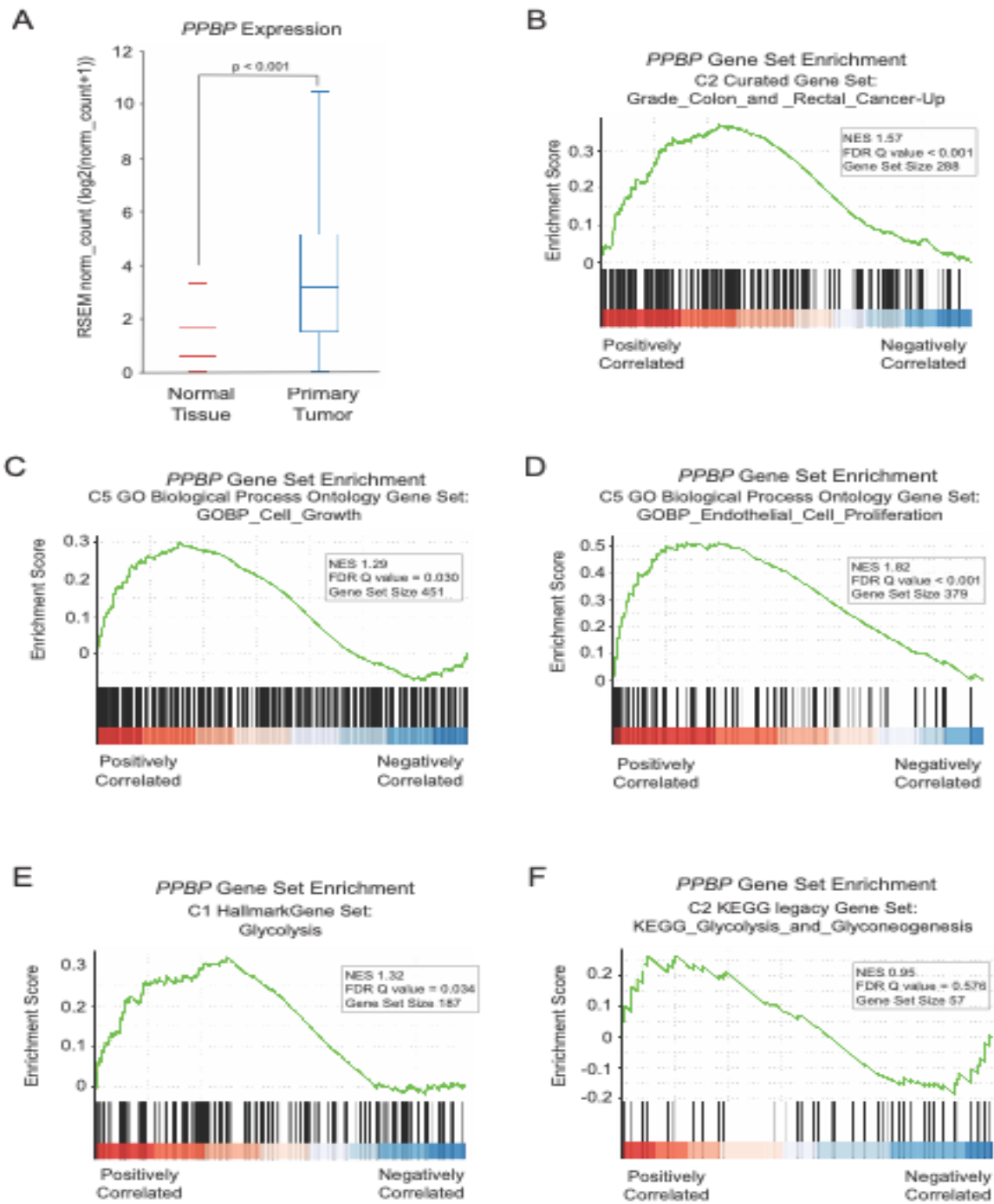


Figure 8. *PPBP* expression is elevated in colon adenocarcinoma and its expression positively correlates with cell proliferation and glycolysis gene sets in colon adenocarcinomas. **A.** RNA seq analysis of *PPBP* expression in normal tissue (n = 41) and primary tumors (n= 286) in the TCGA COAD cohort. **B.** *PPBP* gene set enrichment with the MSigDB Grade_Colon_Rectal_Cancer_Up gene set in the TCGA COAD cohort. **C-D.** *PPBP* gene set enrichment with proliferation-related gene sets in the MSigDB C5 GO Biological Process gene sets (GOBP_Cell_Growth (C)) and (GOBP_Endothelial_Cell_Proliferation (D)) in the TCGA COAD cohort. **E-F.** *PPBP* gene set enrichment with glycolysis-related gene sets in the MSigDB C1 Hallmarks gene set (Glycolysis) (E) and KEGG gene set (Glycolysis_and_Gluconeogenesis) (F) in the TCGA COAD cohort. Normalized enrichment score (NES), FDR q-value, and size of the gene set are shown.

Figure 9

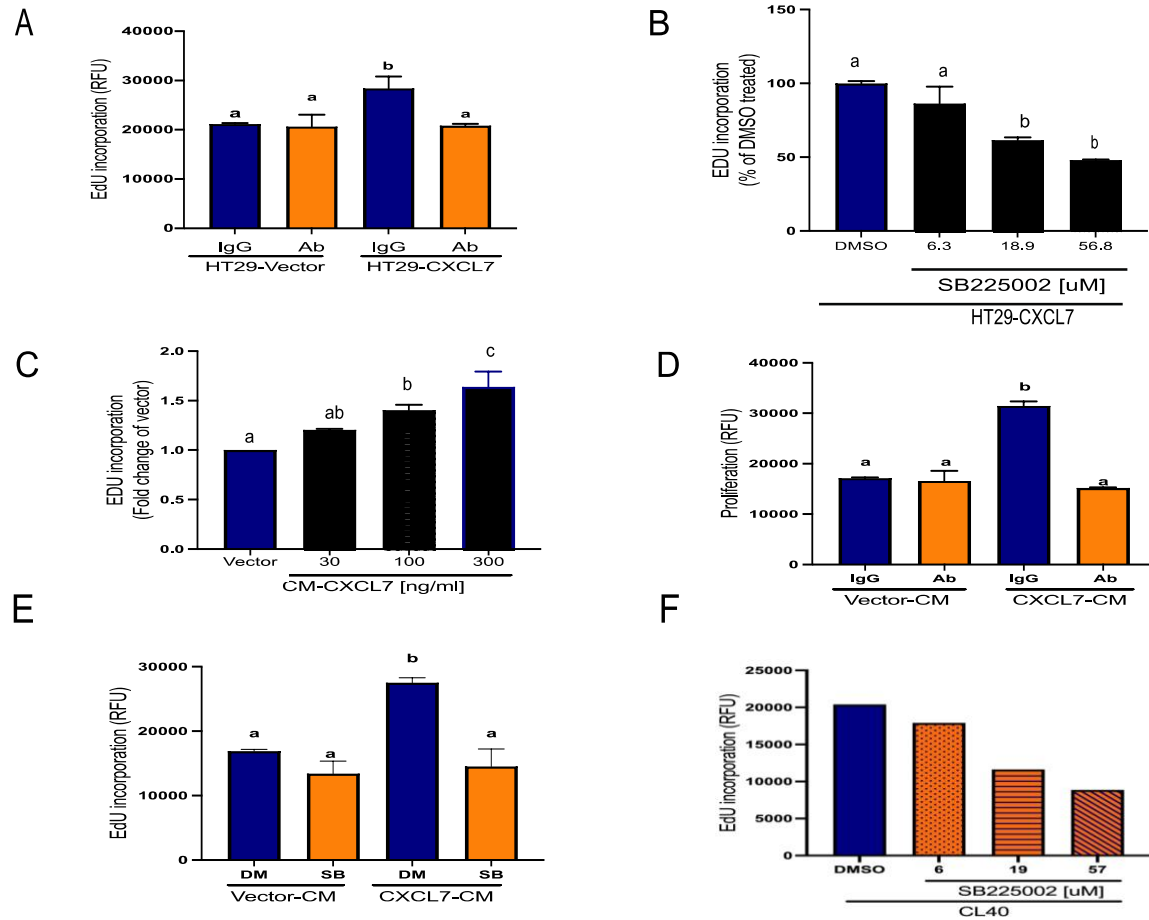


Figure 9. Upregulation of CXCL7 is associated with inducing colorectal cancer proliferation in autocrine and paracrine dependent manner. (A) Proliferation assay results for vector transfected cells (HT-29-Vector) and CXCL7 transfected cells (HT-29-CXCL7) treated with control (IgG) [40ug/ml] or CXCL7 neutralize antibodies (Ab) [40ug/ml]. (B) Proliferation assay showed dose-dependent inhibitory effect for SB225002 (SB) on HT-29-CXCL7 proliferation when treat the cells with three-fold serial dilution of the highest concentration of [56.8uM]. (C) Treating HT-29 cells with conditioned media (CM) from 293T cells transfected with CXCL7 enhances cells' proliferation in dose dependent manner. CXCL7 protein concentration in pooled CM is 860ng/ml

after 48 hrs. (D-E) Proliferation of HT-29 cells with conditioned media (CM) from 293T cells transfected with vector (Vector-CM) or CXCL7 (CXCL7-CM) treated with a CXCL7 neutralizing antibody (Ab) [40ug/ml] Vs. equivalent concentration of control (IgG) or CXCR2 inhibitor (SB) [58.6uM] Vs. equivalent concentration of vehicle, DMSO (DM) .The CXCL7 protein concentration in CM-CXCL7 is (300ng/ml)(F)Three-fold serial dilution for highest concentration of SB225002 (SB) [56.8uM] inhibit proliferation of CL40 in dose dependent manner. (n= 3 independent experiments, different letters represent significant differences (*p<0.05) between groups using a one-way ANOVA followed by the Tukey post hoc test).

Figure 10

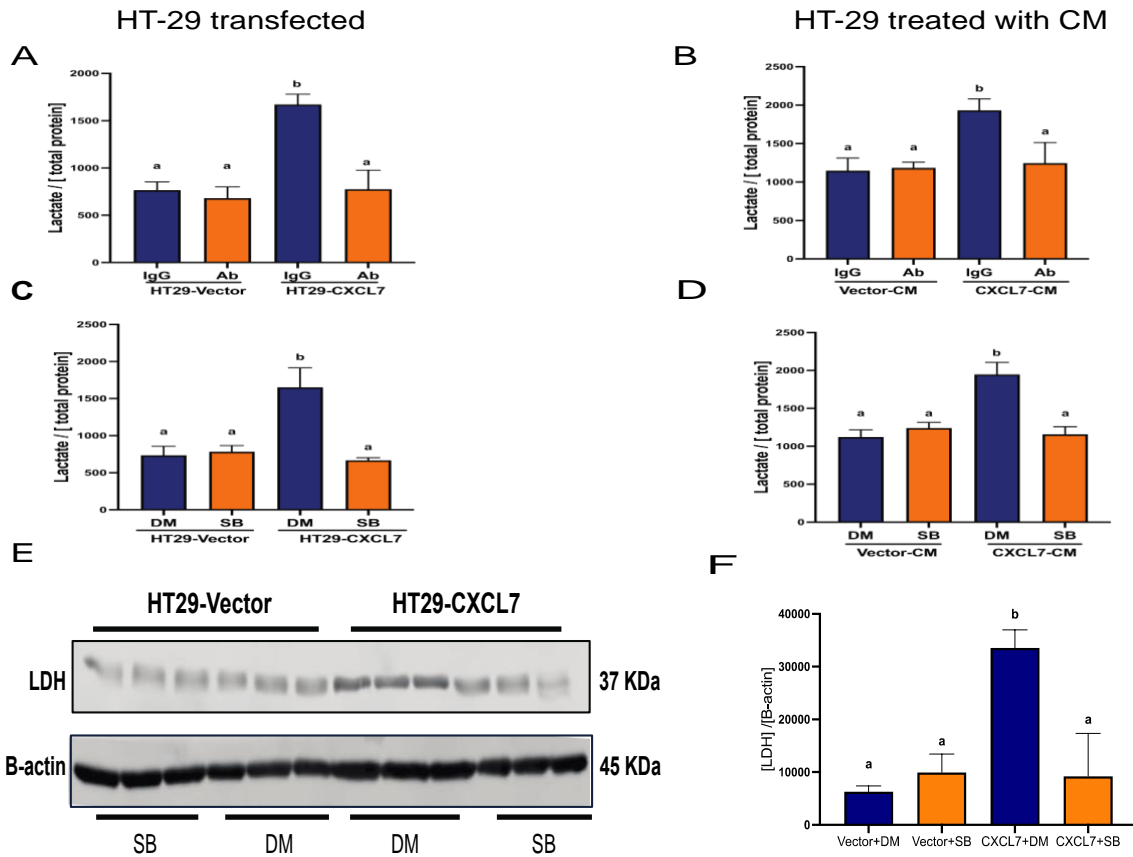


Figure10. Overexpress of CXCL7 was associated with enhance LDH activity and lactate secretion. (A) Lactate secretion in the media of HT-29 transfected cells with either vector or CXCL7 (HT-29-Vector and HT-29-CXCL7, respectively) treated with vehicle (IgG) or CXCL7 neutralizing antibody (Ab)[40ug/ml]. (B) Lactate secretion in the media of HT-29 cells treated with CM from 293T overexpressing vector or CXCL7 (Vector-CM and CXCL7-CM, respectively) treated CXCL7 neutralizing antibody (Ab) [40ug/ml] or equivalent concentration of vehicle (IgG). The CXCL7 protein concentration is 300ng/ml in CM-CXCL7 that have been

used in the experiment. (C) Transfected HT-29 cells were treated with SB225002(SB) [58.6 μ M] or equivalent concentration of vehicle DMSO (DM). (D) HT-29 cells culture with conditioned media from Vector-CM or CXCL7-CM, were treated with SB225002(SB) [58.6 μ M] or equivalent concentration for vehicle DMSO (DM). (E) Western blotting analysis of protein expression for lactate dehydrogenase (LDH) in: (1) HT-29 vector transfected cells (HT-29-Vector) treated with SB225002(SB) [58.6 μ M] or equivalent concentration of vehicle DMSO (DM). and (2) HT-29 CXCL7 transfected cells (HT-29-CXCL7) treated with SB225002(SB) [58.6 μ M] or equivalent concentration of vehicle DMSO (DM). Expression of β -actin was used as an internal control. Western blots are representative of three independent experiments. Pooled CXCL7 concentration in CM is 860ng/ml. Lactate concentration was normalized to the total protein isolated from the cells (n= 3 independent experiments, different letters represent significant differences (p<0.05) between groups using a one-way ANOVA followed by the Tukey post hoc test. Increase proliferation capability for CXCL7 overexpress cells is correlated with increase lactate excretion.

Figure 11

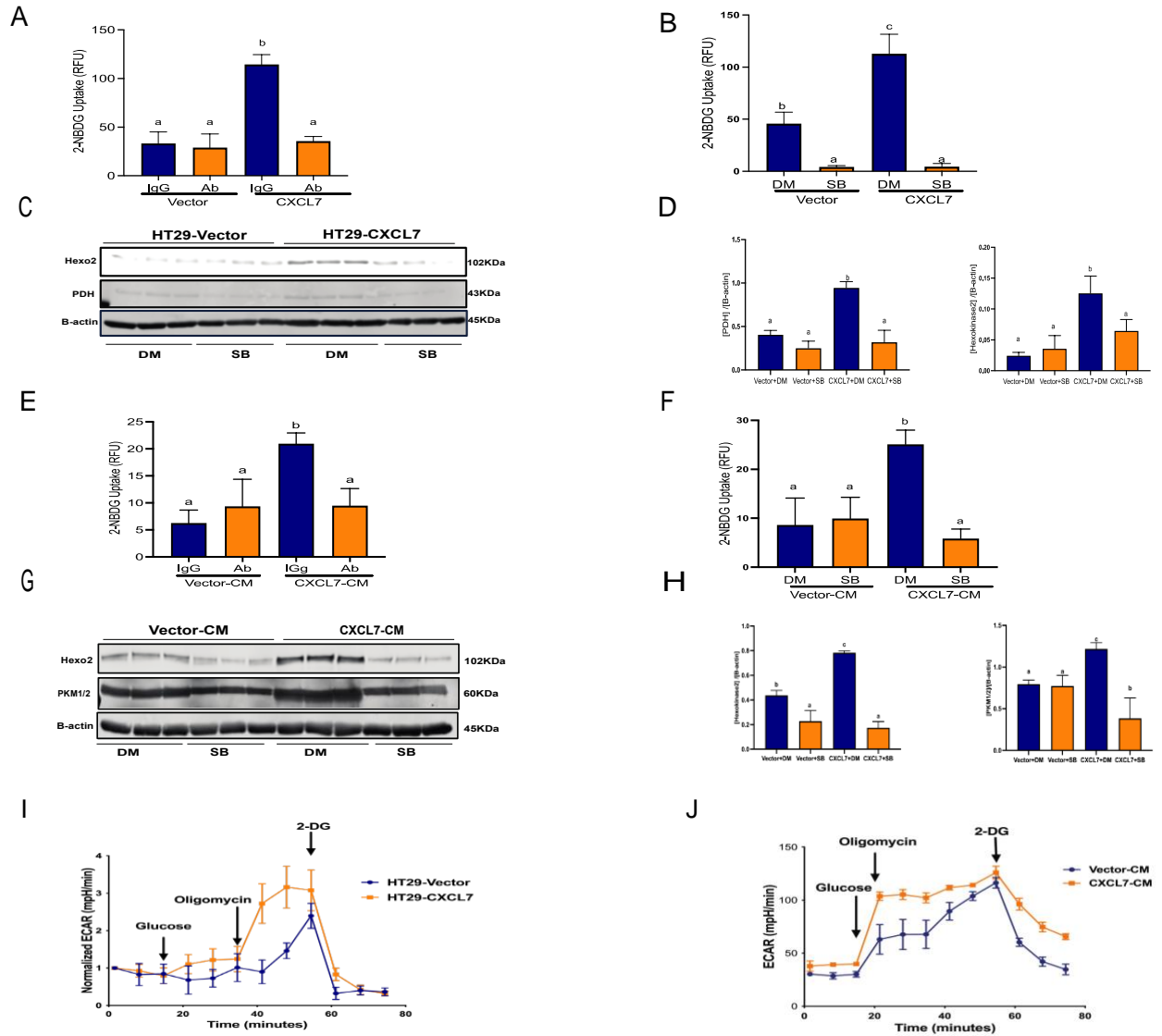


Figure 11. (A-B) glucose uptake assay results for HT-29 transfected cells with either vector or CXCL7 (HT-29-Vector and HT-29-CXCL7, respectively). Cells were treated with vehicle (IgG or DMSO (DM)), CXCL7 neutralize antibodies (Ab), or CXCR2 inhibitor SB225002(SB). (C-D)

Western blotting and quantification for expression of selected glycolytic enzymes; hexokinase 2 (Hexo2) and pyruvate dehydrogenase (PDH) in HT-29-Vector and HT-29-CXCL7 treated with DMSO (Vector-DM) (CXCL7-DM) respectively, or HT-29-Vector and HT-29-CXCL7 treated with CXCR2 inhibitor (SB225002), (Vector-SB) and (CXCL7-SB) respectively ($P < 0.05$). Expression of b-actin was used as an internal control. (E-F) glucose uptake assay results for HT-29 culture with CM from 293T overexpressing vector or CXCL7 (Vector-CM and CXCL7-CM respectively). Cells were treated with vehicle (IgG or DMSO (DM)), CXCL7 neutralize antibodies (Ab), or CXCR2 inhibitor SB225002(SB). (n= 3 independent experiments, different letters represent significant differences ($p < 0.05$) between groups using a one-way ANOVA followed by the Tukey post hoc test). (G-H) Western blotting analysis and quantification for expression of selected glycolytic enzymes; hexokinase 2 (Hexo2) and pyruvate kinase M1/2 (PKM1/2) in Vector-CM and CXCL7-CM treated with DMSO (Vector-DM) (CXCL7-DM) or Vector-CM and CXCL7-CM treated with CXCR2 inhibitor (SB225002), (Vector-SB) and (CXCL7-SB) respectively ($P < 0.05$). Expression of b-actin was used as an internal control. (I-J) Seahorse XF glycolysis stress test revealed that both HT-29 expressing CXCL7 cells and HT-29 cells treated with CXCL7-CM pose higher capacity for glycolysis compared to their control, representative experiment performed in triplicate. For glucose uptake assay, nonspecific uptake was determined in the presence of 10 μ M cytochalasin B and was subtracted from all values. The results shown are mean \pm SD(n=3).

Figure 12.

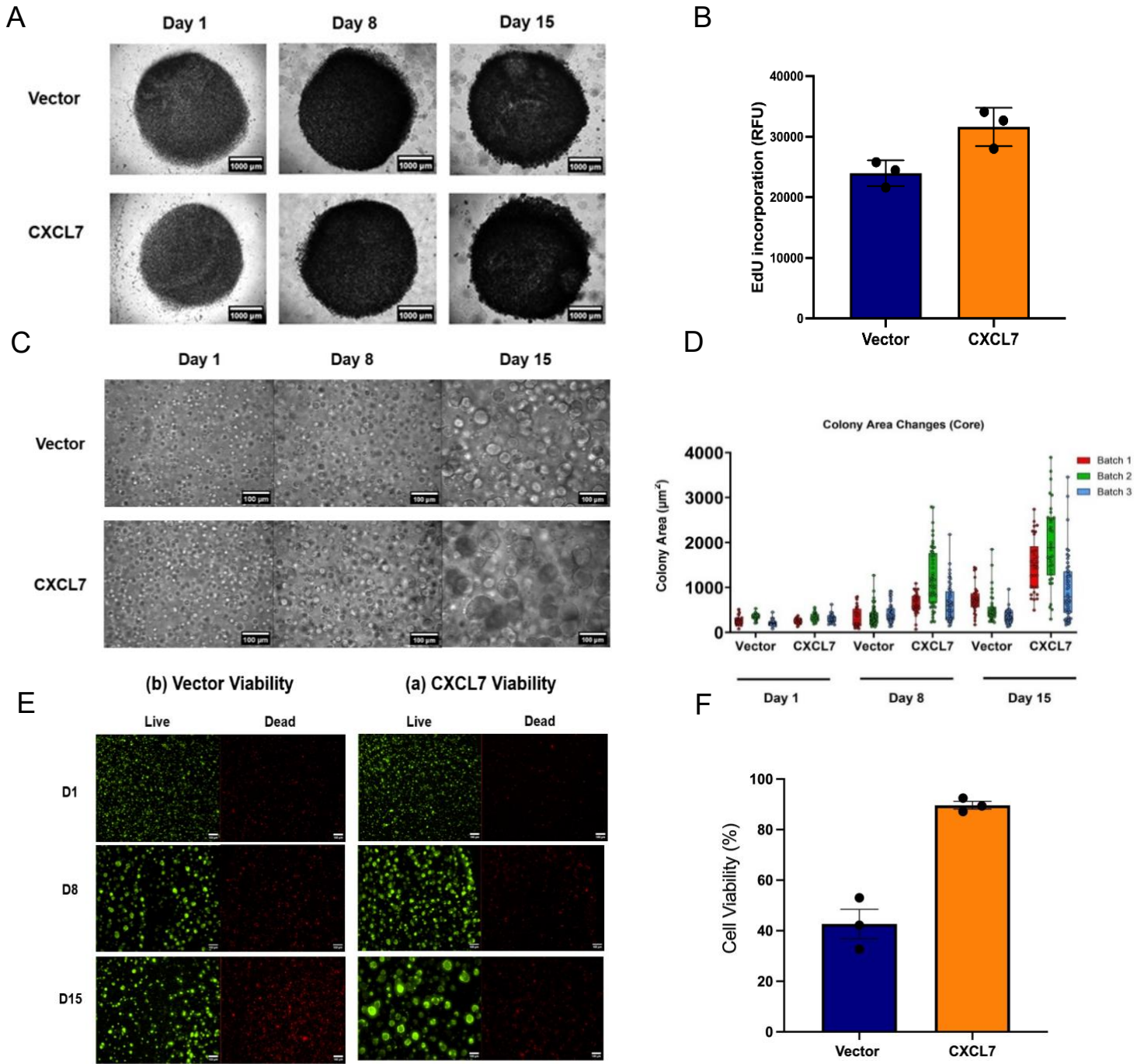


Figure 12. Overexpressing CXCL7 shows enhanced proliferation in 3D engineered colon cancer model. (A) Tissue growth images for 15 days period of time made with PF hydrogel encapsulated with colon cancer cells transfected with CXCL7 and vector. Images of cell encapsulated tissues have been taken on day 1, day 8 and day 15 (2X magnification, scale bar = 1000 μm). (B) Proliferation level for CXCL7 transfected tissues is higher than vector transfected one on day 8 ($*p < 0.05$, $n = 3$ separate batches of cell culture). (C) Colony morphology for 3D model of colon cancer over the time. Images showing core section of the tissue and the colony size increase over the culture period (20X magnification, scale bar = 100 μm). (D) Increasing of average colony area for all three batches over the culture time ($*p < 0.05$, $n=3$ z-stacks from at least three independent tumor constructs). (E) Cell viability in PF-based hydrogels encapsulated with colon cancer cells. Representative fluorescence z-stacks of cells within PF-based tumor constructs stained with calcein AM (Live, green) and ethidium homodimer (Dead, red) on day 1, day 8 and day 15 (10X magnification, scale bar = 100 μm) demonstrate uniform distribution of cancer cells. HT-29 cells transfected with CXCL7 exhibit relatively high cell viability through 15 days of culture ($n=3$ z-stacks from at least three independent tumor construct). (F) Quantitative viability analysis shows differences on day 15 for both CXCL7 and vector transfected tissues ($*p < 0.001$, $n=3$ separate batch).

Figure 13.

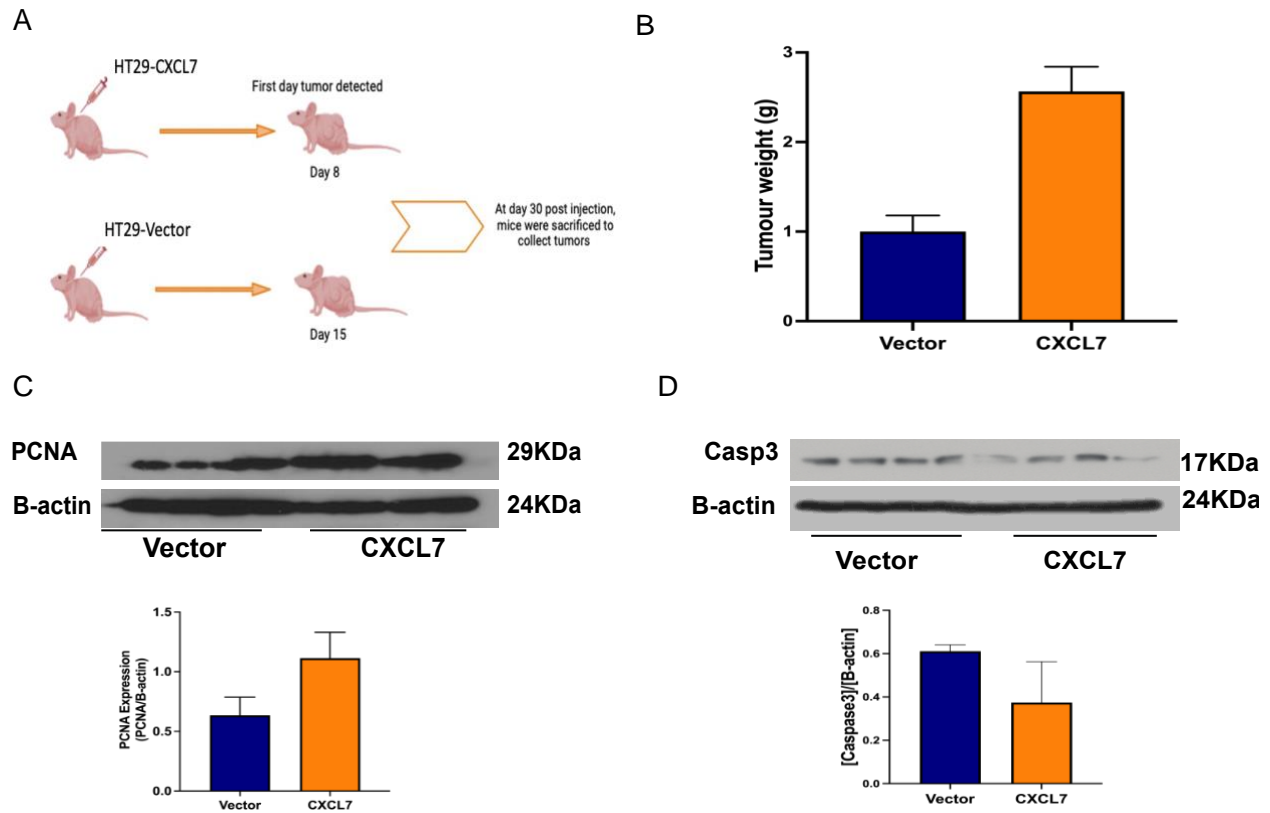


Figure 13. Overexpression of CXCL7 Accelerates the Growth of HT-29 Xenograft Tumors in Nude Mice. (A) Schematic illustration for HT-29 transfected xenograft animal model method. (B) bar graph represents the mean of tumor weights (mice, n=8) of the excised tumour from mice mice injected with either HT-29 cells transfected with vector (vector) or HT-29 cells transfected with CXCL7 (CXCL7) (***p<0.0001). (C) Westering blotting and quantification for protein expression for proliferative cell nuclear antigen (PCNA) in vector and CXCL7 tumors, the specific bands were normalized with the internal control β -actin (*p<0.05). (D)

Western blotting and quantification for protein expression for Caspase3 in vector and CXCL7 tumors, the specific bands were normalized with the internal control β -actin (n=8, p<0.05).

Figure 14.

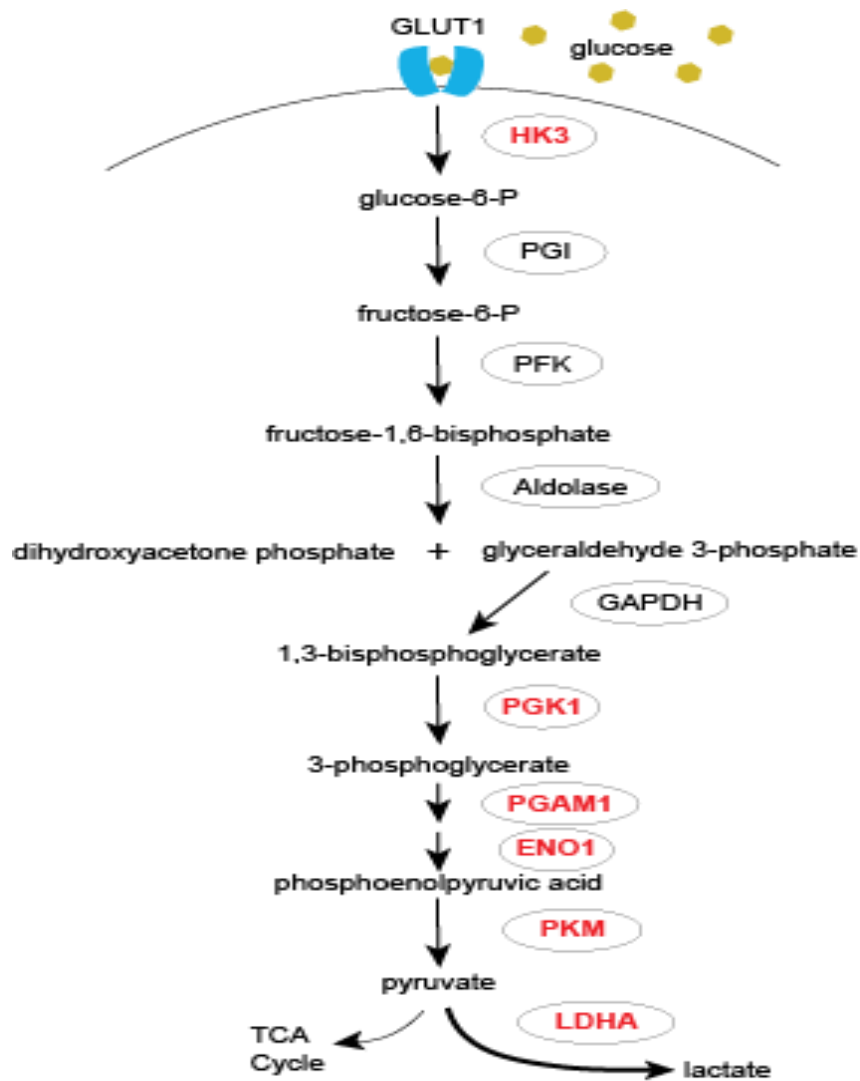
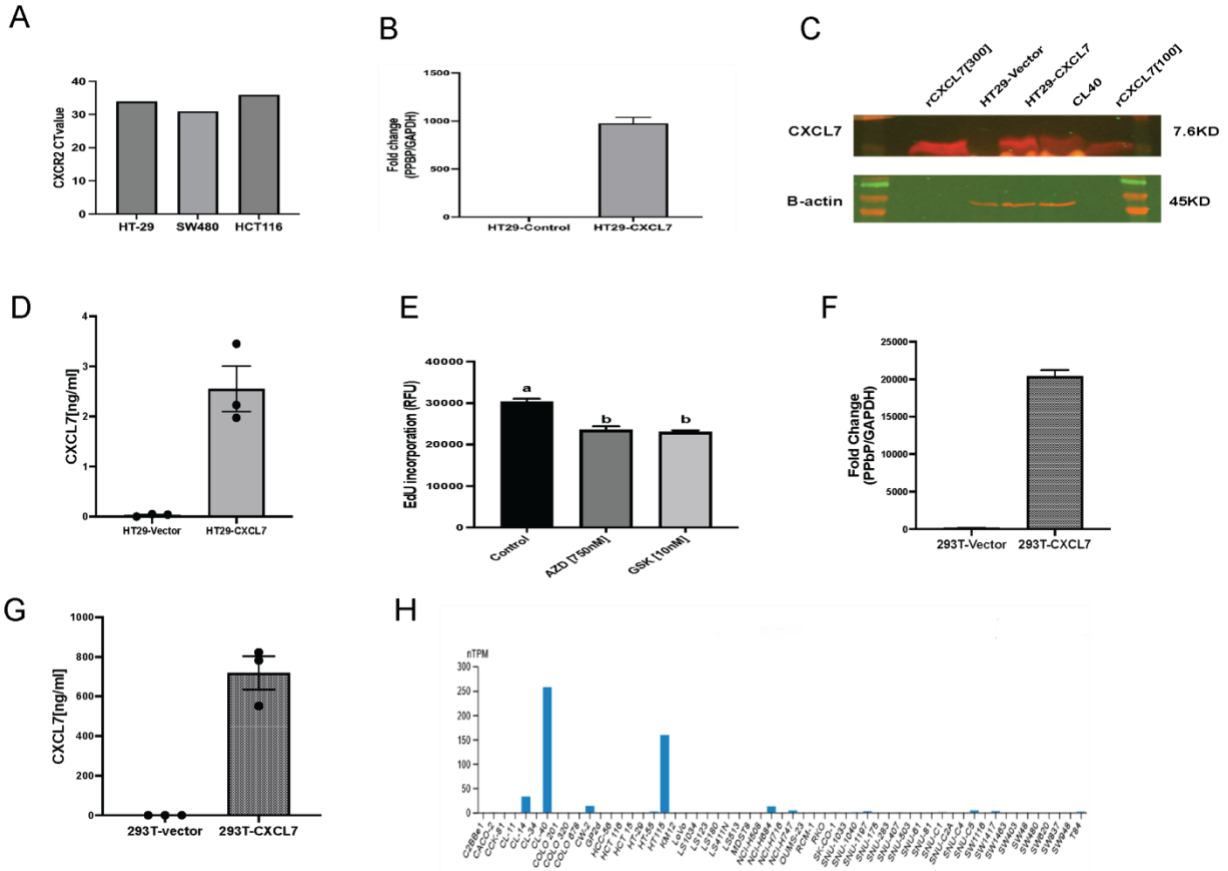


Figure 14. *PPBP* expression positively correlates with gene expression of glycolytic enzymes enriched in glycolysis-related gene sets in colon adenocarcinomas. Glycolytic enzyme genes which were enriched in both the C1 Hallmarks gene set (Glycolysis) and KEGG gene set (Glycolysis_and_Gluconeogenesis) in the TCGA COAD cohort are shown in red text.

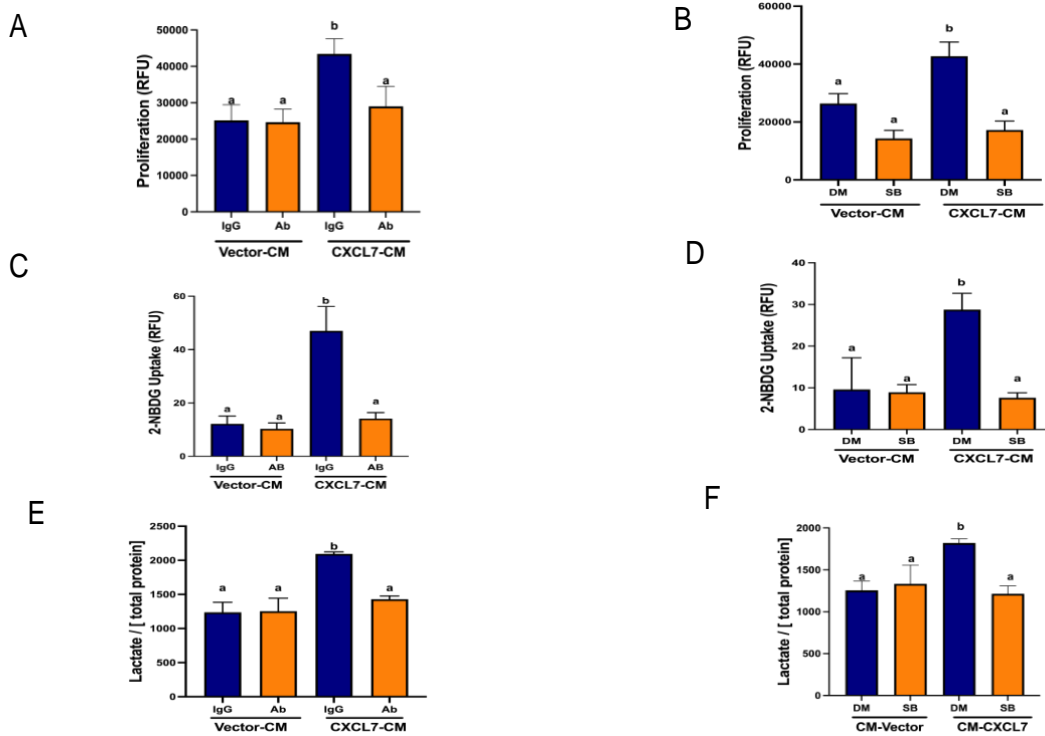
Supplemental Figure 1



Supplemental Figure 1. (A) *CXCR2* mRNA/*B2M* expression among common CRC cell lines HT-29, CL40, SW480, and HCT116. Different letters represent significant difference (B) *PPBP* mRNA/*GADPH* expression was significantly greater in HT-29 cells transfected with CXCL7 (HT20-CXCL7) compared to HT-29 cells transfected with vector (HT-29-vector). Data represent the normalized means \pm SD (n = 3 biological replicates, ****p* < 0.001). (C) Western blotting analysis show protein expression for following samples; from the left first band is positive control/ rCXCL7 [300ng], HT-29 transfected with vector, HT-29 transfected with CXCL7, CL40 WT, and positive control rCXCL7 [100ng]. The cells were grown in experiment media situ 48 h prior to protein extraction and western blot analysis. The western blot membranes were re-

stained with an anti-B-actin (Sigma) for loading control. **(D)**The CXCL7 protein concentration in cultured media from HT-29-vector and HT-29-CXCL7 for 48 h, were determined by ELISA assay. Data represent the means \pm SD (n = 3 biological replicates; ** $p < 0.01$). **(E)** proliferation assay result for HT-29-CXCL7 when using CXCR2 antagonists; AZD-5069 and Danirixin (GSK1325756) show CXCR2 blockers significantly inhibit the proliferation in HT-29-CXCL7 (n = 3 biological replicates, **** $p < 0.0001$). Different letters represent different values. **(F)** *PPBP* mRNA/GADPH expression was significantly greater in 293T cells transfected with CXCL7 compared to vector. (**** $p < 0.0001$). **(G)**The CXCL7 protein concentration in cultured media from 293T-vector and 293T-CXCL7 for 48 h, were determined by ELISA assay. Data represent the means \pm SD (n = 3 biological replicates; **** $p < 0.01$). **(H)** *PPBP* expression among various CRC cell lines (n=57).

Supplemental Figure 2



Supplemental Figure 2. (A-B) Proliferation of SW480 cells with CM from 293T cells transfected with vector (Vector-CM) or CXCL7 (CXCL7-CM) treated with control (IgG) vs a CXCL7 neutralizing antibody (Ab) (left panel) or DMSO (DM) vs. CXCR2 inhibitor (SB) (right panel) (n=4 independent experiments). (C-D) Results from glucose uptake in SW480 cells treated with CM from 293T overexpressing vector or CXCL7 (Vector-CM and CXCL7-CM respectively). Cells were treated with control (IgG) vs a CXCL7 neutralizing antibody (Ab) (left panel) or DMSO (DM) vs. CXCR2 inhibitor (SB) (right panel) (n=3 independent experiments). (E-F) Lactate secretion in the media of SW480 cells treated with CM from 293T overexpressing vector or CXCL7 (Vector-CM and CXCL7-CM, respectively). Cells were treated with vehicle (IgG or DMSO (DM)), a CXCL7 neutralizing antibody (Ab), or CXCR2 inhibitor

SB225002(SB). Lactate concentration was normalized to the total protein isolated from the cells.
(n= 3 independent experiments, different letters represent significant differences ($p < 0.05$)
between groups using a one-way ANOVA followed by the Tukey post hoc test).

Chapter 3: Summary and conclusion

Metabolic reprogramming is an important feature of tumors that has long been the focus of attention and is associated with tumor progression and aggressiveness [236]. The dominant feature of this altered metabolism is manifested by an increase in glucose uptake and fermentation of glucose to lactate, and it is referred to as the Warburg effect [83]. In the present study, we investigated the role of CXCL7 in colon cancer cells proliferation, as well as the role and molecular mechanism of CXCL7 in the regulation of the malignant phenotypes of colon cancer cells through inducing glycolytic capacity of cells. We found that the increase expression of CXCL7 was significantly positively correlated with increasing in cells proliferation and glycolytic capacity. *In vitro* study showed that CXCL7 plays a proliferative role in colon cancer cells (HT-29) when transfected the cells with CXCL7 or treat the cells with exogenous CXCL7 from the CM of CXCL7-overexpressing 293T- cells. In addition, we speculate the specificity of CXCL7-CXCR2 axis role in cells' proliferation by administering CXCL7 neutralizing antibody and CXCR2 antagonist, and we found blocking CXCL7-CXCR2 axis abrogated the proliferative impact of CXCL7. Furthermore, the activity of glycolysis pathway was found to be upregulated after CXCL7 overexpression or treatment.

The outcomes derived from our research highlight the dynamic and impact of CXCL7 expression on various facets of cancer cellular behavior, specifically influencing cells proliferation, colony development, as well as cellular viability and tumor size over the entire experimental duration. This underscores the multifaceted role of CXCL7 in promoting favorable conditions for cancer cellular metabolism and proliferation within the studied context. Moreover, using multi approaches, including 2D cells, 3D models, and HT-29 transfected cells xerograph model, to

identify CXCL7's impact on CRC growth opens avenues for further investigation into its potential as a therapeutic target and biomarker for CRC. However, further understanding of the molecular mechanism underlying the impact of CXCL7-CXCR2 axis on CRC development and progression is urgently needed, which may promote the potential benefit of developing targeted therapy for CRC.

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