Investigating effect of galectin-3 on protease secretion in human colon cancer cells

Thesis submitted in accordance with the requirements of the University of Liverpool for the Doctorate in Philosophy by

Shun Li

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Acknowledgements

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This PhD study started in 2018 and unfortunately has been impacted by COVID-19 pandemic from 2020. The lab work has been impacted greatly due to the restrictions on the maximum capacity of each laboratory and prolonging delivery time for reagents.

Abstract

Proteases are enzymes that are responsible for proteolysis of proteins in living organisms. They regulate multiple biological processes. The catalytic reaction of proteases is irreversible therefore the level of proteases is tightly regulated in physiological condition. In epithelial cancer, the expression and secretion of proteases are often dysregulated, resulting in increased cancer cell growth, invasion, migration, angiogenesis and metastasis.

Galectin-3, a β-galactoside-binding protein expressed by many types of human cells, is often found to be overexpressed in various cancers. Extracellular galectin-3 is known to interact with many cell surface molecules and promotes multiple steps in cancer progression and metastasis.

This project investigated the hypothesis that galectin-3-mediated promotion of cancer progression and metastasis might be associated with regulation of protease expression in cancer cells. Various experiments were used in this study to assess the effect of galectin-3 on protease expression and secretion in human colon cancer cells and the effect of galectin-3-mediated protease expression and secretion on cancer cell invasion and signalling.

It was found that galectin-3 induces the secretion of a number of proteases in colon cancer cells in an autocrine and paracrine way. Higher galectin-3 expression and secretion by cancer cells was associated with higher protease secretion. It was also found that this galectin-3-induced secretion of proteases directly promoted colon cancer cell invasion, disrupted epithelial integrity and caused leakage of the epithelial monolayer. The underlying mechanism of galectin-3 inducing protease secretion is likely via activation of PYK2/GSK3 signalling, at least for cathepsin B in this study.

These discoveries provide insight into the impact of galectin-3 and galectin-3-induced protease secretion on cancer cell behaviour in cancer progression and metastasis and may aid future development of novel therapeutic strategies for cancer.

Abbreviations

ADAM	A Disintegrin and metalloproteinase domain-containing protein
AIIt	Annexin II heterotetramer
ANOVA	Analysis of Variance
APC	Adenomatous polyposis coli
BAI1	Brain-specific angiogenesis inhibitor 1
Bcl2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAFs	Cancer associated fibroblasts
CCL18	Chemokine ligand 18
CEA	Carcinoembryonic antigen
CRLM	Colorectal liver metastases
CTSA	Cathepsin A
CTSB	Cathepsin B
CTSX	Cathepsin X
CRC	Colorectal carcinoma
CRD	Carbohydrate recognition domain
CM	Conditioned media
DPPIV	Dipeptidyl peptidase IV
DMSO	Dimethyl sulfoxide

DMEM	Dulbecco′s Modified Eagle′s Medium
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immunoassays
EMT	Epithelial-mesenchymal transition
ERK	Extracellular regulated MAP kinase
FAK	Focal adhesion kinase
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FCS	Fetal calf serum
G-CSF	Granulocyte colony-stimulating factor
GlcNAc	N-Acetyl glucosamine
ICLiPs	Intramembrane cleaving proteas
IGFs	Insulin-like growth factors
IGF-I	Insulin-like growth factor-I
IL-6	Interleukin 6
IGFBP	Insulin-like growth factor binding protein
LAMP1	Lysosome-associated membrane protein-1
KiSS-1	Metastases suppressor
MAPKs	Mitogen-activated protein kinas

MCP	Modified citrus pectin
MDM2	Mouse double minute 2 homolog
MMPs	Matrix metalloproteases
MMRN2	Multimerin 2
MW	Molecular weight
NG2	Neural/glial antigen 2
NTD	N-terminal domain
OPD	O-Phenylenediamine dihydrochloride
PAR-1	Protease-activated receptors type-1
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PAIs	Plasminogen activator inhibitors
RAS	Rat sarcoma viral oncogene homolog
pRb	Retinoblastoma protein
pY14Cav1	Phosphorylated caveolin-1
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulfate
SLC12A5	Solute carrier family 12 member 5
sICAM-1	Soluble intercellular adhesion molecule-1
SRC	Rous sarcoma oncogene
SPRY4	Sprouty RTK signaling antagonist 4
TCF4	Transcription factor 4
TF	Thomsen–friedenreich
TEMED	Tetramethylethylene-diamine

TEER	Trans-epithelial electrical resistance
TNF-α	Tumour necrosis factor alpha
TGF-β	Transforming growth factor beta
TGF βR	Transforming growth factor- β receptor
TIMP	Tissue inhibitors of metalloproteinases
TPR	Translocated promoter region
TME	Tumour microenvironment
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

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Chapter 1

Introduction: Cancer, Proteases and Galectin-3

1.1 Cancer

Cancer is a leading public health issue and is responsible for a large proportion of deaths annually worldwide. According to the American Cancer Society, 1,958,310 new cancer cases and 609,820 cancer deaths are expected to take place in the United States in 2023 [1]. Cancer is a complex disease, and it originates from the cumulative effect of genome changes, causing upregulation in cell growth including defects in cell apoptosis and cell cycle alteration. Normal cells are monitored and controlled strictly by cellular signals which decide the fate of cells into either cell differentiation or apoptosis. Alternatively, cancer cells acquire the ability to proliferate independently and uncontrollably. The uncontrollable dissemination, proliferation and differentiation of cancer cells eventually causes the poor prognosis of the disease.

1.1.1 Colon cancer

Epithelial cancers, termed as carcinomas, occur and develop from transformed epithelial cells. They are grouped into four broad groups depending on the type of the original epithelial cells, namely basal cell carcinoma, adenocarcinoma, squamous cell carcinoma and transitional cell carcinoma [2]. Adenocarcinoma originates from epithelial cells (particularly glandular cells) and it secretes substances such as mucus and digestive fluids [3]. Most of cancers like colon, breast, and prostate cancer are adenocarcinomas. Squamous cell carcinoma starts in cells in the top layer of the epithelium (or squamous cells) [4], while basal cell carcinoma originates from the cells in the bottom layer of the epithelium (or basal cells) [5], both of which are strongly associated with skin cancer. Transitional cell carcinoma often occurs in the stretchy cells in the urinary tract epithelium, termed transitional cells, which can stretch as the

organ expands. Examples include ureteric and renal pelvis transitional cell carcinoma [6].

Colorectal cancer (CRC) is one of the epithelial cancers, also termed as colorectal carcinomas. According to colorectal cancer statistics in 2023, in the United States of America, there will be approximately 153,020 individuals diagnosed with colon cancer and roughly 34% of this population will die from the disease [7]. The formation of CRC starts with genetic damages that make the epithelial cells in the intestinal mucosa prone to neoplastic transformation [8] The presence of a benign precursor lesion, also known as a polyp, as well as other lesions in the large intestine, known as adenomatous polyps and serrated polyps, is identified in most CRC carcinogenesis. The process of CRC starts with a slight inflammation, then developing adenomatous polyps in the epithelium, and finally developing adenocarcinoma [9]. There are about 1 in fifth of CRC is related to hereditary syndromes such as familial adenomatous polyposis (FAP). In FAP patients, one or two alleles of the adenomatous polyposis tumour suppressor (APC) genes is inactivated by the somatic mutations [10]. There are estimate 95% of FAP patients develop adenoma as early as the age of 35 [11]. In addition, the patients with inflammatory bowel disease might be prone to developing CRC, in which CRC occurs through development of dysplasia towards neoplastic transformation [12].

Over the decades, the risk of cancer-associated death has decreased drastically, mortality rates for all cancers combined have decreased by around a fifth (19%) in the UK since 1970s [13, 14]. Most of the reduction comes from smoking-related cancers, as there has been a decline in smoking in the population. There are also contributions from adjuvant chemotherapies for colon and breast cancer, as well as earlier diagnosis, surgical removal of tumours and targeted therapies. However, there have been very

few breakthroughs in terms of treating metastasis, which is responsible for nearly 90% of cancer related death [15]. Metastasis was first introduced in 1829 by J.C. Recamier to explain the transfer of disease between two distant body parts and is currently described in cancer as: the spread of cancer cells from the primary tumour to surrounding tissues and to distant organs. A successful metastatic spreading includes tumour cells detaching from the primary site, intravasating into blood vessels or lymphatic vessels, escaping immune surveillance, extravasating at distant capillary beds, and colonizing the distant organs [16]. The difficulty of characterizing metastasis comes from its multi-step development and most research nowadays excludes metastasis in the *in vivo* state [17].

In CRC, approximately 25 to 50% of patients develop liver metastases (CRLM) during their illness. The high risk of CRLM might be contributed by the portal vein system directly connecting the liver and colorectal which provides tumour sufficient blood supply [18]. The current standard treatment in CRLM patients are curative resection and chemotherapy [19]. However, the surgery is only applicable in 10% to 20% of cases with a 5-year survival rate as 30% due to factors such as presence of extrahepatic disease, the size and location of the tumour or unresectable disease [20].

1.1.2 Molecular basics of cancer

Tumorigenesis or oncogenesis is the progress that transforms normal cells into cancer cells. In the process of transformation, normal cells acquire autonomy and independence from the local microenvironment and become self-sufficient to survive and ready to invade [21]. The characteristics of tumorigenesis include the cellular, genetic, and epigenetic changes and altered cell division. The initial step of

carcinogenesis results from an irreversible alteration in the genome such as one or more simple mutations, transversions, transitions, and/or small deletions in DNA [22], some of the genetic changes that increases the risk of cancer can be inherited to the next generation, for example, if a mutated *BRCA1* or *BRCA2* is passed to the child, the child will have higher risk of developing breast and other cancers.[23] Genetic changes come from endogenous metabolism or from exposure to exogenous agents which can be chemical or physical such as chemotherapeutic agents or tobacco smoking [24]. Chemicals may cause an adduct with DNA when being metabolized to the reactive intermediates in which the adduction is repairable and result in no DNA damage. However, if the adduction is not repaired, this can lead to a gene mutation, particularly in critical areas such as proto-oncogenes such as *KRAS* in colon cancer or tumour suppressor genes such as *TP53*, and cancer might occur. Endogenous metabolism can also cause an adduct in DNA or DNA damage by producing reactive intermediates or reactive oxygen species. In this case, an unrepaired adduct or damage might lead to cancer as well. Subsequently, these two mechanisms may cause upregulated oncogene expression or downregulated tumour suppressor gene expression. Furthermore, normal cellular signalling can be affected by endogenous or exogenous variables which results in alterations in pathways that regulate proliferation, differentiation, apoptosis, migration, and angiogenesis [25]. Eventually, these cells transform into a malignant neoplasm and cancer occurs. Tumour formation is considered to be a selective process in which it starts with a single gene mutation in a single progenitor cell or in some cases, a mutated gene was inherited from the parents, and it is much more likely to develop the second gene mutation. The offspring of this precursor cell happens with subsequent mutations, further followed by clonal selection

of daughter cell populations that expand and proliferate carrying the accumulated alterations in an unconstrained manner [26].

Colon carcinoma, for example, is characterised by mutations in at least four genes, which collectively provide growth and survival advantages for colon cancer cells. These include mutational activation of *KRAS* oncogene and three allelic deletions of specific regions of chromosomes 5, 17 and 18, which contains tumour suppressor genes. The allelic loss on chromosome 17 is associated with *TP53* gene and deletion of *DCC* gene is located on chromosome 18. The mutated gene identified on chromosome 5 is *APC gene*, and *APC* gene was reported as a key tumour suppressor gene that suppresses Wnt signalling [27]. In the case of CRLM, previous studies have shown that *BRAF, NRAS, PI3KCA, NRAS, KRAS, TP53, EBF1* and *CDK12* might be risk genes of CRLM [28].

Most of the genes involved in cancer progression are categorized into tumour suppressor genes, oncogenes and DNA repair genes. Tumour suppressor genes normally play a protective role against cancer progression, where they constrain cell growth by monitoring the cell differentiation rate, repairing mismatched DNA, and regulating cell apoptosis. Mutation in a tumour suppressor gene is characterized as loss-of-function. For example, the tumour suppressor gene *TP53* is the most mutated gene in cancer, as more than half of cancers include a missing or damaged *TP53*gene. Other major tumour suppressor genes include *p27*, *TP53*, *LRP1B*, *APC*, *CSMD1*, *BRAC-1* and *BRCA-2* [29]. Oncogenes originate from gain-of-function mutations in proto-oncogenes and can promote cell growth, division, and survival. Some of the main oncogenes in carcinomas are *RAS* family, *HER-2*, *c-myc*, *PI3K*, *FLG*, *SPTA* and *ZNF536* [30]. In addition to DNA changes in genes that regulate cell growth, it is prominent that alternations in the genome occur in genes that control apoptosis and

DNA damage repair. In normal physiological conditions, the expression and activity of pro-apoptotic proteins such as Bcl-Xs and anti-apoptotic proteins such as Bfl 1 are tightly regulated [31]. However, in cancer cells, the homeostasis between pro- and anti-apoptotic proteins is disrupted in which anti-apoptotic activities are more favoured [32]. Finally, DNA repair genes are made to correct the errors from DNA replication, and many of them function as tumour suppressor genes. For example,*TP53. BRCA1* and *BRCA2* are all DNA repair genes. Cells have DNA replicating and repairing mechanisms, including high fidelity DNA polymerases with proofreading abilities and base and nucleotide excision repair systems, to repair any replication error or DNA damage from chemicals. It is believed that DNA repair genes are frequently mutated in cancer. For instance, mutated *BRCA1* and *BRCA2* genes, which participate in homologous recombination and Fanconi anemia pathway repair, have been found to increase the susceptibility of developing ovarian and breast cancer [33].

In summary, instability of the genome in cells causes genetic alterations and subsequently allows the cells to acquire proliferative and anti-apoptotic traits which favour cancer progression and metastasis. These characteristics of cells in the progression of cancer have collectively been described as the hallmarks of cancer.

1.1.3 The hallmarks of cancer

The hallmarks of cancer were first proposed by Douglas Hanahan and Robert A. Weinberg in 2000 and these were reviewed again in 2011 and 2022 (Fig 1.1). They characterized cancers with features that favour survival, proliferation, and metastatic dissemination, in which cells which acquire these biological capabilities will progress to a neoplastic state with malignant properties. The hallmarks include: sustaining

proliferative signalling, resisting cell death, evading growth suppressors, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis, deregulating cellular energetics and avoiding immune destruction [34]. The list of hallmarks also includes two enabling characteristics: tumour-promoting inflammation and genome instability and mutation. In 2022, new additions included cellular plasticity, non-mutational epigenetic reprogramming and polymorphic variations in organ/tissue microbiomes [35]. In 2023, Douglas Hanahan suggested the involvement of neuronal signalling in modulating tumour development and neuronal innervation as a common constituent of the tumour microenvironment [36].

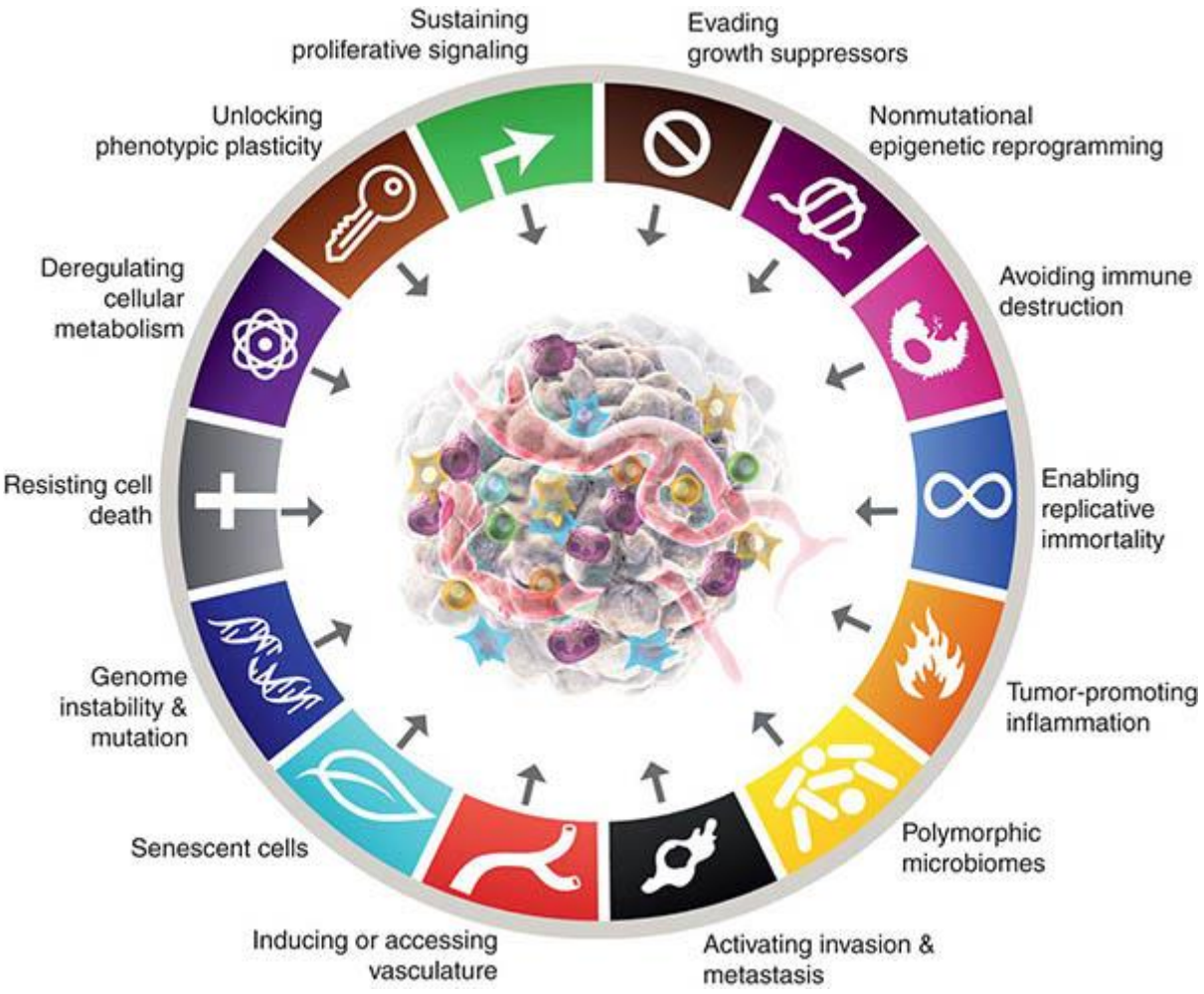

Figure 1.1 The hallmarks of cancer. The figure shows the biological capabilities acquired during cancer progression and adapted from [35].

1.1.3.1 Tumour cell growth

The first defined characteristic of cancer is self-sufficiency in growth signals. Normal cells require mitogenic growth signals from the extracellular microenvironment to proliferate, while tumour cells acquire the ability to activate oncogene which results in the production of their own growth signals with independence of exogenous stimulation [37]. This autonomy with cells independent of the microenvironment disrupts homeostasis. The tumour cells are also insensitive to anti-growth signals which are responsible for maintaining cellular quiescence and tissue homeostasis [38].

In order to escape anti-growth signals, tumour cells adapt to evade growth-supressing factors by disrupting the retinoblastoma protein (pRb) pathway [39]. Tumour cells also have their own mechanisms to avoid programmed cell death like apoptosis and anoikis. The most common strategies tumour cells apply are the loss of p53-mediated apoptotic regulation [40] and production of survival-promoting insulin-like growth factors (IGFs) [41]. These three hallmarks of cancer (resistance to anti-growth signals, growth signal autonomy, and apoptosis) allow tumour cells to escape normal cellular senescence and endow them with replicative immortality.

1.1.3.2 Angiogenesis in cancer

One of the key features of tumour cells is to invade lymphatic or blood vessels and circulate in the intravascular stream, in which sustained angiogenesis plays a critical role in tumour cell function and survival [42]. As cells growth require constant supply of oxygen and nutrients as well as the ability to eliminate the metabolic waste, tumour neovascularisation provides cells with assess to the elements they need [43]. This process includes four steps: (1) There is immediate destruction and hypoxia and the basement membrane in tissues is damaged; (2) Angiogenic factors migrate and activate endothelial cells; (3) Proliferation and stabilization of endothelial cells; (4) Angiogenic process is continuously promoted by angiogenic factors [42].

Angiogenesis is tightly regulated and sustained predominantly by regulation of several proteins. These proteins are produced by endothelial cells, tumour cells, and the surrounding stroma. Several proteins termed proangiogenic factors are considered to induce endothelial cell growth, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), estrogen, angiogenin, and interleukin 8. For example,

it was first characterized that VEGF induces vascular leakage and permeability thus promoting vascular endothelial cell proliferation [44]. However, the effect of proangiogenic factors is countered by inhibitory agents called antiangiogenic factors, such as tissue inhibitors of metalloproteinases (TIMP). thrombospondins, and endostatin. These proteins prevent tumours from increasing their size [45]. For example, endostatin is reported to inhibit endothelial cell migration, which results in increased endothelial cell apoptosis and cell cycle arrest *in vitro* [46]. The overall outcome of angiogenesis homeostasis is determined by both proangiogenic and antiangiogenic factors on the vascular endothelial cell. Previous studies have shown that angiogenic proteins are involved in the growth and spread of tumours. VEGF proteins are expressed in 50% of human cancers [47] and these factors are reported to develop in the uterine cervix [48], endometrium [49], ovary [50] and stomach [51]. Also, correlations between VEGF expression and the poor prognosis of cancers are found in lung cancer [52], colorectal cancer [53], breast cancer [54], head and neck cancer [55], and malignant mesothelioma [56]. These studies have also suggested that the aggressiveness of tumour cell spreading is associated with the expression of angiogenic factors, therefore they are valuable markers to identify high-risk patients who have a poorer prognosis.

1.1.3.3 Tumour metastatic spreading

The ability to invade and metastasis of cancer cells is responsible for the majority of human cancer deaths [15]. For cells to invade, the attachments between cells are often lost as well as their shapes being altered. Cell-cell adhesion molecules such as E-cadherin, which assists with assembling epithelial cell sheets and sustaining the

quiescence of the cells within these sheets, are often downregulated and inactivated [57]. In addition, a regulatory program called epithelial-mesenchymal transition (EMT) plays a key role in transforming epithelial cells into their invasive form [58]. There are several transcriptional factors involved in facilitating EMT and programming invasion, including *Slug*, *Zeb1/2*, *Twist* and *Snail* [59]. The expressions of these transcriptional regulators are responsible for loss of morphology conversion from epithelial to fibroblastic morphology, loss of cell-cell and cell-matrix junctions, expression of enzymes that degrade matrix, increased cell motility and resistance to apoptosis [59], thus, promoting tumour invasion. Interaction between cancer cells and neighbouring stromal cells is also required for activation of EMT where they recruit different cells into the surrounding stroma, including myofibroblasts, fibroblasts, macrophages, granulocytes, lymphocytes, and mesenchymal stem cells [60]. The recruitment of these cells provides an inflammatory environment and assists with release of EMT-inducing signals such as transcriptional factors.

Besides local invasion at the primary tumour site, metastasis is a multistage process involving cancer cells intravasating into nearby blood and lymphatic vessels, extravasating into the parenchyma of distant tissues, and eventually proliferate and establish themselves at the distant site [61].

In order to adapt and conquer the distant site, there are a few steps that circulating cancer cells need to achieve, including penetrating the distant tissue, escaping immune surveillance, surviving in the supportive niches, and eventually replacing the host tissue [62]. Cancer cells from the primary tumour normally undergo apoptosis at the secondary site within 24 hours of extravasation [63, 64], while cells with the ability to obtain mitogenic stimulation from cytokines and growth factors at the secondary site are likely to colonize successfully. These cells are able to self-renew and recruit the

supporting stroma, including a blood supply [65]. Factors from the primary tumours such as VEGF-A have been found to move bone marrow-derived cells towards premetastatic sites [66]. The cells from the premetastatic niche subsequently secrete factors such as SDF-1 that can recruit disseminating tumour cells to help tumour cells adapt at the distant site [66, 67].

1.1.3.4 Tumour microenvironment

The tumour microenvironment (TME) is the ecosystem that surrounds a tumour inside the body and it contains different cells such as immune cells and stromal cells, and there are also non-cell components such as blood vessels and extracellular matrix [68]. A tumour and its microenvironment often interact and affect each other, either positively or negatively, and it is believed that the TME participates in many stages of cancer progression (Fig 1.2). The interaction between cancer cells and TME occurs in the early stage of tumour growth. This allows cancer cells to escape apoptosis and facilitate local invasion [69].

Immune cells in TME can either perform both pro- and anti- tumorigenic effects [70]. Two types of immune cells are known in humans: adaptive immune cells and innate immune cells. Adaptive immune cells such as T-cells and B-cells are responsible for triggering the immune response towards specific antigens while innate immune cells such as dendritic cells and neutrophils are responsible for the non-specific defence mechanism that occur within hours of exposure to foreign antigens [71]. Stromal cells from nearby endogenous tissue stroma are also recruited by cancer cells during tumour formation [72]. Upon being recruited to the TME, stromal cells can release factors such as chemokines to promote tumour proliferation, invasion and metastasis

[73]. Endothelial cells (EC) in TME can help coordinate blood vessel formation, provide water and a nutrient supply, and sustain metabolic homeostasis [74]. They play key roles in angiogenesis in which they secrete proangiogenic factors such as VEGF. VEGF, in turn, stimulates endothelial cells to form new blood vessel lumens in an autocrine and paracrine fashion [75]. During cancer progression, fibroblasts are transformed into cancer associated fibroblasts (CAFs) which are induced by TGF-β and bone morphogenetic protein (BMP) during EMT [76, 77]. CAFs are another important component in TME that contribute to tumour proliferation, neoangiogenesis, ECM remodelling, immunosuppression and metastasis [78]. They are in the tumour stroma and are strongly associated with communication between cancer cells and TME. They often originate from tissue resident fibroblasts and are formed from adipocytes, ECs, pericytes, stellate cells and bone marrow derive mesenchymal stem cells [77]. In the TME, cancer cells and associated stroma cells secrete transforming growth factors to transform fibroblasts into CAFs, such as TGF-β [79]. An abundance of CAFs within the TME is often associated with poor prognosis in many cancer types [80]. For instance, in colorectal cancer, the presence of CAFs is strongly associated with disease recurrence [81]. Many of the extracellular components within the TME are produced by CAFs, such as growth factors, cytokines and extracellular matrix [82, 83], and these promote cancer cell invasion and metastasis [84]. TME also contains non-cellular components such as ECM. ECM has important functions within the TME through providing a physical scaffold for cells and promoting tumour cell dissemination [85]. ECM contains collagen, fibronectin, elastin, and laminin and account for approximately 60% of the mass of solid tumours [86]. Accumulation of collagen can result in desmoplasia which is strongly associated with poor patient prognosis [87-89]. The ECM is a reservoir for cytokines and growth factors, which can be further released

through ECM degradation facilitated by proteases such MMPs, thus promoting cancer progression [90].

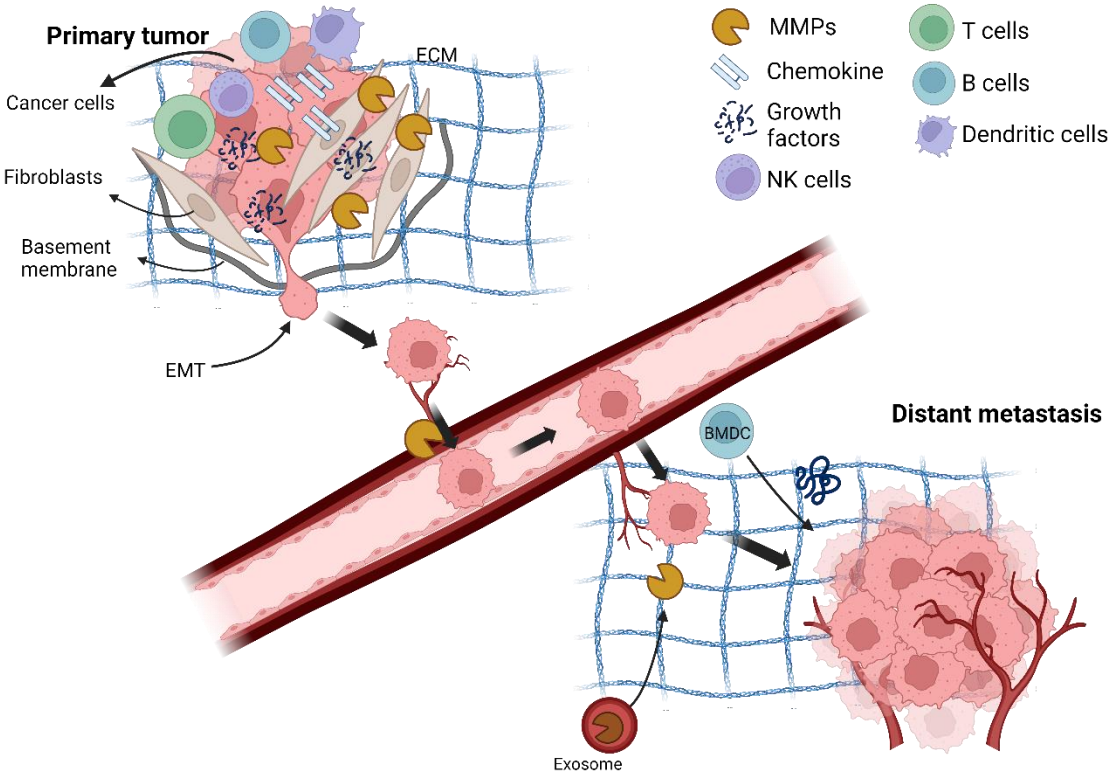

Figure 1.2. The tumour microenvironment regulates cancer metastasis and contributes to every stage of tumour metastasis. TME is a dynamic ecosystem composed of many different cells involved in cancer progression. The communication between cancer cells and other cells allows cancer cell to invade from the primary site, intravasate into blood vessels, extravasate blood vessels and finally colonize the distant site.

1.2 Proteolytic enzymes

It has been over 100 years since proteolytic enzymes were first mentioned by P.A. Levene in his studies on “The cleavage products of proteoses” in the first issue of The Journal of Biological Chemistry [91]. The concept of these proteins was initially defined as destructive enzymes that are essential for protein catabolism generating new amino acids in living organisms. Through the years, studies on proteases have started to reveal their other roles other than demolishing proteins non-specifically, as they catalyse exceptionally specific proteolytic reactions [92].

The fundamental molecular action of a proteolytic enzyme is to cleave a protein or a large peptide and release an amino acid or a peptide [93]. The term “proteolysis” refers to an irreversible process that is often involved in post-translational processing, which can happen immediately after synthesis of a protein or after the protein has translocated [94]. The outcomes of proteolysis allow cells, tissues or organs in living organisms to function properly, and the process is essential to life.

First of all, proteolysis is required to process proteins which allows them to translocate to their destinations, including removing the methionine residue from newly synthesized proteins, removing signal peptides from proteins that are destinated for the cellular secretory pathway and removing targeting signals from proteins that destinated for specific organelles [95]. Second, it is essential for the activation of enzymes or hormones in which proteolysis removes the pro-peptides from the precursor forms of these proteins [92]. It can also break down polyproteins into individual proteins and peptides and release proteins from the cell surface [92]. Moreover, proteolysis terminates the cellular signalling that peptides or proteins recruits by destroying either them or their binding partners [96]. Finally, proteolysis is

essential for organisms to defend against external invasion, including terminating toxic proteins from parasites and pathogens, or releasing antigenic peptides from parasites and pathogens [97, 98].

Table 1.1. Identified proteolytic enzymes in living organisms

Species	**Number of proteolytic enzymes**
Arabidopsis thaliana	678
Bacillus subtilis	190
Caenorhabditis elegans	359
Drosophila melanogaster	477
Escherichia coli	405
Mus musculus	677
Homo sapiens	641
Saccharomyces cerevisiae	117
Thermoplasma volcanium	43

Proteolytic enzymes are encoded in the genomes of almost all living organisms with the exception of particular viruses which hijack host enzymes to do the processing for themselves. Up to date, 641 proteolytic enzymes have been identified in humans and 677 in the mouse (Table 1.2.1) [99].

Nearly all proteolytic enzymes are hydrolases that break a chemical bond by the addition of a water molecule. The current understanding of proteases derives from the collection of studies stating their involvement in many biological processes. Overall, proteases regulate protein localization, protein-protein interactions, cellular signalling, and bioactive molecule synthesis. As a result, proteases are associated with DNA replication and transcription, cell proliferation, tissue remodelling, neurogenesis, angiogenesis, wound repair, fertilization, haemostasis, stem cell mobilization, immunity, inflammation, senescence, autophagy, apoptosis and necrosis [100-104].

1.2.1 Structure of proteases

The simplest protease molecule (example of chymopapain shown in Fig 1.3A) has two subdomains which are separated by the active site. There are the catalytic residues within the active site such as a catalytic dyad or triad, and an additional residue which stabilizes the intermediate state between the enzyme and substrate [105]. Substrate-binding sites are in line with the active sites. When the specific amino acids fit into its exclusive binding site, it establishes the specificity of the enzyme. The specificity can be also determined by residues on either side of the cleavage site in a substrate or an amino acid several residues away from the cleavage site. It is evident that binding sites can cooperate in which a particular substrate occupying one binding site can lead to binding of a neighbouring binding site by another amino acid [105].

In addition, there are binding pockets on the surface or inside of the protein which possess the ability to bind a ligand [106]. The binding pockets in the peptidase are termed as S_1, S_2, S_3… on the N-terminal side of the scissile bond, and S_{10}, S_{20}, S_{30}… on the C-terminal side. There are also residues in the substrate termed as P_1, P_2, P_3… on the N-terminal side of the scissile bond, and P_{10}, P_{20}, P_{30}… on the C-terminal side (Fig 1.3B).

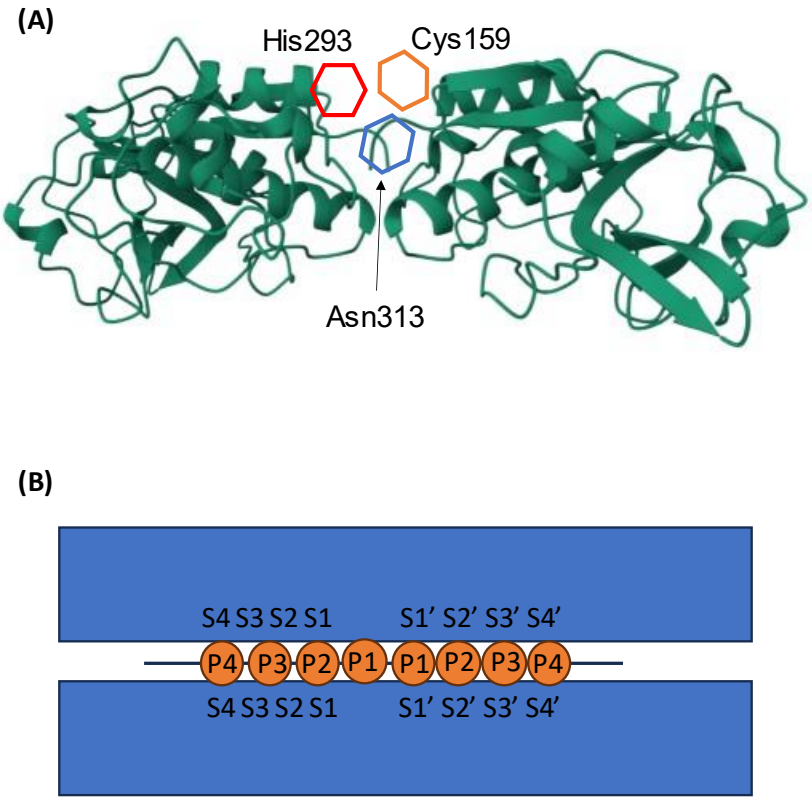

Figure 1.3. The structure of chymopapain and its binding with substrates. (A) Chymopapain (PBD:1YAL) has two subdomains that are separated by an active site, the active site residues are shown by hexagons with residue Cys159 in orange, His293 in red and Asn313 in blue. (B) The binding of peptidase binding site and substrate. The enzyme is shown as a blue rectangle, and the residues in the substrate are shown as orange balls connected by a string. The substrate binding pockets in the peptidase are shown as S4–S1 and S1'–S4'. Residues in the substrate are shown as P4–P1 and P1'–P4'.

1.2.2 Classification of proteases

There are several ways to classify proteases including specificity, catalytic type and homology. By specificity, proteases can be classified into two groups: exopeptidases, with a free N- or C-terminus to facilitate its function, and endopeptidases, with at least one terminal blocked, using internal peptide bonds to facilitate its function [107].

According to the MEROPs database, exopeptidases can be further divided depending on their catalytic action at which terminus, and how many amino acids are released in the catalytic reaction. There are:

1) Aminopeptidases that release one N-terminal amino acid
2) Dipeptidylpeptidases that release a dipeptide from the N-terminus
3) Tripeptidylpeptidases that release a tripeptide from the N-terminus
4) Carboxypeptidases that release one C-terminus amino acid
5) Peptidyl-dipeptidases that release a dipeptide from the C-terminus
6) Dipeptidases that only cleaves a dipeptide

The classification of endopeptidases is less obvious than exopeptidases in which the only unconventional rule to distinguish them is whether they act on proteins of any size or only act on short peptides (termed as oligopeptidases).

Proteases can also be classified based on their catalytic actions. There are serine, cysteine, aspartic, threonine, metalloproteases, glutamic, and asparagine peptide lyases [108]. The groups of proteases including glutamic, aspartic and MMPs use a water molecule as a nucleophile to attack the peptide bond of the substrate, while the other groups of proteases use an amino acid residue as a nucleophile. Moreover,

aspartic peptidases can be further subdivided into two groups base on their structure: pepsins and pepsin-like enzymes and rennet and rennet-like enzymes [109, 110]. Serine proteases are classified into subtilisin-like and chymotrypsin-like based on their structure, and further grouped into 13 clans and 40 families base on their catalytic residues and primary specificities [111]. Cysteine proteases are broadly classified into two subfamilies: cathepsin-B-like and cathepsin-L-like proteases, based on the structures of the prodomain and the mature domain [112]. Glutamic proteases are grouped into two independent families: G1 and G2, where G1 contains Glu and Gln dyad and G2 contains Glu and Asp dyad at their active sites [113]. There are two subfamilies of MMPs: exopeptidases and endopeptidases, where endopeptidase targets internal peptide bonds of a protein while the exopeptidase targets peptide bonds at the terminals [114, 115].

1.2.3 Catalytic mechanisms

The catalytic mechanism of proteases differs from their residues at the active site. Aspartic peptidases have Asp residues at the active site, being ligands of the activated water molecules. Most aspartic peptidases contain two aspartic residues which bind and activate the catalytic water molecule with exceptions being those whose second Asp is replace by residues of other amino acids [116]. Serine endoproteases contain a serine residue at their active sites which can form an acyl-enzyme intermediate by attacking the carbonyl moiety of the peptide bond of the substrate [117]. Cysteine endoproteases contain a Cys-His-Asn triad at their catalytic sites. The cysteine residue can form a tetrahedral thioester intermediate by attacking the carbon of the reactive peptide bond of the substrate, and it releases an amino terminus fragment of the

substrate [118]. Threonine proteases contain a threonine (Thr) residue within the active site. They use the secondary alcohol of their N-terminal threonine as a nucleophile to attack the peptide bond of the substrate to form a covalent acyl-enzyme intermediate [119]. Glutamic proteases contain a glutamic acid residue within the active site where a catalytic dyad: glutamic acid and glutamine presents. The glutamic acid acts as a general base acid, donating a proton to the carbonyl oxygen in the peptide bond of the substrate and further donates another proton to the amide nitrogen, leading to breakage of the peptide bond. Matrix metalloproteases (MMPs) are a family of proteases whose catalytic mechanism involves a metal where most MMPs require zinc while some contain cobalt. The metal ion is connected to the protein through three ligands which varies with histidine, glutamate, aspartate, lysine, and arginine. The catalytic action by MMPs starts with the nucleophilic attack of the water molecule coordinated with zinc to the carbonyl carbon of the substrate, then the glutamic acid residue moves the proton to the amine nitrogen to form a gem-diol reaction intermediate [120].

1.2.4 Functions of proteases in physiological conditions

In the early stage of studying proteases, their predominant role was always considered as degrading proteins. Over decades of study, it is evident that proteases are more than just destructive enzymes, instead they have a range of functions. First of all, proteases are responsible for removing damaged proteins and generating free amino acids that are required to generate new proteins, and they also participate in post-translational modification [121-123], in which post-translational processing determines the fate and functionality of a protein [124]. Proteases are also associated with peptide

processing such as in the cellular defence against external pathogens [125, 126]. In addition, some proteases can have non-proteolytic roles. For instance, non-proteolytic roles in the proteasome are essential for ubiquitin-dependent histone modification, recruitment of activators and co-activators to promoters, elongation of transcription and transport of mRNAs from the nucleus to the cytoplasm which leads to their maturation [127].

Many proteases are lysosomal proteases and the expression of lysosomal proteases is ubiquitous which is in a tissue- or cell-specific manner [119]. There are more than 50 proteases localized in the lysosomal compartment where they facilitate catalytic actions within the lysosome [128]. Considerable numbers of them are intracellular cathepsins, including cysteine cathepsins (cathepsin B), serine (cathepsins A and G), and aspartic (cathepsins D and E) [129]. The non cathepsin lysosomal proteases include the aspartic protease Napsin and the cysteine protease asparagine endopeptidase [130]. The localization of lysosomal proteases is usually towards the inside of vesicles in the endocytic pathway [130], and they are also secreted to the cell surface and into the pericellular surroundings [131]. There are many specific functions that lysosomal proteases facilitate, including degrading bulk proteins, proprotein processing, antigen processing, prohormone processing and extracellular matrix (ECM) degradation, as well as initiating apoptotic processes within the cytosol [130]. Lysosomal proteases are essential for cellular signalling where they modulate and terminate cellular signalling pathways by separating growth factors from their receptors followed by degradation and recycling of proteins to the plasma membrane [132]. For example, cathepsin B has been reported to degrade EGF in the liver as well as internalized EGF receptor complexes [133]. Insulin-like growth factor-I (IGF-I) is also the target of cathepsin-B endolysosomal degradation [134]. Cathepsin D was found

to degrade endosomal insulin [135], while cathepsin L was reported to contribute to degradation of insulin-like growth factor binding protein 3 (IGFBP-3) [136]. Besides lysosomal proteases, other non-lysosomal proteases like MMPs mainly target extracellular substrates such as ECM proteins as well as pro-peptides of other proteases. The main functions of MMPs in physiological conditions are to proteolyze ECM proteins, participate in membrane shedding and chemokine processing, and inactivate or activate other proteases [120].

One of the key roles of proteases in physiological condition is involvement in cellular signalling. Protease signalling is irreversible which differs from other cellular signalling like kinase signalling or receptor signalling. It transmits the signal through the cleavage of proteins, which allows the target protein to be inactivated, activated or altered functionally [137]. Most protease signalling is carried out by protease catalytic activity in which a protease directly cleaves the substrate. For example, cytokines are processed by pro-inflammatory caspases to become their its mature forms [138], and furin cleaves pro-protein to its maturation in the secretory pathway [139]. In other cases, there are some cascade-like signalling pathways involving proteases in which there might be sequential processing of non-protease proteins. For example, regulated intramembrane proteolysis is induced by intramembrane cleaving proteases (ICLiPs) [140], and degradation of intracellular proteins is facilitated by the ubiquitin-proteasome system [141]. There are more complex protease signalling processes such as cascade organization in which the zymogen of one protease is activated by another protease. For example, activation of serine proteases by thrombin occurs in the final steps of the blood coagulation cascade [142].

1.2.5 Regulations of protease activities

Given the irreversible hydrolytic reaction catalysed by proteases in many biological processes, the activities of proteases in living organisms must be tightly regulated. Overall, there are five mechanisms that are responsible for this, including regulating gene expression of proteases, modulating their activation, involvement of endogenous inhibitors, targeting specific compartments and post-translational modification [93].

Transcriptional regulation of gene expression is widely unclarified for most proteases, but there are exceptions such as MMPs, which has great relevance for human diseases. MMPs are reported to be primarily regulated at the transcriptional level and the promoters of MMPs have cis-elements which can be recognized by trans-activators such as AP-1 and PEA3 [143]. Most MMPs contain AP-1 and PEA3 binding sites, and many growth factors or cytokines, such as EGF, VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), interleukins, interferons, tumour necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β) activate cell signalling via binding to AP-1 or PEA3 to induce trans-activation of MMP promoters [144]. Mitogen-activated protein kinases (MAPKs)-mediated phosphorylation is also shown to increase trans-activation of MMP promoters through AP-1 or PEA3 [145]. It was also found that MMP-7 [146], MMP-14 [147], MMP-12 [148], and MMP-26 [149] promoters contain a Tcf-4 site which is for β-catenin/LEF binding and the MMP-9 promoter contains s NF-κB-binding site [150], and they are also regulatory for gene expression. Moreover. it has been shown that Wnt signalling mediates MMP expressions via a Tcf-4 site [151] in which MMP-7 expression is increased by wnt-2 expression via stabilization of β-catenin, leading to an invasive phenotype in cancer cells [152]. It was also found the MMP-9 expression is regulated by TNF-α with the NF-κB-binding site in its promoter [150]. It was also reported that

the metastases suppressor (KiSS-1) blocks NF-κB nuclear translocation, subsequently inhibiting MMP-9 expression in fibrosarcoma cells [153].

All known cellular proteases are produced as zymogens or inactive precursors in order to prevent unnecessary protein degradation. These precursors are suppressed by inactivation mechanisms and some of them require conformational changes to be activated [154]. The activation or conversion of zymogens into the active enzyme normally occurs in a subcellular compartment or the extracellular milieu, where enzyme catalyses proteolysis of an inhibitory “activation segment”. This activation segment is in the N-terminal region and has limited access for substrates to the active site, and it can be only accessed under conditions like pH reduction [154]. For example, in aspartic proteases, a prosegment with 45 residues puts a steric blockage at the active site. These aspartic protease zymogens are also constrained by salt bridges with the active site Asp residue. The activation process for aspartic proteases requires disruption of salt bridges and limited proteolysis of the 45 residue prosegment, which refolds the mature N-terminal region [155, 156]. The activation of zymogens can be carried out by other proteases or additional factors such as the apoptosome which is associated with activating pro-apoptotic caspases [157]. Regulation of protease activation might come from protein cofactors including the tissue factor glycoprotein that initiates the coagulation cascade by binding to serine protease factor VIIa [158].

Endogenous protease inhibitors cause non-specific inhibition of proteases where one protease inhibitor can target several proteases [159, 160]. It derives from the fact that there are considerably lower number of endogenous protease inhibitors than proteases. For example, in the rat genome, there are a total of 183 genes encoding proteases inhibitors, whereas more than 600 protease genes have been annotated in this species [161]. Therefore, the catalytic activities of many proteases are regulated

by other mechanisms other than being blocked by endogenous inhibitors. Nevertheless, there are four groups of endogenous protease inhibitors and these have different mechanisms of inhibition [162, 163]. First, the canonical inhibitors, such as serpins, inhibit their target proteases by mimicking substrate binding and blocking the active site [164, 165]. Second, exosite-binding inhibitors, such as cystatins and thrombin inhibitors, disrupt substrate binding by binding to a region next to the active site [166]. Third, there are protease inhibitors such as TIMPs which combine the previous two mechanisms to facilitate their inhibitions [167]. Finally, allosteric inhibitors disrupt dimerization of the target protease and inhibit its activity through binding to a region that is distantly located from the active site [168].

Other regulatory mechanisms are also involved. For example, it has been found that many microRNAs contribute to MMP regulation and are associated with diagnosis of malignant tumour [169]. Also, protein oxidation can induce protein aggregation or hydrolysis of proteins that directly or indirectly interrupts proteolysis [170]. Taken together, all these mechanisms work in a coordinated manner to ensure that appropriate proteolytic actions are facilitated at the correct time, to prevent potentially harmful outcomes from uncontrolled protease activities.

1.2.6 Proteases in cancer

The dysregulation of protease expression and secretion is widely known to be associated with many diseases including cancer. The dynamic role of proteases in cancer progression has been well established over the decades. Back in 1946, Fisher first proposed that the degradation of the cell matrix and subsequent invasion of tumour to the surrounding tissues could be caused by tumour-associated proteolytic

activity [171]. Later in the 1970s, individual proteases started to be discovered, in which members of the cysteine, serine proteases and MMP families were shown to promote metastatic activities. After two more decades of research, studies revealed that many proteins were involved in regulating cellular invasion and metastasis. To date, we now consider proteolytic enzymes to be involved in all stages of cancer progression, where proteases act as signalling molecules involved in processes including cancer cell proliferation, adhesion, invasion and migration, angiogenesis, metastasis and escape immune surveillance [172]. Intracellular proteases such as lysosomal proteases are normally responsible for the removal of damaged or unwanted proteins and these activities are tightly regulated. Downregulation of these proteases presents in various human cancers, suggesting the cancer suppressor role of these enzymes in the classical sense [173, 174]. On the other hand, extracellular proteases such as MMPs, are considered to actively promote tumorigenesis due to their overexpression in malignant tissues [175]. However, these roles are not fixed, a paradoxical role for proteases has been shown in cancer progression where these enzymes are associated with the hallmarks of cancer where different groups of proteases have been correlated with specific cancer hallmarks shown in Fig 1.4.

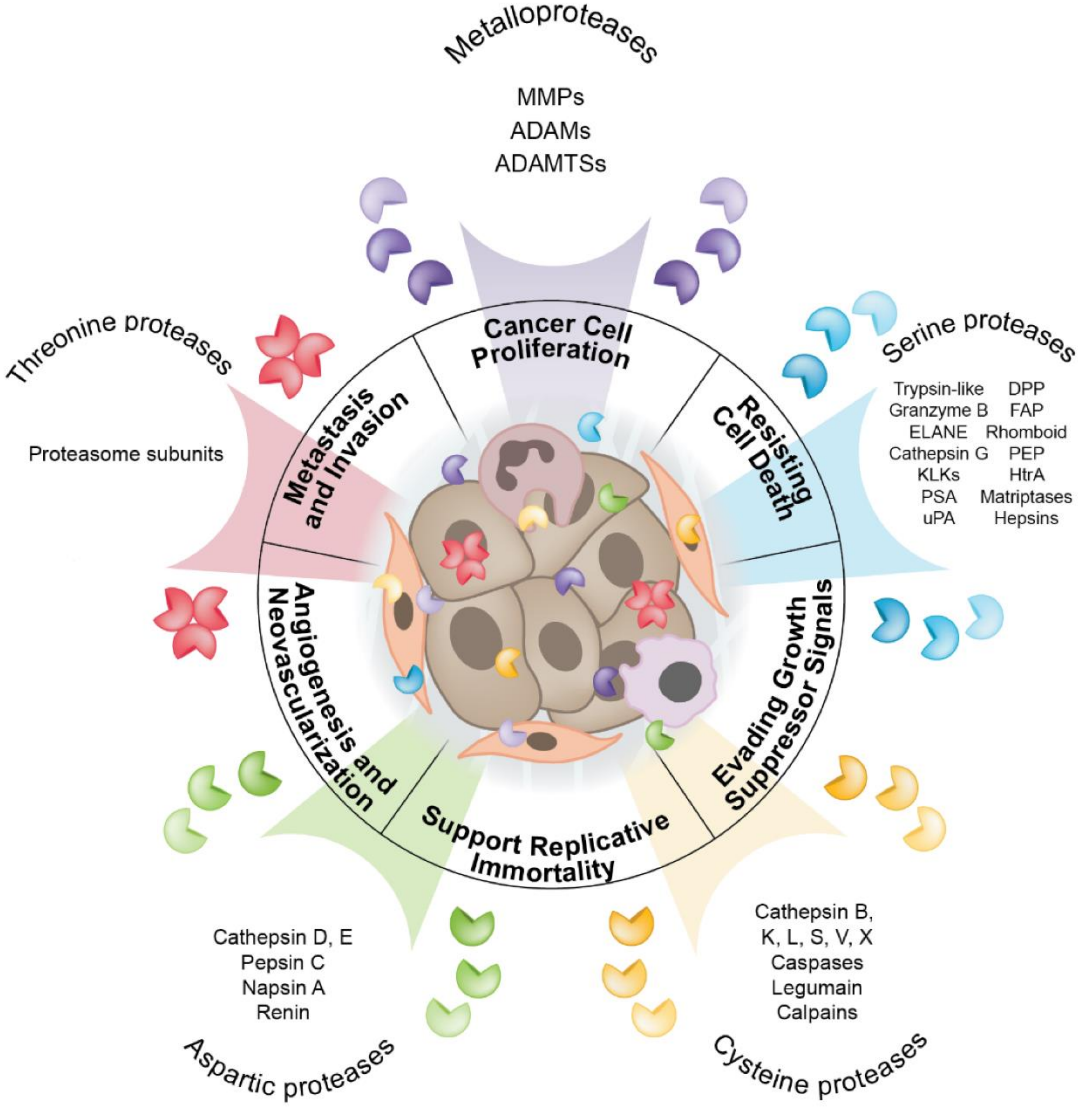

Figure 1.4. Proteases and hallmarks of cancer. Different groups of proteases originate from tumour and other cells in TME, and they contribute to different aspects of cancer progression with their proteolytic activities. Source from [176].

1.2.6.1 Proteases in the tumour microenvironment

As mentioned earlier, dysregulation of protease production and activity is associated with various cancer hallmarks and the association between proteases and TME has been widely demonstrated. Different proteases are produced by either cancer cells or other cells such as stromal cells that are present in the TME [177]. Also, the other key characteristic of proteases in the TME is the differential concentrations in which it can vary from barely detectable amounts to very high amounts [178]. The localization of proteases varies as well in which multi-protein complexes can be found that are composed of proteases and their inhibitors [179]. Finally, different proteases affect each other, for instance, the activation cascade of MMP-9 and MMP-2 is induced by MMP-13 [180, 181]. Taken together, these factors are responsible for establishing an interactive proteolytic network in the tumour microenvironment (Table 1.2).

Table 1.2. The roles of proteases in the TME

Hallmark of cancer	Associated proteases	Functional roles of proteases	References
Tumour growth	MMPs Kallikreins Cathepsins ADAM10	ECM degradation, sustain proliferative signalling pathways, processing of growth factors	[182-189]
Resisting cell death	MMPs Cathepsins Caspases HtrA Granzyme B ADAM	Apoptosis signalling, apoptosis resistance, autophagy recycling, immune system evasion	[190-193]
Evading growth suppressor signals	MMPs Cathepsins Kalikreins ADAMs ADAMTs	Secretion of cytokine and chemokine, receptor signalling, interrupting p53 signalling	[190, 194, 195]
Supporting replicative immortality	MMPs and ADAMs Granyzme B Cathepsins KLK4-7	Maintain growth signalling, immune system hijacking	[182, 185, 187, 192, 196]
Angiogenesis	MMPs Kallikreins PSA Cathepsins Calpains	ECM degradation, growth factor signalling, secretion of cytokines	[187, 197-200]
Tumour invasion and metastasis	MMPs ADAMs Kalikreins PSA FAP, DPPIV, PEP Cathepsins Legumain Calpains	Cancer cell migration, ECM degradation, EMT, receptor signalling, metabolic signalling, kinase signalling	[185, 187, 197-199, 201-205]

1.2.6.2 Proteases in tumour growth

Many proteases, as the key regulators of cell proliferation and apoptosis, play crucial roles in promoting tumour growth. Through digesting ECM, the primary role of MMPs in tumorigenesis is to facilitate the release of encapsulated tumour cells [206]. The follow-up release of activated growth factors after ECM degradation promotes tumour growth, in which MMPs are found to catalyse membrane-bound growth factor precursors [191]. Similarly, MMPs regulate cellular signalling by mediating several tumour growth-associated pathways such as rat sarcoma viral oncogene homolog (RAS), phosphatidylinositol 3-kinase (PI3K), FAK, extracellular regulated MAP kinase (ERK), and rous sarcoma oncogene (SRC) [207]. For instance, FAK activation is mediated by MMPs in which MMPs catalyse integrin and break the cell-ECM adhesion, subsequently promoting oncogenic activities [208]. The interplay between MMPs and Src phosphorylation also contributes to the mitogenic activities [197, 209-211]. Therefore, MMP-mediated pathways in cellular proliferation are associated with tumour progression. In addition, some MMPs disrupt tumour suppression processes, thus promoting tumour growth. MMP-14 is involved in disrupting the production of a tumour suppressor protein, brain-specific angiogenesis inhibitor 1 (BAI1) [212], when MT1-MMP inhibits the tumour suppressor Sprouty RTK signaling antagonist 4 (SPRY4) expression, subsequently favouring cell invasion in melanoma cells through MMP2/RAC1 activation [213]. It has also been shown that MMP-9 is induced by mouse double minute 2 homolog (MDM2) which promotes its tumorigenic activities through inhibiting p53 expression [214], and si-RNA inhibition of MMP-9 results in increased expression of activation of cell cycle inhibitor proteins such as p16 and inducing ERK-mediated senescence [215].

Cysteine protease like cathepsins and caspases are most directly involved in tumour growth. Cathepsins such as cathepsin B can induce tumour growth via activating various growth factors, such as TGF-β [216]. Lysosomal cathepsin B is also associated with apoptosis, acting as a downstream effector of caspases [217] [218]. Caspases are known to activate apoptosis in cells and the association between caspase expression and cancer cell sensitivity is shown to affect apoptosis [219]. It was first found that the caspase 8 gene was downregulated in neuroblastomas [173], lung, head and neck, gastric and colorectal carcinomas [220-222], leading to increased tumour growth. Further studies have revealed that other caspases like caspases -3, -5, -6, -7 and -10 are also downregulated in various cancers [223-228], which in turn is associated with increased tumour growth. Taken together, these results suggest that the downregulation of caspases is significantly associated with tumour growth via deregulated cancer cell apoptosis.

In humans, the most involved aspartic protease in tumour growth is renin. Renin is the key component of the renin-angiotensin system, where elevated activity of RAS promotes cell proliferation [229]. The introduction of selective synthetic renin inhibitors to glioblastoma cells was found to reduce cell proliferation and induce apoptosis [230]. In addition, the inhibition of threonine proteases such as proteasomes is reported to reduce cancer cell proliferation via stimulating pro-apoptotic proteins and disrupting NF-κB pathway, therefore suppressing tumour growth [231].

1.2.6.3 Proteases in tumour invasion and metastatic spread

A considerable number of proteases contribute towards tumour invasion and metastasis. First, MMPs are the main contributors of ECM degradation. The ECM is

an essential component in TME that holds cancer and other cells together in which it provides signals and contributes to cell-cell communication [232]. The cancer cells are not able to pass through ECM due to the pore size of ECM being less than 7 μm^2 [233], therefore degradation of ECM by proteases is essential to allow cells to invade, and MT1-MMP is one of the main proteases responsible for ECM degradation [234, 235]. There are other MMPs that mediate ECM degradation during cancer progression [236]. MMP-1 and -2 have been reported to promote associated angiogenesis and metastasis in breast cancer [237]. MMP-3 overexpression has been reported to correlate with increased lung cancer metastasis [238]. MMP-13 overexpression induced by TNF-α is also associated with enhanced lung cancer metastasis [239]. MMP-1 from fibroblasts that targets protease-activated receptors type-1 (PAR-1) was also shown to activate signalling cascades to promote breast cancer cell invasion [240]. In addition, the NF-κB/MMP-7 signalling pathway promotes tumour invasion and metastasis in human bladder urothelial carcinoma through disrupting the expression of solute carrier family 12 member 5 (SLC12A5) [241]. The overexpression of MMP-9 is reported to contribute to EMT in prostate cancer and its metastasis [242].

Many cathepsins are also involved in promoting tumour invasion and metastasis, and cysteine cathepsins are the most involved cathepsins in cancer progression. Cathepsin B was found to promote the metastatic spreading of squamous carcinoma cells [243]. Cathepsin B-deficient transgenic mice showed reduced migration and invasion of tumour cells *in vitro* [244]. Cathepsin B is reported to degrade many components of ECM and basement membrane to promote tumour progression [245]. The introduction of selective cathepsin B inhibitor CA-074 diminishes ECM degradation and inflammatory breast cancer invasion [246] as well as reduced metastasis in tumour- bearing animals [247]. In addition, siRNA inhibition of cathepsin

B in tumour cells decreased collagen I degradation *in vitro* and bone metastasis *in vivo* [247]. Overexpression of cathepsin B was observed in thyroid carcinoma with increased invasion and metastasis along with a high level of degradation of types I and IV collagen [248]. A previous study showed that the plasma membrane in invasive EJ cells contained more active cathepsin B, while non-active cathepsin B stayed in lysosomes in non-invasive RT4 cells [249]. Cathepsin B and D are both reported to promote AGR2-mediated dissemination of pancreatic cancer cells [250]. A previous study showed that inhibition of cathepsin H by binding with its antibody decreased the invasion of glioblastoma cell lines [251], and cathepsin H also affected cell migration by affecting integrin activities [252]. The expressions of MMP-9 and cathepsin K were upregulated by coronin 3 and further promoted gastric cancer invasion and metastasis *in vitro* and *in vivo* [253]. It has been reported that invasive squamous cell carcinoma (SCC) has higher cathepsin K expression in the stroma than in other epidermal tumours [254]. Co-culture with cathepsin K expressing fibroblasts increased the invasion of breast-tumour epithelial cells and this invasion was inhibited by cathepsin K inhibitors [255]. Moreover, cathepsin K is reported to be overexpressed in primary and cutaneous melanoma metastases in which it promotes melanoma invasion and metastasis via degrading matrix proteins [256]. Cathepsin K also promotes prostate tumour metastasis to bone [257]. Cathepsin S has been found to promote cell invasion in various cancers including colorectal [258], gastric [259], and hepatocellular carcinoma [260]. siRNA inhibition oof cathepsin S expression disrupted tumour invasion in pancreatic islet cell cancer [261]. Upregulation of cathepsin X was found to increase cell invasion in gastric cancer *in vitro* [262]. Recent studies also showed that cathepsin X-mediated inhibition of the tumour suppressive function of profilin 1 is required for cancer cell adhesion, migration, and invasion in various types of cancer

[263]. Cathepsin Z is reported to be associated with the upregulation of MMP-2, MMP-3 and MMP-9, thus promoting hepatocellular carcinoma invasion and metastasis [264].

Other cathepsins like serine and aspartic cathepsins are also associated with tumour progression. Cathepsin A expression was found to be elevated in primary malignant melanoma lesions in comparison to normal pigmented nevi [265]. Cathepsin B has been shown to contribute to fibroblast-mediated invasion in esophageal cancer invasion into the ECM [266]. Cathepsin D is reportedly associated with facilitating various type of cancer cell proliferation and local dissemination [267] as well as Cathepsin L and other lysosomal proteins via the lymphatic system [268]. Overexpression of cathepsin D has been reported to promote fibroblast motility and invasion in breast cancer [269].

In addition, other proteases such as urokinase-type plasminogen activator (uPA) also play a key role in cancer invasion and metastasis. This protein belongs to the uPA system which also includes the uPA receptor and plasminogen activator inhibitors (PAIs) [270]. uPA activates plasminogen to plasmin which subsequently activate pro-MMPs to MMPs (such as MMP-9), therefore promoting cancer cell invasion and metastasis [271]. The uPA system regulates the Ras-ERK pathway and p38 MAPK pathway in breast cancer to facilitate metastasis [272].

1.2.6.4 Proteases in angiogenesis

Angiogenesis is regulated by the net outcome between the activities of angiogenic and anti-angiogenic factors. Proteases such as cathepsins may regulate the production of angiogenic activators and inhibitors. Overexpression of cathepsin B is closely associated with the increased intensity of angiogenesis in cancer cells in resected

colon adenocarcinoma [273]. Stromal cathepsin D expression has been shown to correlate with microvessel density in ovarian tumours [274]. In addition, in breast cancer, cathepsin D overexpression in host stromal cells is associated with increased vascular density [275]. It has been found that Cathepsin H is associated with forming functional tumour vasculature and promoting hepatoma cell metastasis, while cathepsin H knockdown reduced angiogenic switching of pre-malignant hyperplastic islets [276, 277]. Moreover, cathepsin H-deficient RT2 mice have reduced tumour size, which is associated with less blood vasculature and increased apoptosis [278]. siRNA inhibition of cathepsin S in MHCC97-H cells reportedly reduced angiogenic islet formation and tumour growth and also caused decreased cell proliferation, invasion and angiogenesis [279]. It was also found that cathepsin S produced by cancer cells and CAFs promoted neovascularization and tumour growth in a syngeneic colorectal carcinoma murine model [280].

MMPs can also facilitate angiogenesis by degrading basement membrane and pro-angiogenic signals [281, 282]. MMP-9 is reportedly responsible for releasing angiogenic cytokines such as VEGF and chemokines such as CXCL5, 8, 6 to promote angiogenesis [283, 284]. Other MMPs such as MMP-2, -13 and -14 are also found to induce VEGF expression [285, 286]. In addition, MMP-1, −3, −7, −16 and −19 are seen to cleave and release matrix-bound VEGF to induce angiogenesis in cancer [287]. It was also found that MMP-9 and MMP-2 are associated with degradation of the angiostatic protein Multimerin 2 (MMRN2) produced by endothelial cells which subsequently promotes angiogenesis and restores pro-metastatic ability [288].

1.3 Galectin-3

Galectins are a family of lectins characterized by affinity towards β-galactosides and contain one or two conserved carbohydrate-binding domains [289]. There are 15 galectins discovered in mammals and they are also found in many other species [290]. Galectin-3 is a 32kDa protein of the galectin family and was first identified on the surface of murine macrophages [291]. Galectin-3 is a chimera-type galectin due to its N-terminal domain being adjacent to the carbohydrate recognition domains. The binding partners of galectin-3 are often glycoconjugates with N-acetyllactosamine, but its affinity toward ligands varies with additional saccharides near to the galactose residue [292]. In physiological conditions, galectin-3 expression is found in cells such as epithelial, immune, and sensory neuron cells [293]. The galectin-3 null mice model exhibits various phenotype such as decreased inflammation [294], increased tissue damages in kidney [295], and decreased gastric tumorigenesis [296], suggesting the key functional roles of galectin-3 in the body. Galectin-3 expression is also found in various cancers in which the level of galectin-3 depends on the degree of tumour progression, invasiveness and metastatic ability.

Overall, as a multifunctional protein, galectin-3 is characterised by its varied binding properties and affinities for ligands, altered localization at cellular level and direct association with cancer progression. Therefore, galectin-3 has drawn large interest in cancer research over the last decades.

1.3.1 Galectin-3 structure

Galectin-3 contains three domains: a glycine- and proline-rich long N-terminal domain of 130 aa, a conserved short N-terminal domain of 12 aa, and a carbohydrate

recognition domain (CRD) of approximately 130 aa (Fig 1.4) [289]. the short N-terminal domain might play a role in the secretion and apoptosis of galectin-3 as the deletion of the short N-terminal domain inhibits galectin-3 secretion [297] while mutation in Ser6 in the short N-terminal domain induces galectin-3 apoptosis [298].

The short N-terminal domain is thought to play a role both in secretion of galectin-3 and in the prevention of apoptosis. Deletion of this domain is demonstrated to inhibit galectin-3 secretion [297], while mutation of Ser6 to alanine (S6A) and to glutamic acid (S6E) promotes apoptosis of breast carcinoma cells [298].

There is a collagen like-N-terminal domain in the long N-terminal domain of galectin-3 which contains 7-14 repeats of a 9-aa sequence Pro-Gly-Ala-Tyr-Pro-Gly-X-X-X (Fig 1.4). The multimerization or homodimerization of galectin-3 is driven by its long N-terminal domain, and it can be catalysed by MMP-2 and -9 into a fragment with CRD and a polypeptide containing the amino terminal end of intact galectin-3, which further results in tighter binding to glycoconjugates as well as preventing the multimerization of the protein [299, 300]. The C-terminal domain of galectin-3 usually forms a globular structure with 6 stranded β-sheets arranged in a β-sandwich and contains the CRD structure responsible for lectin activity. It was found that galectin-3 has a motif named NWGR in the CRD which also exists within the BH1 domain of the B-cell lymphoma 2 (Bcl2) family of proteins. This motif has been shown to be associated with anti-apoptotic properties in both Bcl-2 and galectin-3 [301], by interacting with apoptosis regulator Bax [302].

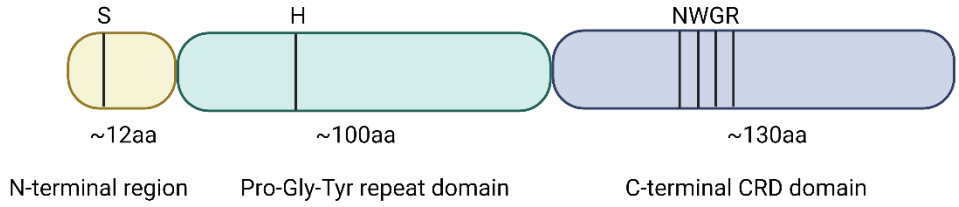

Fig 1.4. Galectin-3 structure. Galectin-3 contains a C-terminal carbohydrate recognition domain (CRD) of 130 aa which contains the NWGR motif and a long N-terminal domain which contains a short N-terminal domain of 12 aa with a serine 6 (S) phosphorylation site and a collagen like-N-terminal domain of repeats of Pro-Gly-Tyr.

1.3.2 Characterization of galectin-3 by its binding properties

Previous studies have considered N-acetyllactosamine as the preferential ligand of galectin-3 [303, 304]. It was also evident that long oligosaccharides such as polylactosaminoglycans can also be the biding partner of galectin-3 due to its extended binding site [305]. It was also found that the Arg144 residue of human galectin-3 (or Arg139 in hamster) is associated with binding of molecules connected to O-3 of a terminal β-linked galactose residue [306]. When galectin-3 or its CRD interacts with carbohydrate ligands, a conformational change of the protein occurs [303] which rearranges the structure of the backbone near the binding site [307]. In

addition, the phosphorylation of Ser6 of galectin-3 can influence its ability to recognize carbohydrate motifs, potentially affecting its the downstream biological effects [308].

Furthermore, galectin-3 is considered as a monomeric protein but it can assemble into homodimers through its CRD without a ligand to regulate its biological functions [309]. Excess ligand can lead to galectin-3 assembling into a pentameric structure through interacting with its N-terminal domain [310]. The assembly of oligomers interacts with its ligands on the cell surface and initiates cell surface signalling [311, 312].

Despite all galectins binding to β-galactoside, their binding affinity towards ligands varies depending on their unique galactose-containing structures. For instance, galectin's binding affinity is up to 10 times stronger towards N-acetyllactosamine (Galβ1,4GlcNAc) than towards lactose [313-316]. Also, the binding affinity of galectin-1 towards Thomsen–Friedenreich (TF) antigen is nearly 100 times lower than that of galectin-3 [317]. Cytoplasmic galectin-3 is found to interact with many proteins including β-catenin [318, 319], fibronectin [320], laminin [321], Bcl-2 [322], and α1β1 integrin [320] through protein-carbohydrate or protein-protein interactions.

1.3.3 Characterization of galectin-3 by its localization

Localization of galectin-3 is well established in which intracellular, extracellular or circulating galectin-3 affect cell and tissue functions in different manners. Intracellular galectin-3 is responsible for assisting cells to escape apoptosis through a carbohydrate-recognition independent manner [323], while extracellular galectin-3 facilitates cell–cell and cell–matrix interactions and induces apoptosis by interacting with cell surface glycoconjugates [324].

The presence of galectin-3 in the cytoplasm is associated with cellular functions such as apoptosis and many ligands for galectin-3 have been identified. Bcl-2 was first found as the ligand for galectin-3 *in vivo*, in which galectin-3 binds Bcl-2 through its CRD [325]. There are other apoptotic signalling molecules identified as binding partners for galectin-3 including CD95 (APO-1/Fas), a member of the death receptor family [326]. Moreover, apoptosis proteins such as Nucling and Alix/AIP1 were also known to be novel ligands for galectin-3 [327] [328]. Galectin-3 is also a selective binding partner of activated K-Ras and Akt proteins, suggesting its involvement in regulating cell proliferation and differentiation [329-332]. Nucleic galectin-3 was found to correlate with ribonucleoprotein complexes [333] where it acts as a pre-mRNA splicing factor and is involved in spliceosome assembly [334].

Extracellularly, galectin-3 exhibits its functions in a paracrine and autocrine manner. Generally, extracellular galectin-3 mediates cell-cell and cell-matrix adhesion [335] and can also serve as the chemoattractant for macrophages and monocytes [336]. To this notion, galectin-3 influences many biological processes such as immune reactions, cellular homeostasis, angiogenesis and organogenesis.

1.3.4 Galectin-3 expression in physiological conditions

In physiological conditions, galectin-3 plays an important role in tissue development in which it mediates the processes of apoptosis, cell proliferation and adhesion, and angiogenesis [337-339]. Galectin-3 expression is seen to correlate with mouse [340] and human [341] tissue development and embryogenesis. A previous study showed that galectin-3 expression first occurs on Day 4 of gestation, and after Day 8, expression occurs in the notochord cells in mouse embryogenesis. It was also found

that galectin-3 expression primarily occurs in cells which have a high growth rate including the endodermal end of the bladder, epidermis, oesophagus, and larynx [289]. In humans, galectin-3 expression is primarily found in epithelial cells including colon [342], small intestine [343], kidneys [344], cornea [345], thymus [346], lungs [347], prostate [348], and breast [349]. Galectin-3 is also found to be expressed in ductal cells such as pancreas [350], salivary gland [351], and hepatocellular biliary ducts [352], and kidneys [353].

1.3.5 Galectin-3 in cancer

As mentioned in the last section, as important as galectin-3 in physiological conditions, the altered expression of this protein can result in many pathological conditions including cancer. In several epithelial cancers, galectin-3 expression is increased [354], as well as the concentration of the cytoplasmic galectin-3 [355], and the levels of circulating galectin-3 which has been shown to be up to 30-fold increased [356]. The high expression of galectin-3 is tightly associated with poor prognosis in many cancers (Table 1.3).

Table 1.3. Galectin-3 expression in cancers and its clinical significance

Cancer types	Galectin-3 expression	Clinical significance	References
Colorectal	Upregulated	Poor prognosis	[357]
Tongue carcinoma	Upregulated	Poor prognosis	[358]
Prostate	Upregulated	Poor prognosis	[359]
Ovarian	Upregulated	Poor prognosis	[360]
Melanoma	Upregulated	Poor prognosis	[361]
Thyroid	Upregulated	Poor prognosis	[362]

Particularly, in colorectal cancer, galectin-3 shows increased expression and great effect on promotion of cancer progression. It was found that expression of galectin-3 was 5-fold higher in colorectal cancer tissues than normal tumour-adjacent intestinal epithelial tissues [363]. Previous studies also show that patients with detectable expression of galectin-3 in the CRC tumour have more distant and lymph node metastases as well as venous invasion and wall invasion than galectin-3-negative patients [364]. The circulating galectin-3 has up to 5-fold increase in the bloodstream of CRC patients [365], and it has been found to induce cytokines secretion and act as a pro-inflammatory mediator during CRC metastasis [366, 367]

Accumulating evidence has shown the important role of galectin-3 in cancer cell activities including transformation, apoptosis inhibition, tumour growth, cell adhesion, cell invasion, anoikis resistance, and angiogenesis. In terms of promoting cancer cell invasion and metastasis, extracellular galectin-3 primarily influences tumour cells and

stromal cells present in the TME, while intracellular galectin-3 is associated with mRNA splicing and apoptosis promotion (Fig 1.5).

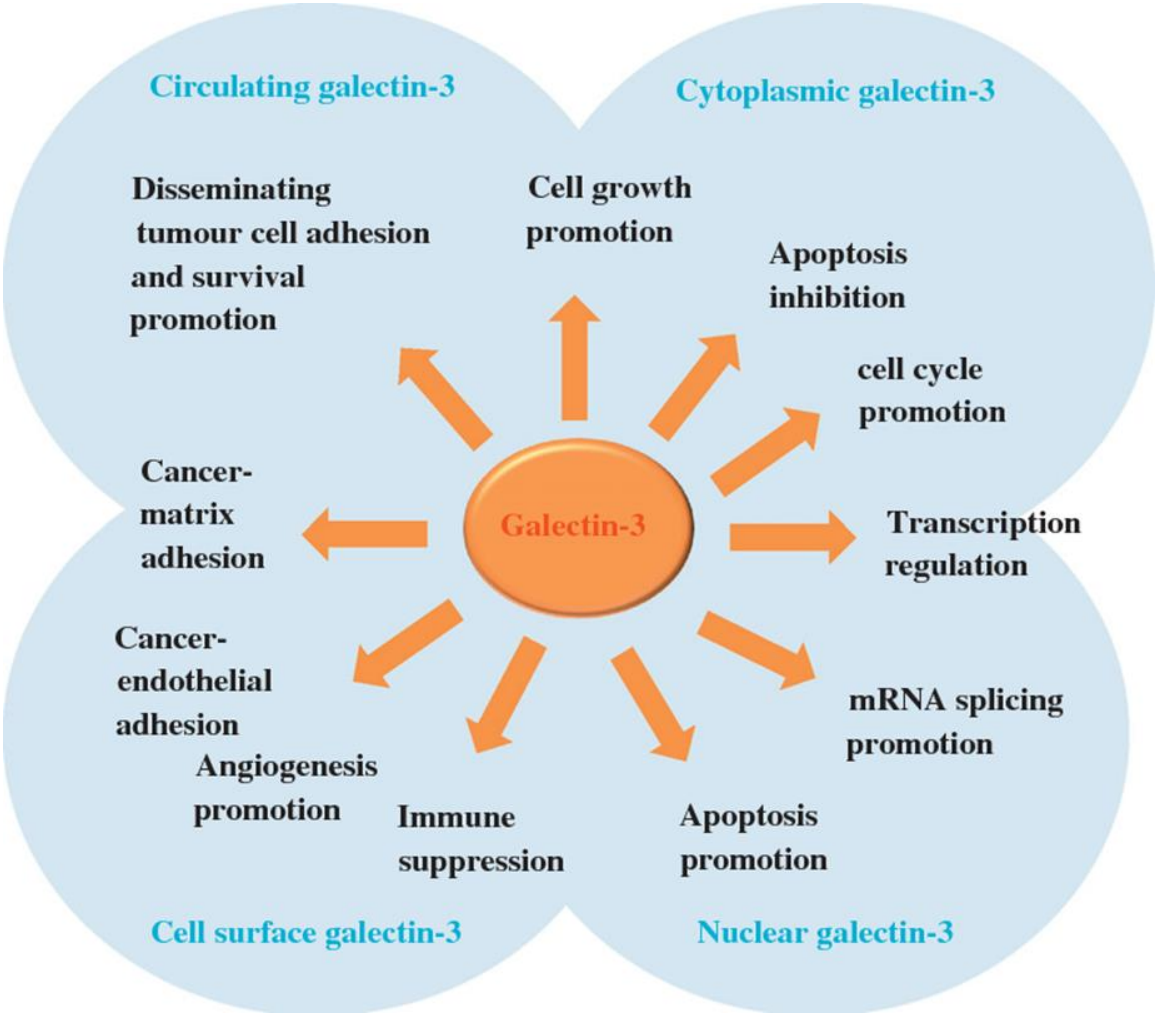

Figure 1.5. Differential effects of intercellular and extracellular galectin-3 on cancer cell behaviours. Source from [368].

1.3.5.1 Galectin-3 in tumour transformation

There is mounting evidence that supports a critical role for galectin-3 in neoplasmic transformation [369, 370]. downregulation of galectin-3 expression in MDAMB-435 cells caused a decrease in cell malignancy as well as morphology, and cells were unable to grow in a serum-free environment. Moreover, inhibition of galectin-3

expression resulted in tumour growth suppression *in vivo* [371]. Similarly, galectin-3 overexpression was required to maintain the characteristic transformed phenotype, suggested by anchorage-independent growth, in thyroid papillary carcinoma cells, as antisense cDNA galectin-3 silence resulted in reduced resistance to anoikis [372]. In addition, introduction of galectin-3 cDNA into normal thyroid cells provided them with anoikis resistance and serum-independent growth [373]. Taken together, these studies suggest that the galectin-3 overexpression in melanoma and thyroid cancer cells is strongly associated with a tumorigenic phenotype.

The role of galectins in cell transformation has not yet been fully identified. However, galectin-3-mediated transformation has been partially acknowledged with the ability to recognise and interact with oncogenic K-Ras [374]. These interactions usually lead to K-Ras-dependent activation of PI3K and Raf-1 and demolish ERK activation via upregulating ERK phosphatases. The activation of Raf-1 and PI3-K further activate several signalling cascades and mediate gene expression [375]. In addition, the interaction between galaectin-3 and β-catenin induces expression of cyclin D and c-MYC and promotes cell cycle progression [376].

Taken together, these studies show the involvement of galectin-3 in cancer hallmarks of evading growth suppression, maintaining proliferative signalling and resisting cell death.

1.3.5.2 Galectin-3 in cancer cell adhesion and migration

Upregulated galectin-3 expression has been shown to promote tumour invasion via assisting the escape of tumour cells from the primary site. Increased tumour cell adhesion to ECM proteins such as fibronectin, collagen IV, elastin and laminin

promotes the metastatic spread of tumour cells [90]. The interactions between galectin-3 and Mgat5-N-glycans, carcinoembryonic antigen and lysosome-associated membrane glycoproteins reportedly contribute towards cancer cell adhesion and migration [377]. Galectin-3 has also been found to interact with transforming growth factor- β receptor (TGF-βR) and epidermal growth factor receptor (EGFR) which results in the endocytosis of these factors, in turn, promoting cell adhesion and migration [378]. It has been shown that galectin-3 interacts with extracellular carcinoembryonic antigen (CEA) promotes colon cancer cell migration. Overexpression of galectin-3 increases migration of colon cancer cells through the activation of the K-Ras-Raf-Erk1/2 [379].

It was first found that galectin-3 interacts with TF in human prostate and breast cancer, in which the interaction participates in cell adhesion to vascular endothelial cells and cell-cell homotypic aggregation. It was also found galectin-3 and TF antigen interaction mediates cancer cell-endothelium adhesion in mice and *in vitro* [380-382]. Also, galectin-3 has been reported to interact with cancer-associated TF (CATF) epitope which affect the early stages of cancer cell adhesion to the endothelium [383-385]. The introduction of anti-galectin-3 antibody, anti-TF antibody, lactulosyl-L-leucine or modified citrus which disrupted the interaction of galectin-3 and TF, inhibited human breast cancer cells adhesion to endothelial bone marrow cells and HUVECs *in vitro* and in an *ex vivo* model [380]. Cell-surface galectin-3 upregulation by CATF antigen-induced endothelial and cancer cell interaction, promotes prostate and breast cancer cell adhesion to the endothelium of intact micro-vessels [386]. TF antigen-expressing breast cancer cells exhibited migration of the cell surface galectin-3 in endothelial cells in response to metastatic, but not in TF antigen-null cells [387]. Taken together, these results indicate that cancer cell-associated TF acts as a binding partner for galectin-3

in promoting cell adhesion, and also induces galectin-3 to cell surface for subsequent binding to tumour cells during cancer progression.

Galectin-3 secretion was shown in breast carcinoma cells in the processes of detachment of the cells and changing into a rounder phenotype. The cells which expressed and secreted more galectin-3 were prone to adhere and spread faster than cells which had less galectin-3 expression, which suggests that extracellular galectin-3 promotes cell adhesion and spreading [388]. Likewise, the introduction of exogenous galectin-3 to breast carcinoma cells promoted the migration of cells through Matrigel proteins [389].

Finally, there are studies which have shown an association between galectin-3 and proteases in promoting cancer cell migration and invasion. MMP-1 expression was upregulated by galectin-3 via phospho-paxillin, which further promotes cell invasion and migration in gastric cancer [390]. Similarly, in mouse melanoma cells, MMP-1 expression was upregulated by galectin-3 via its direct binding to the AP-1 site, further promoting the motility of cells [391].

1.3.5.3 Galectin-3 in metastatic spread

As mentioned previously, galectin-3 favours the initial steps during the metastatic spread of tumour cells such as promoting cells escaping from the primary tumour, and adhesion of cells to the ECM or endothelium. When tumour cells reach the circulation, the interactions of circulating galectin-3 with CATF play vital roles in metastasis. In many cancers, MUC1 expression and occurrence of the TF antigen carried by MUC1 are both upregulated and the levels of circulating galectin-3 are also significantly higher in metastatic tumours than localised tumours [392]. It was shown that the

concentrations of circulating galectin-3 were increased up to 950 ng/ml in metastatic breast cancer comparing to 20-313 ng/ml in healthy individuals [392]. The production of galectin-3 was found to be facilitated by tumour tissues, stromal cells, macrophages and fibroblasts [392]. In the past decade, studies have shown that the interactions between upregulated circulating galectin-3 with TF carried by MUC1 on the cancer cell surface make great contributions to the dissemination of cancer cells to secondary sites. Interactions of galectin-3 with MUC1 lead to cell surface polarization of the mucin which further results in the open access of adhesion molecules like CD44 and E-selectin [393, 394]. This cell surface event further promotes adhesion of disseminating tumour cells to the blood vascular endothelium [394]. Moreover, the galectin-3-mediated redistribution of MUC1 on the cell surface induces cancer cell homotypic aggregation and the formation of circulating tumour aggregates [395]. This transformation of cells into cell clusters allow cells to escape anoikis, apoptosis induced by loss of cell-cell/matrix contacts, therefore favouring cell survival [395]. Interestingly, with only 3% of tumour cell clusters in the circulating tumour population, this is responsible for over 50% of the metastasis in a mouse metastasis model, and with 5% of tumour cell clusters, there was a significant reduction of overall survival in breast and prostate cancer patients [396]. It is also reported that overexpression of galectin-3 had counter-effect towards anoikis and other apoptotic stimuli such as the chemotherapy agent cisplatin [397]. This protective role of galectin-3 allows cells to have more prolonged life in the bloodstream during the cancer dissemination process. Galectin-3 has also been reported to assist tumour cells escaping nitric-oxide induced apoptosis. Tumour cells often face ischemia reperfusion injury and lack of oxygen as a result of tumour embolism, and it has been demonstrated that high metastatic tumour cells show resistance to reoxygenation injury [398]. Subsequent studies also found

that galectin-3 expressing breast carcinoma BT549 cells had more resistance to apoptosis induced by nitric oxide than galectin-3 null cells. It was also found that galectin-3 expressing cells formed metastatic colonies in liver while galectin-3 null cells did not [399].

In addition, galectin-3-MUC1 interactions have been shown to activate MUC1-mediated signalling pathways such as the MAPK and PI3K/Akt pathway, resulting in increased proliferation and motility of human colon cancer HCT116 cells, while shRNA mediated galectin-3 knockdown caused reduced cell proliferation and motility [400]. This galectin-3-mediated signalling requires the full-length galectin-3 protein where only C-terminal galectin-3 showed no effect.

1.3.5.4 Galectin-3 in angiogenesis

Exogenous galectin-3 promotes endothelial capillary tubule formation on Matrigel and chemotaxis of HUVECs and enhance neovascularisation *in vivo* [401]. The introduction of lactose and modified citrus pectin (MCP), as galectin-3 inhibitors, inhibited the angiogenesis in cancer cells, suggesting that galectin-3 carbohydrate binding activity was required for induction of angiogenesis [402]. There are several pro-angiogenic activities of galectin-3 such as interactions of galectin-3 with transmembrane chondroitin sulphate proteoglycan, neural/glial antigen 2 (NG2), and integrins. It was found that NG2 induces angiogenesis by binding to galectin-3 and α3β1 integrin on the cell surface of endothelial cells and activates transmembrane signalling via α3β1 integrin [403]. This signalling was further enhanced by galectin-3-mediated cross-linking of the proteoglycan.

Galectin-3 was also reported to act as a regulator of bFGF and VEGF signalling, as well as promoting the clustering of integrin and activated FAK, resulting in activation of the signalling cascade which triggers blood vessel formation [401]. It was also found that increased circulating galectin-3 promoted endothelial secretion of cytokines such as soluble intercellular adhesion molecule-1 (sICAM-1), granulocyte colony-stimulating factor (G-CSF) and interleukin 6 (IL-6), in which the interactions of these cytokines with vascular endothelial cells in an auto/paracrine manner caused increased tumour cell-endothelium associations and cell migrations and endothelial tubule formation [366].

Finally, previous studies have shown an association between galectin-3 and proteases in promoting tumour angiogenesis. MMP-2 and MMP-9 can digest galectin-3 *in vivo* which further induces angiogenesis [404]. Interactions of galectin-3 with a cell surface enzyme aminopeptidase N/CD13 have also been shown to contribute to the early stages of neovascularisation [405].

1.4 Interactions between galectin-3 and proteases in cancer

Given that galectin-3 plays a key role in cancer progression, it is likely that the crosstalk between galectin-3 and proteases occurs during the processes of tumour invasion and metastasis. Galectin-3 has been reported to be a substrate for human matrix metalloproteinases-2 and-9 [406]. The coordination of galectin-3 and MMP-9 expressions has shown to be involved in the development of hepatocellular carcinoma [407]. It was also reported that galectin-3 accelerates cell motility in gastric cancer by up-regulating protease-activated receptor-1 and MMP-1 [408]. Galectin-3 has been reported to increase the expression of MMP-1 in mouse melanoma cells which increases cell motility [409]. It was also reported that galectin-3 initiates epithelial cell-cell disassembly and disrupts cell-cell contact by inducing MMP-9 expression in a CD147-dependent manner [410].

1.5 Hypothesis

Galectin-3-mediated promotion of cancer progression and metastasis are associated with regulation of protease expression in cancer cells

1.6 Aims

To investigate the effects of galectin-3 on protease expression and secretion in human colon cancer cells

To investigate the effects of galectin-3-mediated proteases expression/ secretion on cancer cell invasion and signalling

Chapter 2

Materials and methods

2.1 Materials

Acrylamide, 30% solution (A3699), Ammonium persulfate (A3678), Crystal violet solution (V5265), Dimethyl sulfoxide (DMSO) and Tetramethylethylene-diamine (TEMED) were obtained from Sigma (Gillingham, UK). Bovine Serum Albumin (BSA, 5217) was purchased from Tocris (Bristol, UK). Glycerol (56-81-5), Glycine (56-40-6), Methanol (67-56-1), Sodium dodecyl sulfate (SDS, 151-21-3), Tris Base (BP152-1) and Tween 20 (BP337-500) were obtained from Fisher (Loughborough, UK). Precision Plus Protein Kaleidoscope (1610375) was purchased from Bio-Rad (Watford, UK). Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS) (10010-015), Penicillin-Streptomycin solution, and 0.5% Trypsin-EDTA (10X) was obtained from Gibco (Loughborough, UK).

Nitrocellulose membrane (B3-0012, Geneflow, Staffordshire, UK), Transwell permeable support, 8.0µm PET membrane (CLS3422, Corning, ME, USA), Transwell permeable support, 0.4µm Polyester membrane (CLS3470, Corning, ME, USA), Proteome Profiler Human Protease Array (ARY021B, R&D system, Abingdon, UK), Proteome Profiler Human Phospho-Kinase Array Kit (ARY003C, R&D system, Abingdon, UK), Human MMP-13 DuoSet ELISA (DY511, Abingdon, UK), Human Cathepsin-B DuoSet ELISA (DY2176, Abingdon, UK), Human MMP-1 DuoSet ELISA (DY901B, Abingdon, UK). All cell culture plastic ware (plates and flasks) and Falcon 8 um pore filters were from Thermo Fisher Scientific (Waltham, USA).

2.2 Antibodies

Biotinylated-anti-galectin-3 (BAF1154) antibody and antibodies against galectin-3 (MAB1154), STAT1 (MAB1490), Phospho-STAT1(MAB2894), STAT3 (MAB1799), Phospho-STAT3 (MAB4934), PYK2 (AF4589), phosphor-PYK2 (MAB6210), GSK3α/β (AF2157), Phospho-GSK3α/β (AF1590) were all purchased from R&D Systems (Abingdon, UK). Peroxidase-conjugated secondary antibodies were purchased from Cell signalling (Massachusetts, USA).

2.3 Cell culture medium and cell lines

The summary of cell lines used in this project is listed in Table 2.1. Human colon cancer SW620, HCT116, Caco-2 and HT29 cells, were all obtained from European Collection of Cell Cultures (Salisbury, UK). All the cells (except HCT116) were cultured in DMEM containing 200 mM L-Glutamine, 0.4% penicillin and streptomycin and 10% FCS. HCT116 cells were cultured in McCoy's medium containing 200 mM L-Glutamine, 0.4% penicillin and streptomycin and 10% foetal calf serum. The cell lines were last authenticated in 2020 by DNA profiling at the Cell Line Authentication Facility, University of Liverpool. Galectin-3 knockdown SW620-shGal3 and control SW620-shCon cells were generated using galectin-3 shRNA and control shRNA from SW620 as previously described. Briefly, SW620 cells were transferred with MISSION shRNA plasmid DNA (TRCN0000029305, Sigma) or empty vector control (SHC002, Sigma). The transfected cells were selected by treatment with puromycin. Galectin-3 expressing (SW620shCon) and knockdown (SW620shGal3) colonies were selected. This experiment was done by Dr. Paulina Sindrewicz in her previous work [366].

Table 2.1 Summary of cells used in the thesis

Cell line names	Property of the cell line
SW620	Human Caucasian colon adenocarcinoma cells, it was derived from a metastasis of the same tumour from which the SW480 was derived.
SW620shCon	SW620shCon was obtained from previous experiment as describe before, with insertion of empty vector control
SW620shGal3	SW620shGal3 cells were generated with insertion of shRNA to knock down galectin-3 expression
SW480	Human Caucasian primary adenocarcinoma of the colon
HCT116	Human colorectal carcinoma cell line initiated from an adult male. The cells are adherent with an epithelial morphology.
Caco2	Human colorectal carcinoma cell line. It has the ability to spontaneously differentiate into a monolayer of cells with many properties typical of absorptive enterocytes with brush border layer as found in the small intestine.
HT29	Human colorectal adenocarcinoma cell line with epithelial morphology. It is known to be heterogeneous.

2.4 Cell culture and passaging

In order to keep cells alive and healthy, cells were destined to be passaged once the cell confluence reached 100% (Cells cover the whole surface of the culture flasks). Due to limited space and nutrition in the flask, cancer cells would survive for a considerable amount of time, but the condition of the cells would be less ideal for our

experiments, and eventually cells died from starvation and detached from the flask. Sub-confluent cells also needed to be detached from the flask to be analysed throughout the project. 0.25% Trypsin-EDTA was used to detach the cells from the flask. Trypsin is an enzyme that cuts amino acids such as lysines or arginines, while EDTA acts as a divalent cations chelator in the trypsin solution. The presence of Trypsin-EDTA allows cells to separate from each other by removing calcium from the cell solution as well as cadherins, and also detach from the surface of the tissue culture plastic.

Briefly, cells were washed with PBS for three times followed by addition of 0.5 to 1 ml of 0.25% Trypsin-EDTA to the flask. The cells were then incubated at 37 degrees for roughly 5 min until the cells were visually floating inside the flask. By gently pipetting or applying the cell strainer, cells were resuspended with 5 ml of complete medium thoroughly to avoid large cell clusters and transferred to the new 50 ml Falcon tube. The cell suspensions were then centrifuged at 352g for 5 min to remove the trypsin in the solution. The fresh medium was then added to the tube and 1 or 2 ml of newly obtained cell suspensions were transferred to the new flask with complete medium. In order to avoid any genetic mutation, the cells we used were up to 25 generations and new vial of pre-frozen cells were obtained from the nitrogen tank. To freeze cells, detached cells were resuspended with cell freezing solution which composed of dimethyl sulfoxide (DMSO), FBS and appropriate medium (ratio of 1:3:6) and vials of cells were stored in -80 °C for overnight before being transferred to nitrogen tank.

2.5 Cell counting

After cells were released from the flask, cell counting was performed in order to calculate the number or concentration of the cells. 10 µl of cell suspension was transferred onto a hemocytometer and covered with a cover glass. The number of cells in all four outer squares were counted and the mean number of cell/squares was obtained (Fig 2.6.1). The final number of cells/ml in the suspension equal to the number of cells per square x 10000.

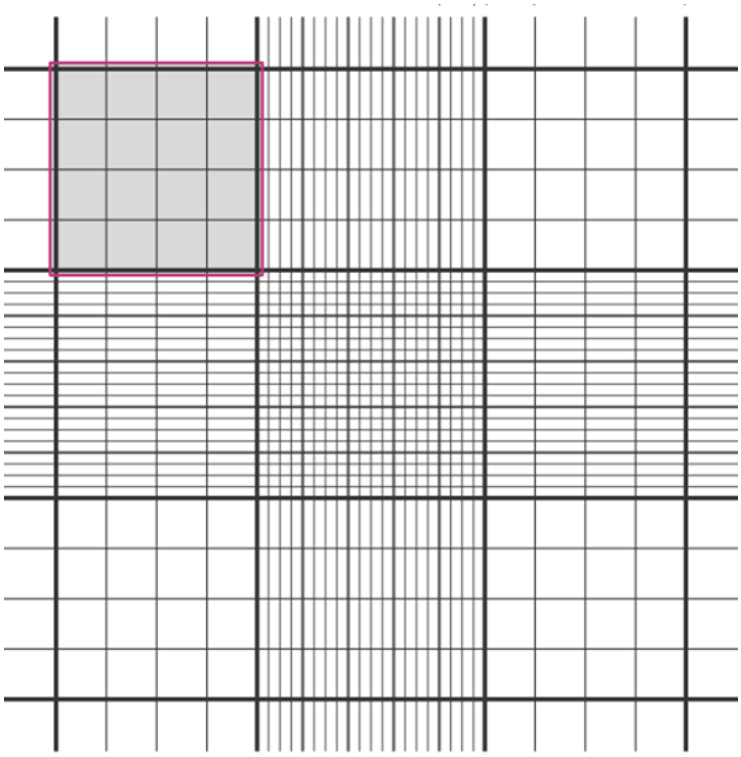

Fig 2.6.1 A representative image of the hemocytometer under the microscope. Number of cells in a 1mm^2 square (red) was counted as well as other 3 outer squares.

2.6 Human Protease profile array

To assess the expression profile of proteases, the Human Protease Array was used. The Human Protease Array is a rapid, sensitive, and economical tool to simultaneously

detect expression of multiple proteases. The array contains 35 human proteases. Cells at 50% confluent were incubated with 10 µg/ml galectin-3 or BSA for 24 hr at 37℃ in serum-free medium. The culture medium was collected and centrifuged at 1956g for 10 min. Cell debris were removed, and the supernatants were transferred to new tubes ready for array analysis.

To use the array, the array membranes were placed in the 4-well multi-dish and incubated for one hour with blocking buffer that provided in the kit. While the membrane was in blocking solution, 15 µl of reconstituted protease detection antibody cocktail was added to each prepared sample and incubated at room temperature for one hour. The sample/antibody mixtures were then added to the corresponding membranes and incubated overnight in the cold room on a rocking platform shaker. Following the overnight incubation, the membranes were washed three times with the 1X wash buffer that was provided in the kit. Each membrane was then incubated with 2 ml of 1X Streptavidin-HRP for 30 min at room temperature on a rocking platform shaker. After three washes, the membranes were developed using chemiluminescence SuperSignal™ kit (Thermo Fisher, Warrington, UK) and visualized with Molecular Imager® Gel Doc™ XR System (Biorad, Hempstead, UK). Density of the blots was quantified using Imagelab version 3.0.1 (Biorad, Hempstead, UK).

2.7 Measurement of proteases secretion by slot blot

Slot blot was one of the methods used to assess protease secretion in this study. Cells ($1.5x10^6$) were cultured in 6-well plates until 80-90% confluent. The cells were washed with PBS and introduced with serum-free medium containing 1% BSA with different

concentration of galectin-3 or BSA. After 24 hours culture, the medium was collected, and 250 µl was loaded onto a slot blot. After one wash with PBS, the nitrocellulose membranes were incubated with blocking buffer (1% BSA/PBS) for 1 hr at room temperature before incubation with antibodies against Cathepsin-B (20 µg/ml), MMP-13 (2 µg/ml), or MMP-1 (40 µg/ml) overnight at 4℃. The blots were washed three times with 0.05% Tween-20 in PBS and incubated with peroxidase-conjugated secondary antibody (1: 5000) for 1 hr. After six washes with 0.05% Tween-20 in PBS, the blots were developed using chemiluminescence SuperSignal™ kit and visualized with Molecular Imager® Gel Doc™ XR System. The density of the blots was quantified using Imagelab version 3.0.

2.8 Electrophoresis and Immunoblotting

Western blotting was used to determine the expression level of different proteins throughout the project.

To prepare the samples, cells of interest were grown in 6-well dishes up to approximately 90% confluency. The cells were washed twice with PBS and lysed with 0.1 ml per well of SDS sample buffer (the receipt shown in table 2.9.1) on ice for 20 minutes. The cells were additionally scraped off from the flasks using cell scrapers and the lysates were then transferred into 1.5 ml Eppendorf tubes, boiled for 10 minutes at 100 ℃ and stored in – 80 ℃ till use.

Table 2.1 SDS-sample buffer recipe

	X2 Sample buffer
0.5% Tris-HCL	2.5ml
Glycerol	1.0ml (20%)
20% SDS	1.0 ml
1% Bromophenol blue	50 µl
β -Mercaptoethanol	0.5ml (10%)

SDS-PAGE

SDS-PAGE was performed following standard laboratory protocol using gels prepared with 1.0- or 1.5-mm thick glass plates.

Resolving gel was prepared at three different concentrations according to the molecular size of proteins of interest: 8% gel for protein molecular weight (MW) above 100 kDa, 10% for MW range from 40 to 100 kDa, and 12.5% for MW below 40 kDa.

The resolving gel preparations (for a 10 ml solution) are shown in the table below:

Table 2.2 Resolving gel recipe

Resolving gel percentage	8%	10%	12.5%
Deionized RO water	5.2 ml	4.1 ml	3.2 ml
1.5M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml
30% Acrylamide	2.2 ml	3.3 ml	4.2 ml
10% SDS	100 µl	100 µl	100 µl
10% Ammonium persulfate	75 µl	75 µl	75 µl
TEMED	10 µl	10 µl	10 µl

The stacking gel (5 ml) was always prepared at 4% and contain:

- 3.05 ml of deionized RO water
- 1.25 ml of 0.5 M Tris-HCl buffer at pH 6.8
- 50 µl of 10% SDS
- 0.665 ml of 30% Acrylamide
- 50 µl of 10% Ammonium Persulfate
- 5 µl of TEMED

Running buffer and transfer buffer were also prepared following the standard laboratory protocol shown in table below:

Table 2.3 Running buffer and transfer buffer recipe

	Running Buffer	Transfer Buffer
Tris-Base	30.67 grams	12.12 grams
Glycine	64.04 grams	57.65 grams
SDS	2.2 grams	
Methanol		800 ml
To make 4L of solution with water	4L	4L

On the day of the analysis, the samples as well as Kaleidoscope Precision Plus Protein Standards were defrosted and boiled once again before being loaded to the gels. Electrophoresis was run at 60 V for 30 min to allow the samples to pass through the stacking gel and then 100 V for approximately 60 minutes until the visible blue line reached the bottom of the glass. The gel was removed from the glass cassette and was assembled into transfer sandwich. The proteins separated on the gel were transferred at 100 V for an hour onto nitrocellulose membrane in 1 X transfer buffer.

Immunoblotting

Following the transfer, the membrane was incubated with blocking solution: 1% BSA in 0.1% Tween 20 with PBS for an hour at room temperature on a rocking platform. The membrane was subsequently probed with 10 ml of primary antibodies: MMP-1 (1 µg/ml), MMP-13 (0.5 µg/ml), Cathepsin B (0.5 µg/ml), Galectin-3 (1 µg/ml), phospho-STAT1 (0.5 µg/ml), phospho-STAT3 (0.5 µg/ml), phospho-PYK2 (0.5 µg/ml), or phospho-GSK3α/β (0.2 µg/ml), for overnight in the cold room on a rocking platform.

The next day, the blot was washed three times with washing buffer (0.1% Tween-20 in PBS) and then incubated with 10ml of secondary antibody: goat polyclonal anti-mouse HRP-conjugated antibody at a final concentration of 35 ng/ml in the blocking buffer for an hour at room temperature on a rocking platform. Finally, the membrane was washed six times with the washing buffer and the blots were developed using chemiluminescence SuperSignal™ kit and visualized with Molecular Imager® Gel Doc™ XR System. The blots were stripped by stripping buffer (Tris-HCl 62.5mM, Mercaptoethanol 100 mM and SDS 2%) and re-probed with antibodies against PYK2 (1 µg/ml), GSK3α/β (0.1 µg/ml), STAT1 (1 µg/ml), or STAT3 (0.1 µg/ml). The density of the blots was quantified using Imagelab version 3.0.

2.9 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used as one of the main methods to examine protease secretion from cells. The assays were performed using 96-well high binding microplates (full volume). The plates were coated overnight at room temperature with specific capture antibodies: MMP-13, Cathepsin B, MMP-1 and galectin-3 (concentrations shown in the later chapters). The capture antibodies were diluted with 1 x PBS to the final concentrations. A few wells were coated with 1 x PBS and served for background subtraction. To avoid non-specific binding, the wells were first incubated with blocking buffer: 1% BSA in PBS for 45 min to 1 hour at room temperature followed by three washes with 0.05% Tween 20 in PBS. Before loading the samples onto the plate, the cell supernatants were mixed thoroughly. Depend on the protein of interest, different volumes of cell supernatants were loaded onto the plate. The levels of MMP-13 and MMP-1 secretions were relatively low in the colon cancer cells, thus 150 µl of cell supernatant for each

well were required while 50 or 100 µl of cell supernatants were enough to detect Cathepsin B and galectin-3 from those cells. After loading the samples, the plates were placed on the rocking platform with gentle agitation at room temperature for at least 2 hours. The plates were then washed with 0.05% Tween 20 in PBS for three times in which the excessive liquid need to be avoid inside the wells. Following the washes, 100 µl/well of the detection antibodies were loaded onto the plates with final concentration of MMP-1 (250 ng/ml), MMP-13 (100 ng/ml), Cathepsin B (50 ng/ml) and galectin-3 (0.1 µg/ml), diluted using 1% BSA in PBS. The incubation time of detection antibodies was 2 hours at room temperature and followed by three washes. The plates were then loaded with 100 µl/well of 1:40 Streptavidin-HRP diluted by 1% BSA in PBS and incubated for 30 minutes at room temperature followed by three washes. SIGMAFAST™ OPD (o-Phenylenediamine dihydrochloride) tablets were used as a substrate for the detection of peroxidase activity in enzyme immunoassays (EIA). OPD is the EIA substrate of choice as it exhibits high sensitivity. Each tablet set dissolved in 20 mL deionized water yields a ready-to-use buffered solution containing OPD and urea hydrogen peroxide. 100 µl/well of dissolved OPD solution was loaded onto the plate and incubated for at least 20 minutes till the colour being visible. Finally, 50 µl of the stop solution (4M H_2SO_4) were added to each well to stop the reaction. The optical density of each well was determined immediately, using a microplate reader at 450 nm and a wavelength correction at 570 nm.

2.10 Investigation of cancer cells invasion through matrix proteins

Corning® Matrigel® Matrix was used to examine a cell's ability to invade through matrix proteins. Matrigel was allowed to thaw overnight in a fridge before use. Pipette

tips were also cooled in the fridge before used to handle the matrix gel. The final concentration of Matrigel was 20 µg/ml (1:10 dilution from the vial of stock). The dilution was done using ice-cold serum-free medium and performed on an ice box. The diluted Matrigel was then loaded onto Transwell insert at 100 µl/well to coat the 8.0 µm pore size insert membrane and the plates were incubated at 37°C for 2 hours. The cells investigated include HT29, SW620 and HCT116 cells. These cells were released from the culture flask by 0.25% Trypsin-EDTA, washed with PBS and spun to remove the solution. The cell pellets were then resuspended in 2 ml of 1% FBS medium or serum free medium. After cell counting, the cell suspension was made to the final density of $1x10^5$ cells/ml cell suspension with 1% FBS medium or serum free medium. 150 µl/well cell suspensions were loaded into upper chamber of the coated Transwell inserts and 600 µl of 10% FBS medium were added to the bottom chamber. To assess the effect of galectin-3 on cell invasion, 10 µg/ml BSA (control) or 10 µg/ml recombinant galectin-3 were added to the Transwell insert of each cell type. After 16 hr incubation at 37°C, the matrix and uninvaded cells inside the inserts were gently removed with cotton swabs. The inserts were washed once with PBS before fixed in 2% formaldehyde/PBS for 20 min. After one wash with PBS, the inserts were stained with 0.5% crystal violet solution for 5-10 min. The cells at the bottom side of the membrane were then counted in three to five randomly selected fields of view (FOVs) under a microscope (Olympus B51, Olympus, Tokyo, Japan) with a 20x objective.

2.11 Assessments of cell monolayer integrity and permeability

Caco2 cells ($5x10^5$/ml) were seeded into 0.4 µm pore size Transwell insert and cultured for various times at 37℃ for cell monolayer formation. The monolayer integrity

was measured with an ohmmeter until trans-epithelial electrical resistance (TEER) reached plateau (approximately 3000 Ωcm^2).

Cells ($1x10^5$/ml) were cultured in 24-well plates for 3 days at 37°C. The culture medium was collected and centrifuged at 1956g for 5 min and used as conditioned media (CM). The culture medium in the trans-wells was removed and replaced with 0.5 ml/well CM. The cells in trans-wells were cultured at 37°C for various times and monolayer integrity was measured with an ohmmeter.

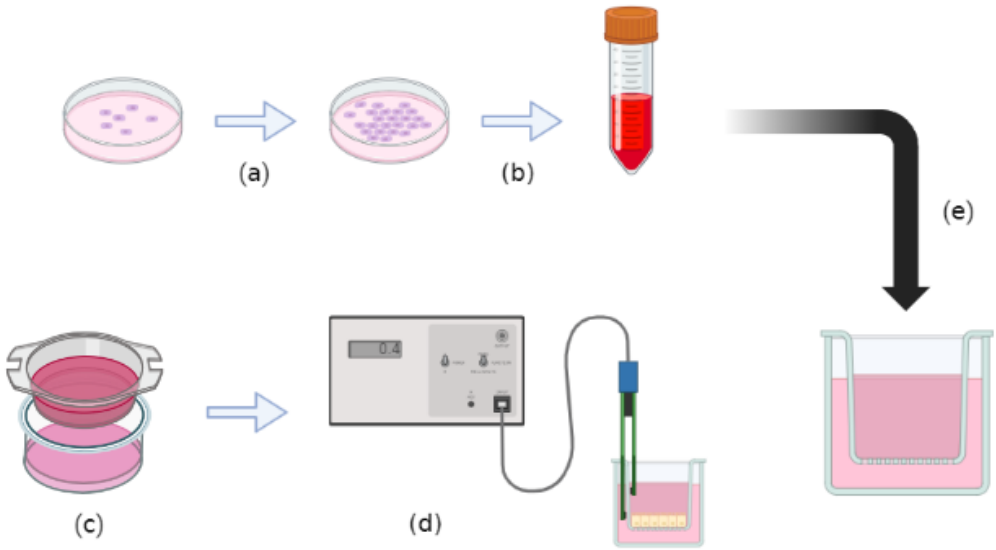

Fig 2.12.1 Schematic diagram of measurement of cell monolayer integrity. (a) Cells were cultured on the plates for 3 days and (b) conditioned media from each cell line were collected and stored. (c) Caco2 cells were introduced into upper chamber with culture medium in the lower chamber and (d) the monolayer integrity was measured with an ohmmeter until TEER reached plateau. (e) The media in the upper chamber was then replaced with the conditioned media from (a).

For assessment of the cell monolayer permeability, 1 mg/ml FITC-dextran (20 kDa) was introduced to the tight cell monolayers (TEER, approximately 3000 Ωcm^2) in the

trans-wells for 30 min at room temperature. The culture medium in the bottom wells was collected and centrifuged at 1956 g for 5 min and the fluorescence intensity in the supernatants were measured using a fluorescence microplate reader GENios Plus (TECAN, Reading, UK).

2.12 Human Protein kinase profile array

The Human Phospho-Kinase Array is a rapid, sensitive, and economical tool to simultaneously detect the levels of phosphorylation of 37 kinases and 2 related total proteins without performing numerous immunoprecipitations and Western blots. In this experiment, SW620 cells were seeded in 24 well plate at 1 x 10^5 cells per well in DMEM medium and incubated overnight at 37°C. The medium in each well was then replaced by serum-free medium with 10 ug/ml BSA or recombinant galectin-3 for 30 minutes at 37°C. The cells were lysed by SDS lysate buffer as described above and the lysates were stored at -80 degree for future use.

The Human Phospho-Kinase Array is divided into two parts (A and B). Both parts were incubated with blocking buffer (1% BSA) for 1 hour at room temperature. Part A and Part B membranes were then incubated in the same lysate preparation but in separate wells of the 8-Well Multi-dish. Before use, the sample lysates were diluted with blocking buffer to make the final volume of 2 ml and each part was incubated with 1ml of lysate dilutes. The incubation took place in the cold room for overnight on a rocking platform shaker. The membranes were then washed three times and incubated separately as: Part A membrane with 1:50 reconstituted detection antibody cocktail A and Part B membrane with 1:50 reconstituted detection antibody cocktail B, for 2 hours at room temperature on a rocking platform. After three washes, the membranes were

the incubate with Streptavidin-HRP for 30 minutes at room temperature on a rocking platform. After three washes, the membranes were developed using chemiluminescence SuperSignal™ kit and visualized with Molecular Imager® Gel Doc™ XR System. The density of the blots was quantified using Imagelab version 3.0.

2.13 Data analysis and statistics

One-way Analysis of Variance (ANOVA) with either Tukey or Dunnett's test was used to compare differences in multiple groups as indicated in the figures. Two-way ANOVA with Sidak post-hoc test was used in analysis of time- and dose- dependent protease secretion (compare two different groups at different time or dose). t-test was used in analysis of comparing cell invasion between two groups. Differences were considered significant when $p<0.05$.

Chapter 3

Assessment of galectin-3-mediated proteases secretion in human colon cancer cells

3.1 Introduction

Proteases contribute to many stages of cancer progression and metastasis. The primary action of many proteases in cancer progression is to degrade ECM proteins as a result of their secretion to outside the cells or association with the cell surface. In human colon cancer, cysteine proteases such as cathepsins, serine proteases such as urokinase- (uPA) and tissue-type plasminogen activator, MMPs like MMP-9 and MMP-2, are overexpressed and play crucial roles in colorectal carcinoma invasion and metastasis [179].

In normal human cells, it is well known that the primary enzymes degrading extracellular proteins are endopeptidases. Among the families of endopeptidases, cysteine endopeptidase, MMPs and serine peptidase catalyse proteins extracellularly. The catalytic actions of serine peptidase and MMPs favour a neutral pH and take place in the extracellular space. On the other hand, cysteine proteases perform catalytic actions in acidic conditions such acidic lysosomes, therefore, they are often found in lysosomes inside the cells, but are also secreted to fulfil important tasks in the direct pericellular surrounding in specific cell types.

The secretion of proteases can be regulated at different levels (Fig 3.1). First, higher expression of proteases by the cells likely leads to higher protease secretion. Several extracellular factors as growth factors or cytokines induce MMPs and uPA gene transcription [179, 411-413]. However, the cathepsins are exceptional due to their weak regulation at the transcriptional level.

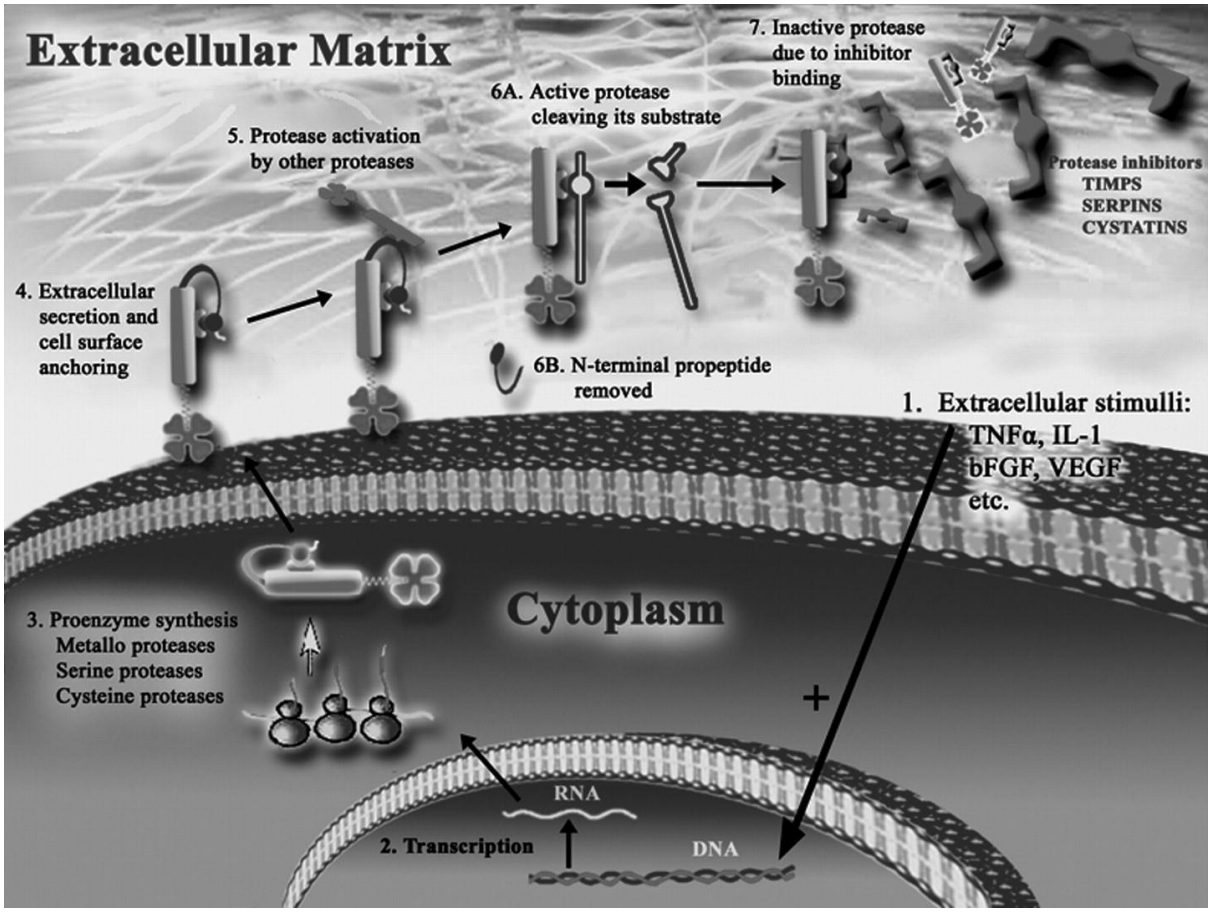

Figure 3.1. The regulation of protease expression and secretion at different levels. The expression of proteases is induced by many extracellular factors such as TNF, IL-1, bFGF etc. Proteases are synthesized as proenzymes from nuclear transcriptions followed by the secretion of proenzyme to the cell surface or pericellular region, and the activation of proenzyme is carried out by other proteases. The catalytic activity of active protease is regulated by its endogenous inhibitors such as TIMPs. Image is sourced from [414].

Second, in most cases, endopeptidases like MMPs, extracellular cathepsins and uPA are expressed and secreted as inactive proenzymes [415-418], and they remain in the pericellular region or attached to the cell surface. This prevents unexpected protein degradation from the catalytic activity by only catalysing the substrates located in the pericellular region and near the cell surface whenever the proteases are activated. Moreover, proenzymes anchoring the cell surface allow them to have easy access to their activators, which results in effective activation as well as being away from the

inhibitors. For instance, pro-uPA is secreted into the leading edge of the cell surface which later leads to the formation of uPA-uPAr complex. The complex is responsible for increased plasminogen activation with surrounding cellular plasminogen receptors, as well as subsequent plasmin-dependent proteolysis [419]. Similarly, the interaction between pro-MMP-2 and MT1-MMP as well as TIMP-2 on the cell surface is essential for the activation of pro-MMP-2 [420].

In tumour cells, there are a great number of proteases present in the lysosomes in the perinuclear region, and these lysosomal proteases are often translocated to the cell surface or secreted during cancer development. For example, tumour cells secrete pro-cathepsin B as well as active forms of cathepsin B. Pro-cathepsin B secretes as the expression of the protein increases [421], whereas secretion of active cathepsin B is likely to involve more complicated signal transduction and remains to be clarified. Secretion of lysosomal enzymes in cancer including pro-cathepsin B can occur due to the combination of high expression and defects in the normal lysosomal trafficking pathways [422-424], while secretion of active cathepsin B from tumour cells can be induced selectively or non-selectively as would be expected for secretion by lysosomal exocytosis [421]. The mechanisms underlying selective secretion remains unclear. Previous studies have shown cathepsin-B secretion can be induced by the arachidonic acid metabolite 12-S-hydroxyeicosatetraenoic acid, an activator of protein kinase C, in human and murine tumour cells [425].

Accumulating evidence has revealed significant roles of galectin-3 in cancer progression and metastasis. Recent studies have shown that overexpression of galectin-3 increases cancer cell motility in colon [379], gastric [426] and thyroid cancer [427]. Galectin-3 overexpression has also been shown to increase tumour cell invasion in various types of cancers including colorectal, ovarian, thyroid, melanoma and the

neuroendocrine tumour pheochromocytoma [428]. Many of these cancer-promoting actions of galectin-3 are associated with galectin-3 interaction with galactoside-terminated cell surface glycoproteins. For example, it has been demonstrated that galectin-3 overexpression promotes tumour cell adhesion to ECM and subsequently the escape of tumour cells from the primary sites [312]. These cancer-promoting activities mediated by galectin-3 can be partially attributed to its interactions with oncofoetal TF-antigen-expressing MUC1 and MUC4 [429, 430]. These galectin-3-MUC1/4 interactions lead to mucin cell surface polarisation and result in introduction of much smaller adhesion molecules, which further promotes adhesion of disseminating tumour cells to the vascular endothelium and assists the formation of tumour emboli [431]. Moreover, secretion of cytokines such as G-CSF, sICAM-1, and IL-6, which promotes metastasis, is also enhanced by circulating galectin-3 [366]. The circulating galectin-3 also promotes cancer proliferation, migration and angiogenesis [390].

Given the broad influence of galectin-3 on cancer progression, it is possible that galectin-3 expression by cancer cells may influence cellular secretion of specific proteases in galectin-3-mediated promotion of cancer progression. This part of the study aims to determine the relationship between galectin-3 expression and secretion and protease secretion in human colon cancer cells.

3.2 Hypothesis and Aims

3.2.1 Hypothesis

Galectin-3 expression and secretion by cancer cells promote cellular secretion of specific proteases.

3.2.2 Aims

The main aims of this part of the study are to assess whether galectin-3 influences the secretion of proteases in human colon cancer cells.

3.3 Methods

3.3.1 Assessment of protease secretion using Proteome Profiler Human Protease Array

The colon cancer SW620, SW480 and HCT116 cells were cultured at 1 x 10^6 cells/ml in a 6-well dish and incubated at 37°C for 24 hours to reach 100% confluence. The culture media were then replaced with serum-free medium containing 0.05% BSA. Immediately after that, the cells were introduced with 10 µg/ml of galectin-3 or BSA and incubated at 37 °C for 24 hours. The culture supernatants were collected and analysed using Proteome Profiler Human Protease Array Kit from R&D system according to manufacturer's instructions. Briefly, the membrane was blocked for one hour with 1% BSA followed by overnight incubation with mixture of detection antibody and samples (1:100 dilution). The membrane was then washed and incubated with 1x Streptavidin-HRP for 30 min. After washes, the membrane was developed as described in chapter two.

To generate heatmap of protease secretion profile, pixel densities on the developed blot were collected and analysed using Imagelab and data output was calculated as follow, the normalized pixel densities of the protease of interest is equal to:

$$\frac{\overline{A} - \overline{N}}{\overline{R} - \overline{N}.}$$

$\overline{A}$ is the mean value of pixel densities of corresponding duplicate spots. $\overline{R}$ is the mean value of the pixel intensities of the reference spots. $\overline{N}$ is the averaged background value.

Subsequently, the normalized pixel density of each individual protease was input into GraphPad Prism 9.5.1 to generate the heatmap.

3.3.2 Assessment of secretion of selected proteins using ELISA

To assess the effect of galectin-3 on protease secretion by ELISA, human colon cancer cells were cultured in a 6-well dish at 1 x 10^6 cells/ml at 37°C overnight. After cells reached 70 to 80% confluence, the media were replaced by the serum-free medium with 0.05% BSA. The cells were then treated with 10 µg/ml galectin-3 or BSA at 0, 24, 48 and 72 hr. The culture supernatants were analysed using corresponding ELISA kits as described in chapter 2.

3.3.3 Assessment of secretion of selected proteins using slot blotting

To assess the effect of galectin-3 on protease secretion by slot blotting, SW620 and HCT116 cells were cultured in a 6-well dish at 1 x 10^6 cells/ml at 37°C for 24 hours to allow cells to reach 90 to 100% confluence. The media were then replaced by serum-free medium with 0.05% BSA, followed by treatment with galectin-3 or BSA at different concentrations (2.5, 5 and 10 µg/ml). The plates were incubated at 37 °C for 24 hours and the supernatants were then collected and analysed using slot blotting as described in chapter 2. The antibodies used were Cathepsin B (20 µg/ml), MMP-13 (2 µg/ml), MMP-1 (40 µg/ml), MMP-12 (5 µg/ml), Cathepsin A (10 µg/ml), and Cathespin X (10 µg/ml).

3.3.4 Assessment of galectin-3 inhibitors on protease secretion

In order to assess the effect of galectin-3 inhibitors on proteases secretion in different cells, each group of cells were cultured in a 6-well dish at 1 x 10^6 cells/ml and incubated at 37°C for 6 hours. After above 90% of the cells were attached to the plate, galectin-3 inhibitors at various concentrations were introduced to the wells and the plates were incubated for 24 hours. The culture supernatants collected and analysed using ELISA.

When assessing the effect of galectin-3 inhibitors on recombinant galectin-3-mediated protease secretion in different cells, the cells were cultured as described previously and incubated at 37°C for overnight, the media were replaced with serum-free medium. The cells were then introduced with 10 µg/ml galectin-3 without or with inhibitors at the same time and incubated at 37°C for 24 hours. The culture supernatants were analysed using corresponding ELISA kits according to manufacturer's instructions.

3.3.5 Assessment of protein expression using western blotting

The expression of six proteases (MMP-1, MM-12, MMP-13, Cathepsin A, B and X) and galectin-3 were examined using western blotting as described previously. The corresponding antibodies used here were anti-MMP-1 antibody (1 µg/ml), anti-MMP-12 antibody (1 µg/ml), anti-MMP-13 antibody (0.5 µg/ml), anti-cathepsin-A antibody (1 µg/ml), anti-cathepsin-B antibody (1 µg/ml), anti-cathepsin-X antibody (1 µg/ml), anti-galectin-3 antibody (1 µg/ml).

3.3.6 Separating HT29 cells into invasive HT29 and non-invasive HT29 cells subpopulations

To separate HT29 cells into invasive and less invasive subpopulations, a transwell with permeable support of 8.0µm PET membrane was used in the experiment. Matrigel was diluted to make the final concentration of 20 µg/ml with serum-free medium. The diluted Matrigel was then loaded onto Transwell insert at 100 µl/well to coat the insert plates and the plates were incubated at 37°C for 2 hours. HT29 cell suspension (1 x 10^6 cells/ml) was then placed in the upper chamber and incubated for 24 hours at 37°C. The cells invaded to the membrane of the lower chamber and the cells remained in the upper chamber were collected separately as invasive HT29 cells and non-invasive HT29 cells, respectively.

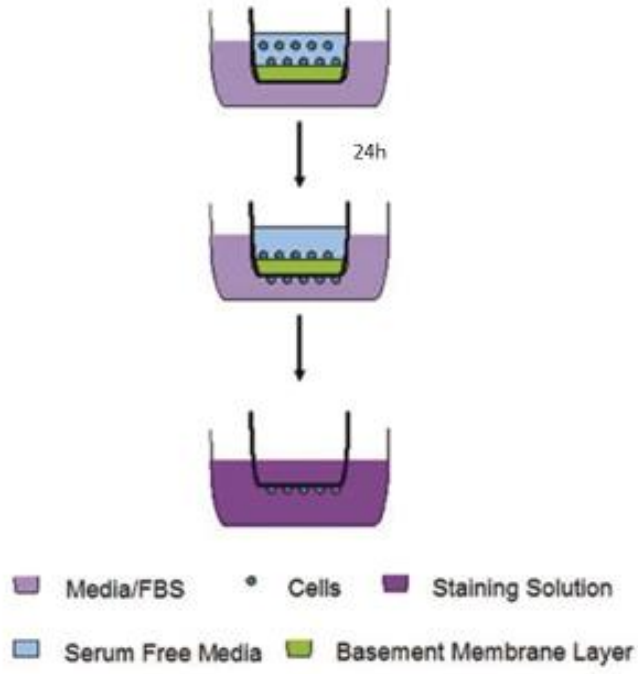

Figure 3.2 HT29 cells subpopulation culture. The HT29 cells were introduced onto the upper chamber and incubated for 24 hr before two sub-populations were collected separately in the upper and lower chambers.

The following assessment of cell invasion was conducted using the protocol described previously in chapter 2.

3.4 Results

3.4.1 Galectin-3 induces protease secretion in colon cancer cells

To assess the effect of galectin-3 on protease secretion from cancer cells, human colon cancer SW620 and SW480 cells were first analysed with a protease array that covers 35 common proteases. SW480 and SW620 colon carcinoma cell lines are derived from primary and secondary tumours resected from a single patient. As such, they are often used to examine genetic changes late in colon cancer progression. The primary tumour-derived SW480 cells have an epithelioid morphology *in vitro*, while metastasis-derived SW620 cells have a fibroblast-like appearance. SW620 and SW480 cells were cultured in the presence or absence of 10 µg/ml galectin-3, a concentration that is close to that seen in the circulation of colon cancer patients with metastasis [366], for 24 hours, and the concentrations of proteases in the media was measured by the Proteome Profiler Human Protease Array. In comparison to cells treated with control BSA, treatment of the cells with galectin-3 resulted in increased secretion of several proteases from SW620 cells (Figure 3.3). Among the 35 proteases, the 10 proteases shown the highest increases in response to galectin-3 were MMP-12 (2.25-fold), Cathepsin B (2.04-fold), MMP-1 (2.04-fold), MMP-13 (1.97-fold), Kallikrein 13 (1.94-fold), DPPIV/CD26 (1.89-fold), Cathepsin A (1.82-fold), MMP-2 (1.82-fold), Cathepsin X (1.68-fold) and Kallikrein 3 (1.65-fold) (Fig 3.4).

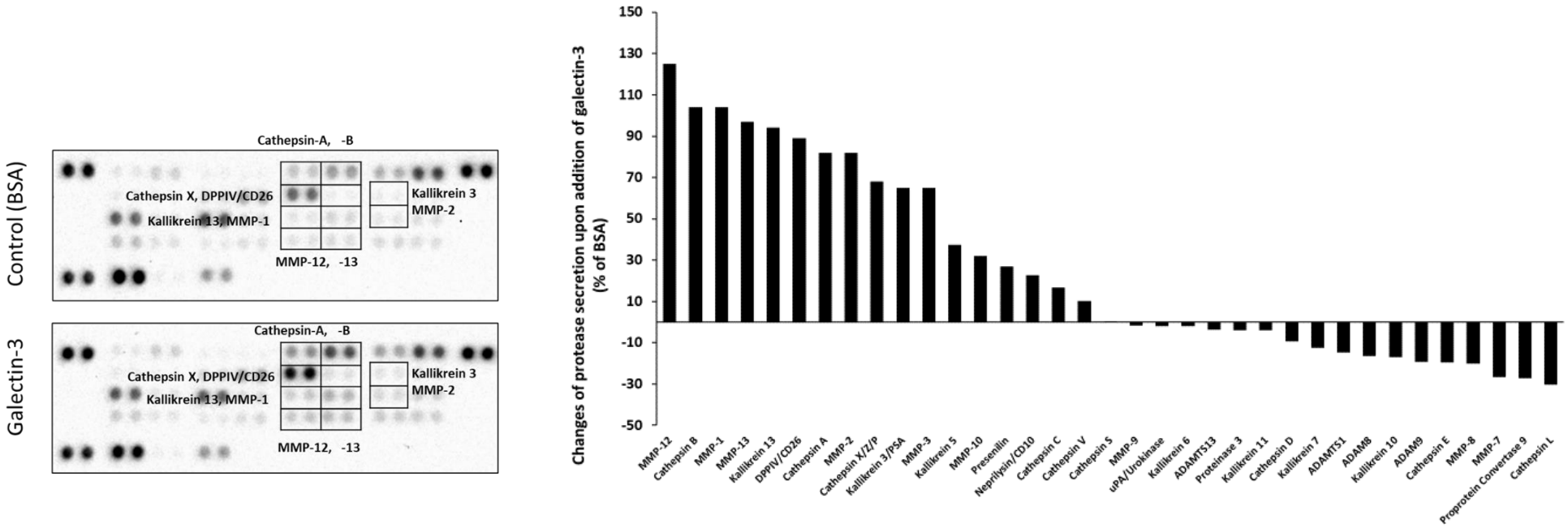

Figure 3.3. Galectin-3 induces the secretion of a number of proteases in human colon cancer SW620 cells. SW620 were treated with 10 µg/ml recombinant galectin-3 or BSA for 24 hr before the levels of 35 proteases were analysed by Proteome Profiler Human Protease Array. This experiment was performed once.

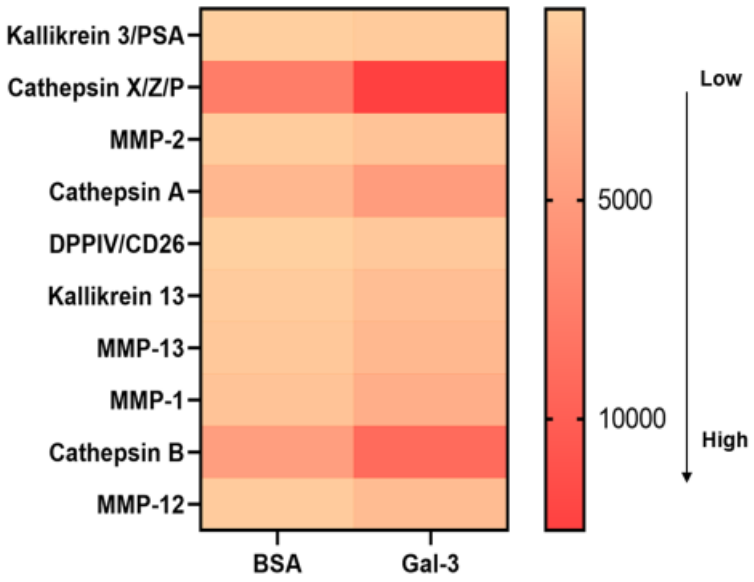

Figure 3.4. Galectin-3 induced several protease secretions in SW620 cells. The 10 most affected proteases in response to galectin-3 are shown in heatmaps (with changes ranked from lowest to highest).

Meanwhile, comparing to SW620 cells, in SW480 cells, there were much less increases of secretion of the 10 most affected proteases seen in SW620 cells with galectin-3 treatment (Fig 3.5). Instead, proteases with the highest increase in secretion in response to galectin-3 in SW480 cells were Naprilysin (1.48-fold), followed by Proteinase 3 (1.47-fold), Presenilin (1.46-fold), MMP-10 (1.33-fold), uPA (1.32-fold), Cathepsin L (1.3-fold), MMP-3 (1.3-fold), Kallikrein 11 (1.26-fold), Kallikrein 7 (1.24-fold), MMP-2 (1.24-fold) and ADAMTS1 (1.23-fold) (Fig 3.4.1.3).

To see if this effect of galectin-3 also occurred to other human colon cell line, human colon cancer HCT116 cells was analysed using the same methods. Human HCT116 cells is a human colorectal carcinoma cell line initiated from an adult male with an epithelial morphology. The proteome profile in HCT116 cells in response to galectin-3

exhibited similar increases when comparing to SW620 cells, in which the secretions of 10 most affected proteases seen in SW620 were also found to be upregulated in HCT116 cells (Fig 3.6). In HCT116 cells, the ten proteases with highest increases in secretion in response to galectin-3 were: MMP-1 (4.66-fold), Kallikrein 13 (4.1-fold), DPPIV/CD26 (2.62-fold), MMP-13 (1.94-fold), MMP-2 (1.93-fold), MMP-12 (1.84-fold), MMP-3 (1.62-fold), Cathepsin B (1.29-fold), Cathepsin A (1.21-fold) and Cathepsin X (1.2-fold).

Taken together, the presence of galectin-3 induces secretion of several proteases in SW620 cells and HCT116 cells but less so in SW480. These proteases include cysteine proteases such as cathepsin B, cathepsin A and cathepsin X, and MMPs such as MMP-1, MMP-12, and MMP-13.

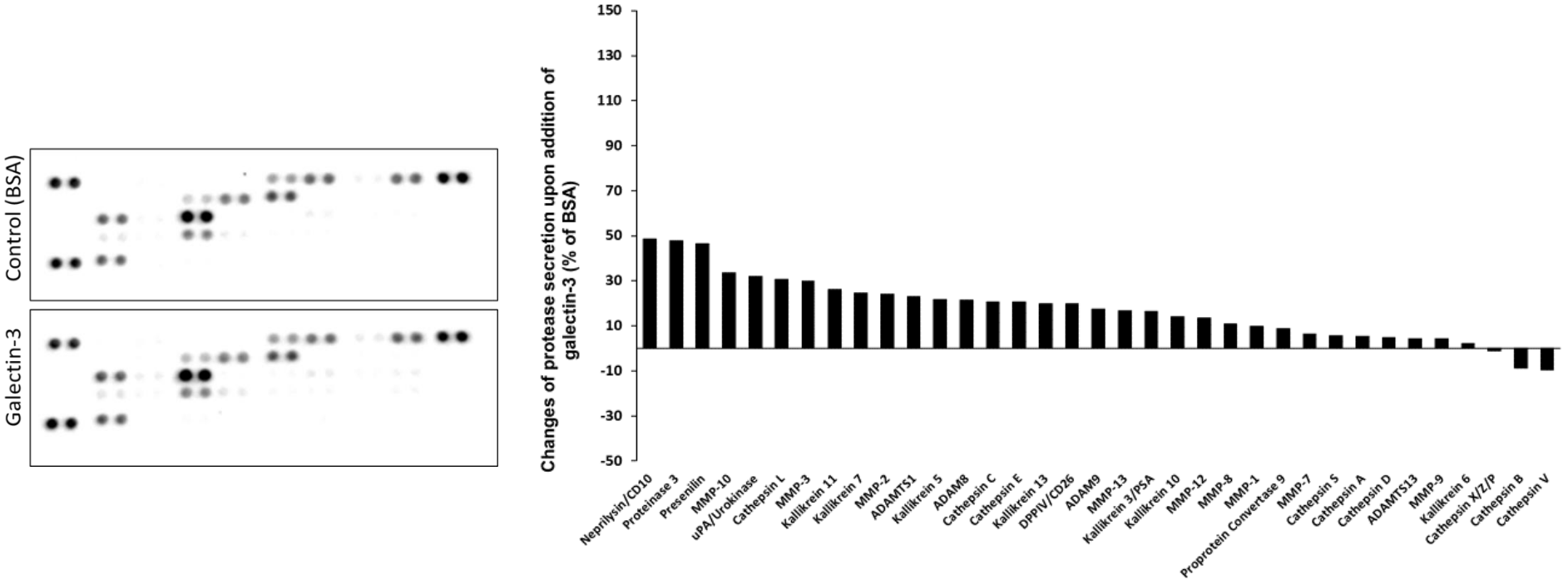

Figure 3.5. Galectin-3 caused much less changes of protease secretion in human colon cancer SW480 cells. SW480 were treated with 10 µg/ml recombinant galectin-3 or BSA for 24 hr before the levels of 35 proteases were analysed by Proteome Profiler Human Protease Array. This experiment was performed once.

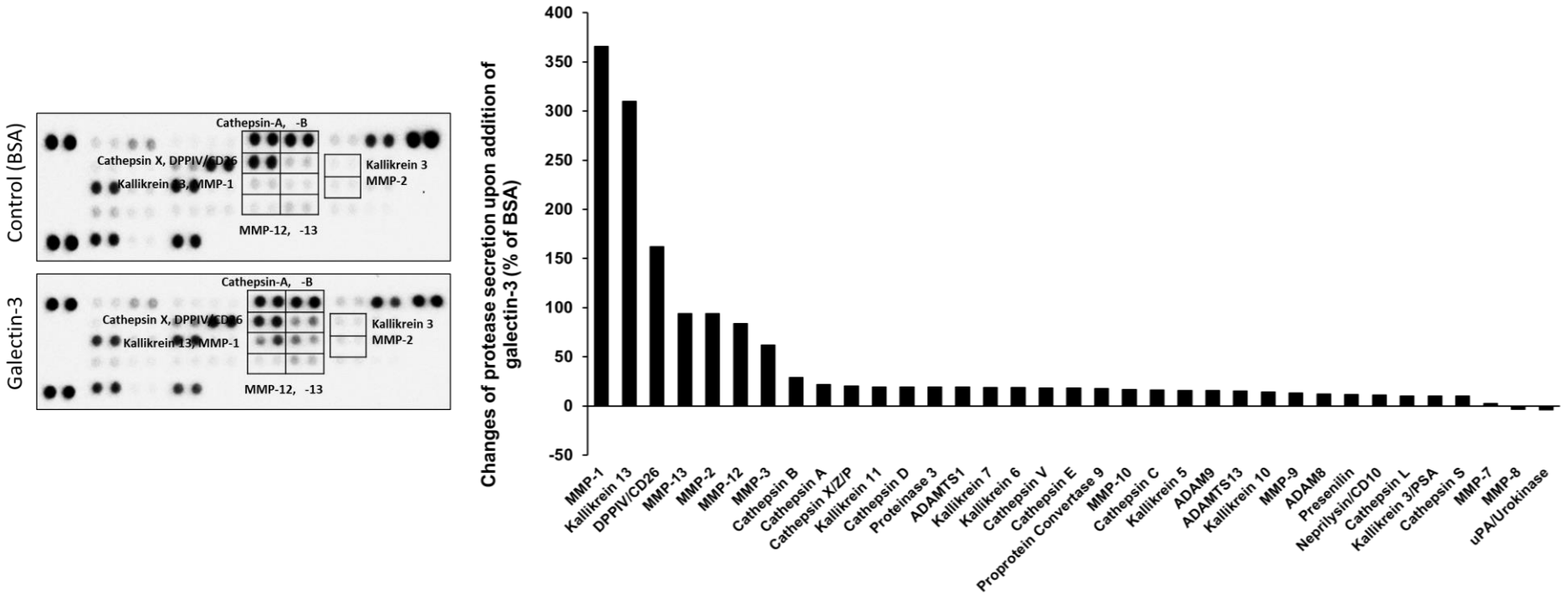

Figure 3.6 Galectin-3 induces the secretion of a number of proteases in human colon cancer HCT116 cells. HCT116 were treated with 10 µg/ml recombinant galectin-3 or BSA for 24 hr before the levels of 35 proteases were analysed by Proteome Profiler Human Protease Array. This experiment was performed once.

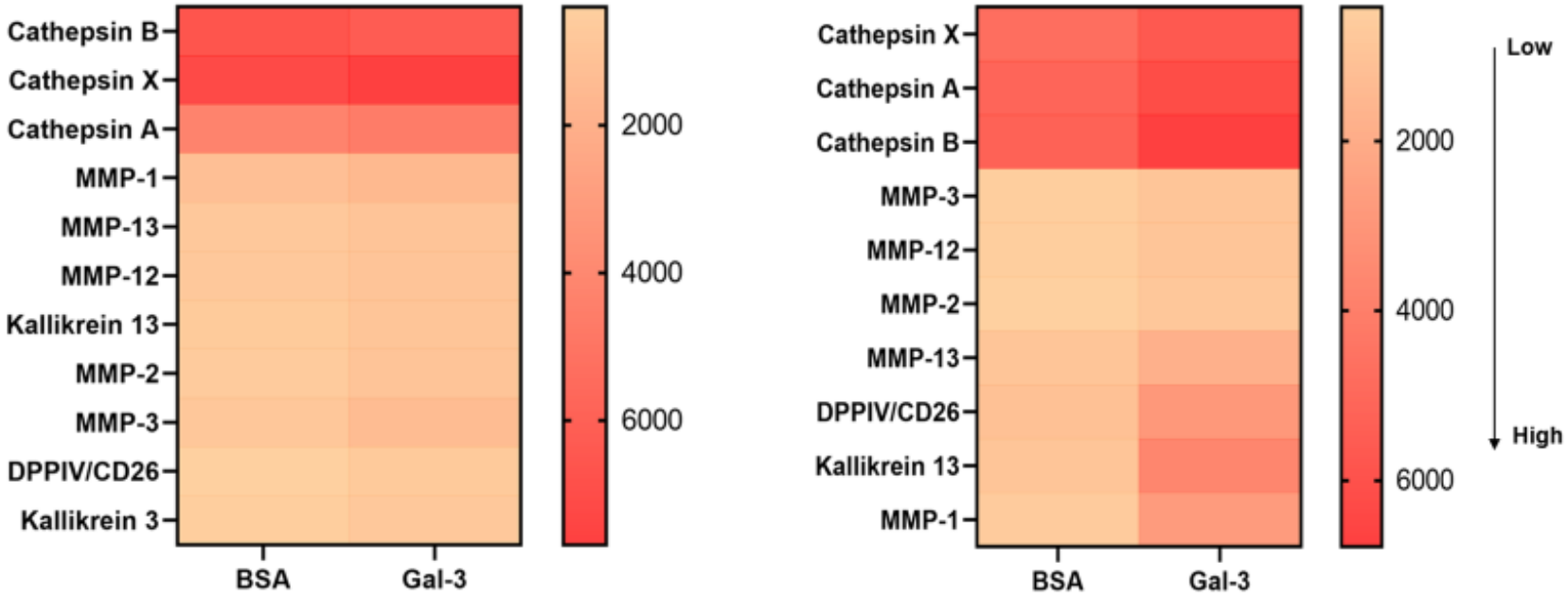

Figure 3.7 Galectin-3 induces the secretion of selected proteases in human colon cancer HCT116 cells but much less in SW480 cells.
The selected 10 proteases from SW620 proteome profile in response to galectin-3 in SW480 (left) and HCT116 (right) are shown in heatmaps.

3.4.2 Galectin-3 mediates protease secretion in both time- and dose-dependent manners

To further investigate the effect of galectin-3 on protease secretion, SW620 and HCT116 cells were treated with 10 μg/ml galectin-3 for various times and the secretion of the three most highly affected proteases MMP-1, MMP-13 and cathepsin-B in SW620 were analysed by ELISA. The presence of galectin-3 caused time- (Fig 3.8E, F) dependent increases in cathepsin-B secretion in both SW620 and HCT116 cells. After 24 hr treatment with 10 μg/ml galectin-3, a 2.1- and 2.0-fold increase was seen in SW620 and HCT116 cells, respectively. Meanwhile, after 24 hours treatment, the levels of MMP-13 secretion by SW620 (Fig 3.8C) and HCT116 cells (Fig 3.8D) were below the accurate measurement threshold by ELISA. However, at 48 hours, MMP-13 secretion showed 1.53-fold increase, and 2-fold increase at 72 hours in response to galectin-3 in SW620 cells, while it showed 2.47-fold increase at 48 hours and 1.73-fold increase at 72 hours.

The levels of MMP-1 secretion in HCT116 were also below accurate measurement threshold (Fig 3.8B) by ELISA. On the other hand, in SW620 cells, after 24, 48 and 72 hours, the levels of MMP-1 secretion showed 1.39-fold, 1.81-fold and 1.51-fold increase in response to galectin-3, respectively.

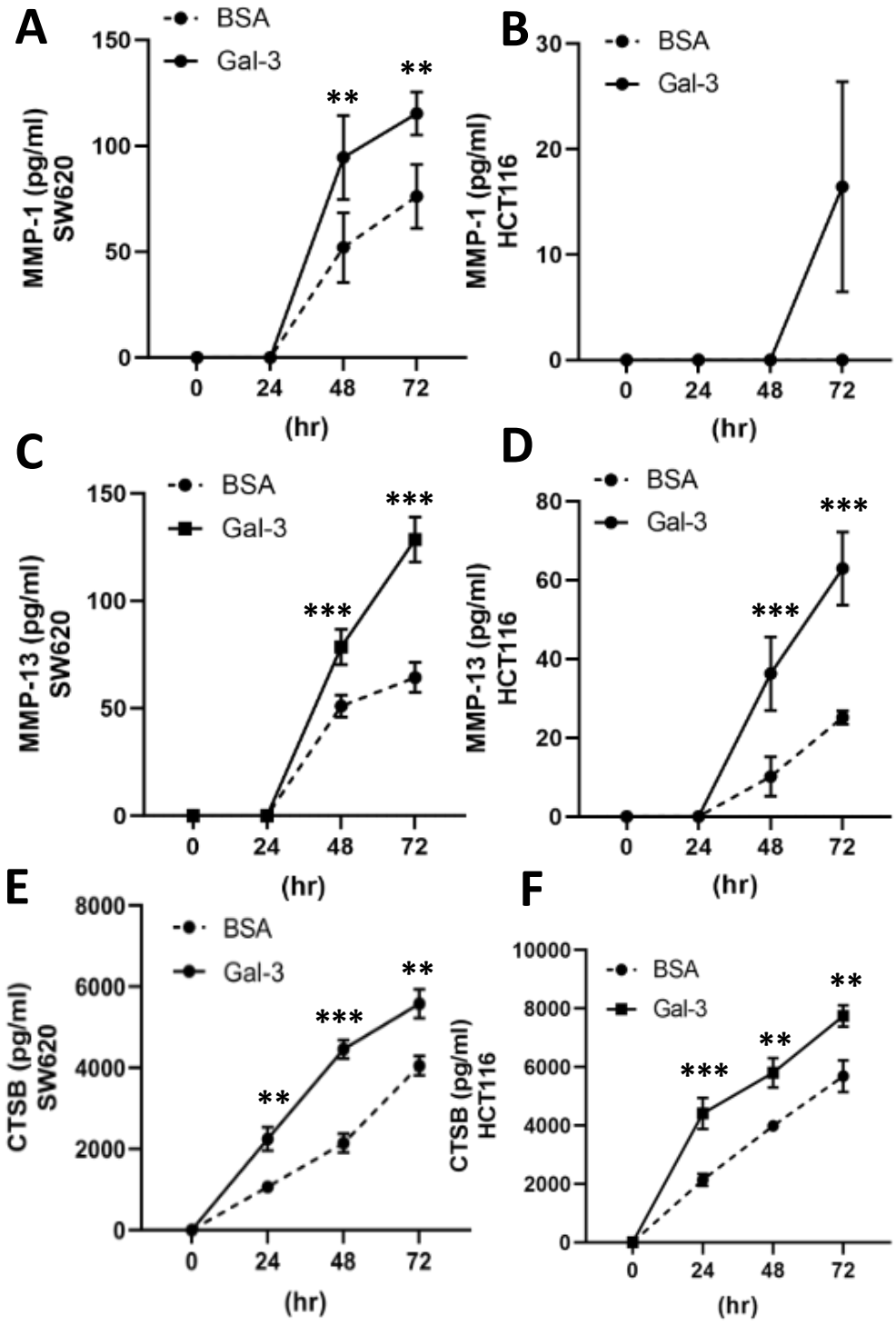

Figure 3.8. Galectin-3 induces protease secretion in human colon cancer cells in a time-dependent manner. SW620 and HCT116 cells were treated with galectin-3 or BSA (10 µg/ml) for 24, 48 and 72 hr before the concentrations of MMP-1(A, B), MMP-13 (C, D) and cathepsin B (E, F) were analysed using ELISA. Data are presented as mean ± SD of three independent experiments (B are from two independent experiments), each in triplicate. *** $p < 0.001$, ** $p < 0.01$ (ANOVA). Šídák post-hoc correction was applied.

To overcome technical difficulty in ELISA and to measure accurately, the levels of MMP-1, MMP-13 and Cathepsin B in response to different pathological galectin-3 concentrations was measured by slot blotting. The presence of galectin-3 caused dose-dependent increases in cathepsin-B secretion (Figure 3.9C) in both SW620 and HCT116 cells. After 24 hr treatment with 10 µg/ml galectin-3 in comparison to control cells, the secretion of cathepsin B shows a 2.5- and 1.7-fold increase in SW620 and HCT116 cells, respectively. The presence of galectin-3 also caused dose-dependent increases in MMP-1 and MMP-13 secretion in both SW620 and HCT116 cells (Fig 3.9A and B). At 5 µg/ml, galectin-3 induced 1.25- and 1.36- fold increases of MMP-13 and 1.73- and 1.98- fold increases of MMP-1 secretion from SW620 and HCT116 cells, respectively.

In addition to MMP-1, MMP-13 and cathepsin-B, we also examine other highly affected proteases by galectin-3 treatment using slot blotting, including MMP-12, cathepsin-A, and cathepsin-X (Fig 3.10). The presence of galectin-3 induced dose-dependent increase of MMP-12, cathepsin-A, and cathepsin-B secretion in SW620 cells. At 5 µg/ml, galectin-3 caused 1.55-, 1.66- and 1.43-fold increases of MMP-12, Cathepsin A and Cathepsin B in SW620 cells, respectively. And at 10 µg/ml, galectin-3 induced 2.11-, 2.06- and 2.14-fold increase of MMP-12, Cathepsin A and Cathepsin X, respectively. However, galectin-3 showed much less or no effect on the secretion of these proteins in SW480 cells.

Taken together, these results suggest that galectin-3 induces protease secretion (MMP-1, -12, 13, Cathepsin A, B and X) in both dose- and time- dependent manner in SW620 and HCT116 cells.

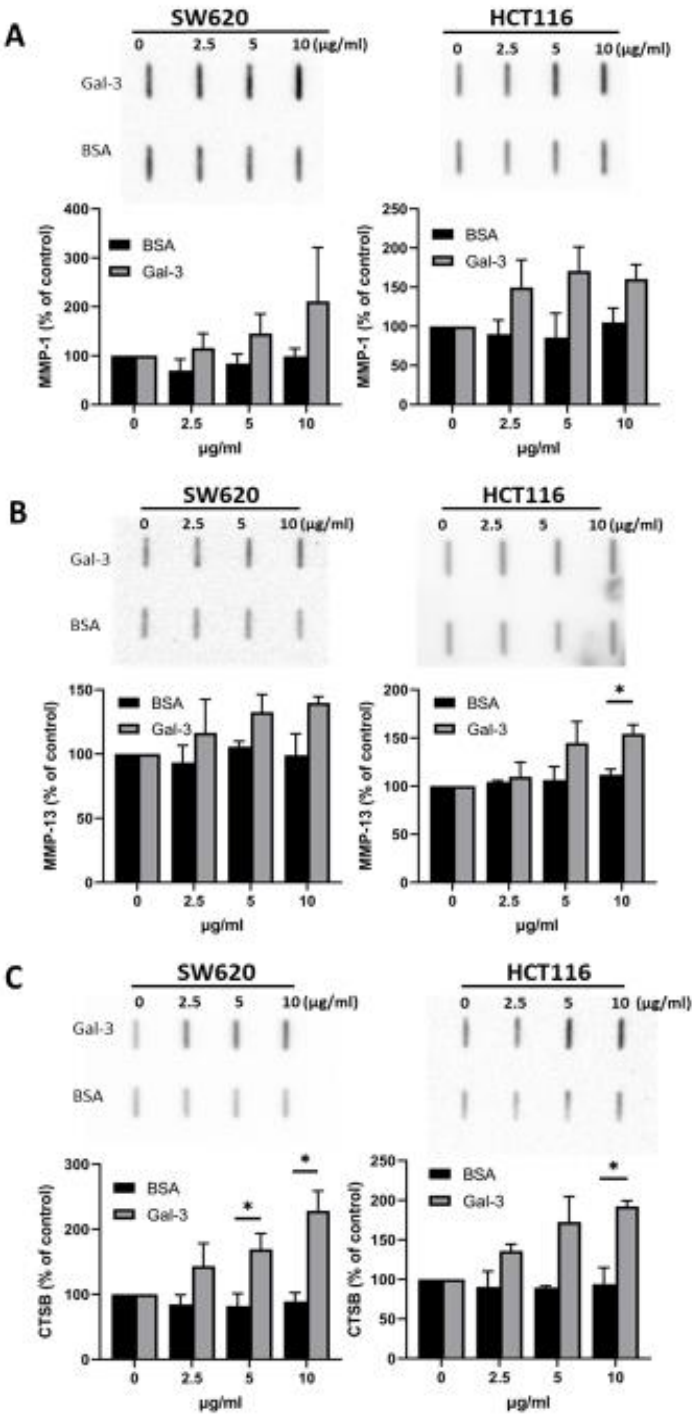

Figure 3.9. Galectin-3 induces protease secretion in human colon cancer cells in a dose-dependent manner. SW620 and HCT116 cells were treated with different concentrations of galectin-3 for 24 hr before the levels of MMP-1 (A), MMP-13 (B) and Cathepsin B (C) in the medium were analysed by slot blotting. The slot densities from three independent experiments were quantified and are shown in the bottom panels. Data are presented as mean ± SD of three independent experiments. * $p < 0.05$ (ANOVA with Šídák post-hoc).

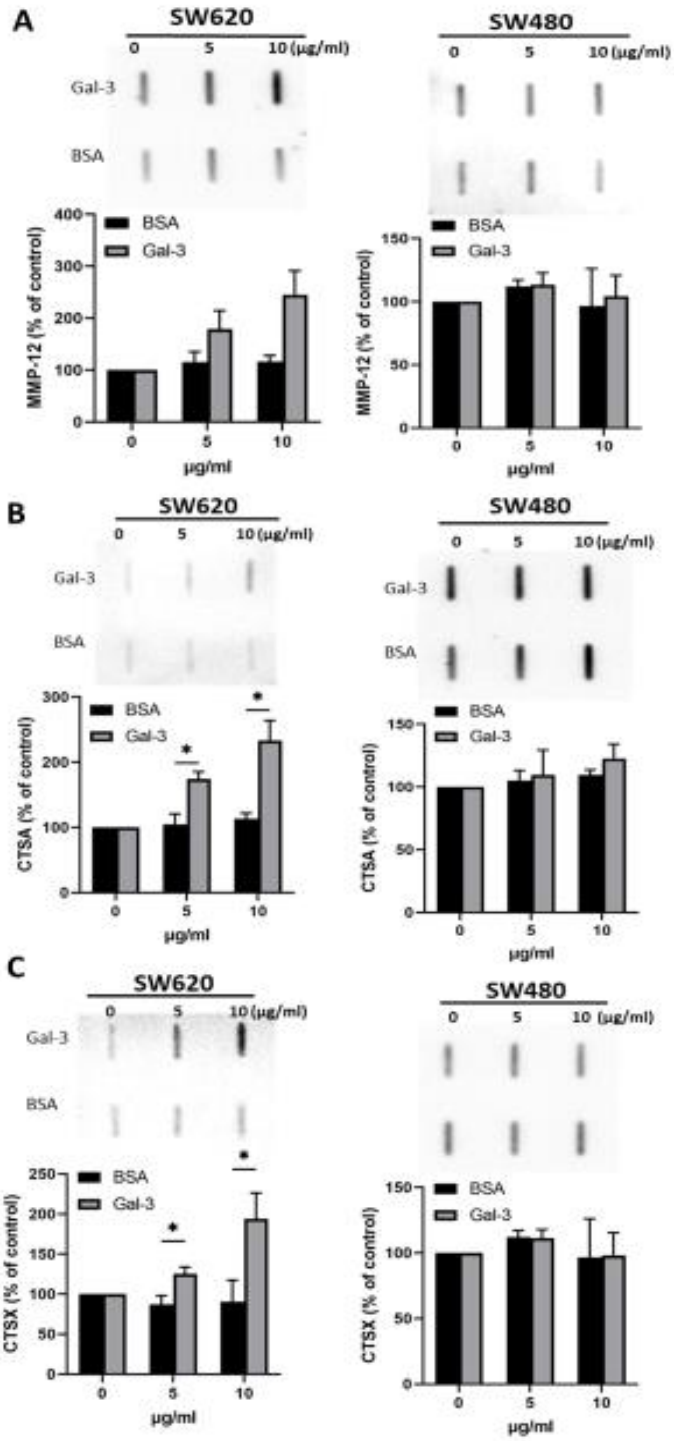

Figure 3.10. Galectin-3 induces protease secretion in human colon cancer cells in a dose-dependent manner. SW620 and SW480 cells were treated with different concentrations of galectin-3 for 24 hr before the levels of MMP-12 (A), Cathepsin A (B) and Cathepsin X (C) in the medium were analysed by slot blotting. The slot densities from three independent experiments were quantified and are shown in the bottom panels. Data are presented as mean ± SD of three independent experiments. * $p < 0.05$ (ANOVA with Šídák post-hoc).

3.4.3 Galectin-3 does not affect the expression of the proteases in cells

The increased secretion of proteins is often caused by higher protein expression in the cells. There are many signalling pathways in cells that regulate the expression of proteases. In order to determine whether galectin-3 mediates protease secretion through increasing their expressions, the expression level of 6 proteases MMP-1, -12, -13, cathepsin -A, -B and -X in cells were examined using western blotting in SW620 and SW480 cells (Fig 3.11). Cells were treated with galectin-3 at 0, 5 and 10 μg/ml for 24 hours before the cells were lysed and the expression of the proteases were analysed. The results showed MMP-1 and MMP-13 were expressed at the same level by SW480 and SW620 cells while SW620 cells expressed more cathepsin B, cathepsin A and MMP-12 than SW480 cells. On the other hand, SW480 cells expressed more cathepsin X than SW620 cells. It was also found the expressions of MMP-1, 12, -13, cathepsin -A, -B and -X in both SW620 and SW480 cells were not affected by the presence of galectin-3 at any concentration. This suggests that galectin-3 induces protease secretion in those cells but does not affect the expressions of those proteins.

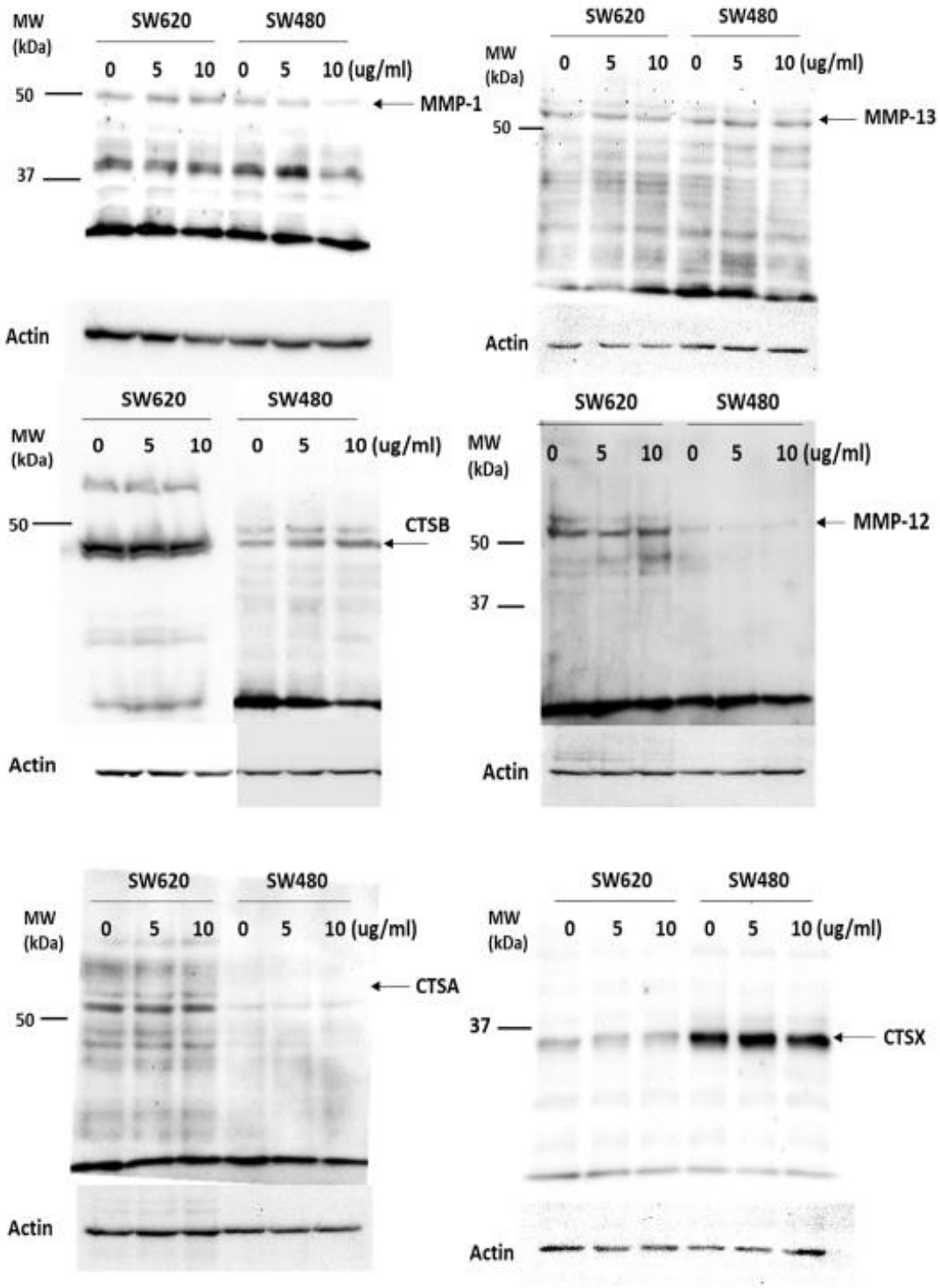

Figure 3.11. Galectin-3 does not affect the expressions of Cathepsin B, MMP-1, MMP-13, MMP-12, Cathepsin A and Cathepsin X in colon cancer cells. SW620 and SW480 cells were treated with 0, 5, and 10 µg/ml galectin-3 for 24 hr and cell lysates were analysed by immunoblotting. The introduction of galecitin-3 had no effect on proteases expressions. This experiment was performed once.

3.4.4 Galectin-3 expression and secretion are associated with proteases secretion in cancer cells

The above investigations were done with exogenous galectin-3. To test the relationship observed with exogenous galectin-3 in protease secretion occurs with endogenous galectin-3, galectin-3 expression in SW620 cells was suppressed using shRNA [432]. Galectin-3 shRNA suppression led to 99% reduction of galectin-3 expression in the cells in comparison to transfection with control shRNA (Fig 3.12A). when galectin-3 secretion in those cells were compared, galectin-3 shRNA suppression led to significant reduction of galectin-3 secretion (13-fold) over 72 hours (Fig 3.12B).

We then compared protease secretion in these cells. SW620-shGal3 and SW620-shCon cells were seeded at the same cell numbers and levels of Cathepsin B and MMP-13 secretion were measured by ELISA after 24, 48 and 72 hours. The results showed the suppression of galectin-3 expression resulted in significant reductions in Cathepsin-B and MMP-13 secretion by 40% (Fig 3.12C) and 49% (Fig 3.12D) at 48 hr culture, and lower levels of cathepsin-B and MMP-13 were produced by SW620-shGal3 than SW620-shCon cells after 72 hr culture. Together, these results suggest that higher galectin-3 expression and secretion is associated with increased protease secretions.

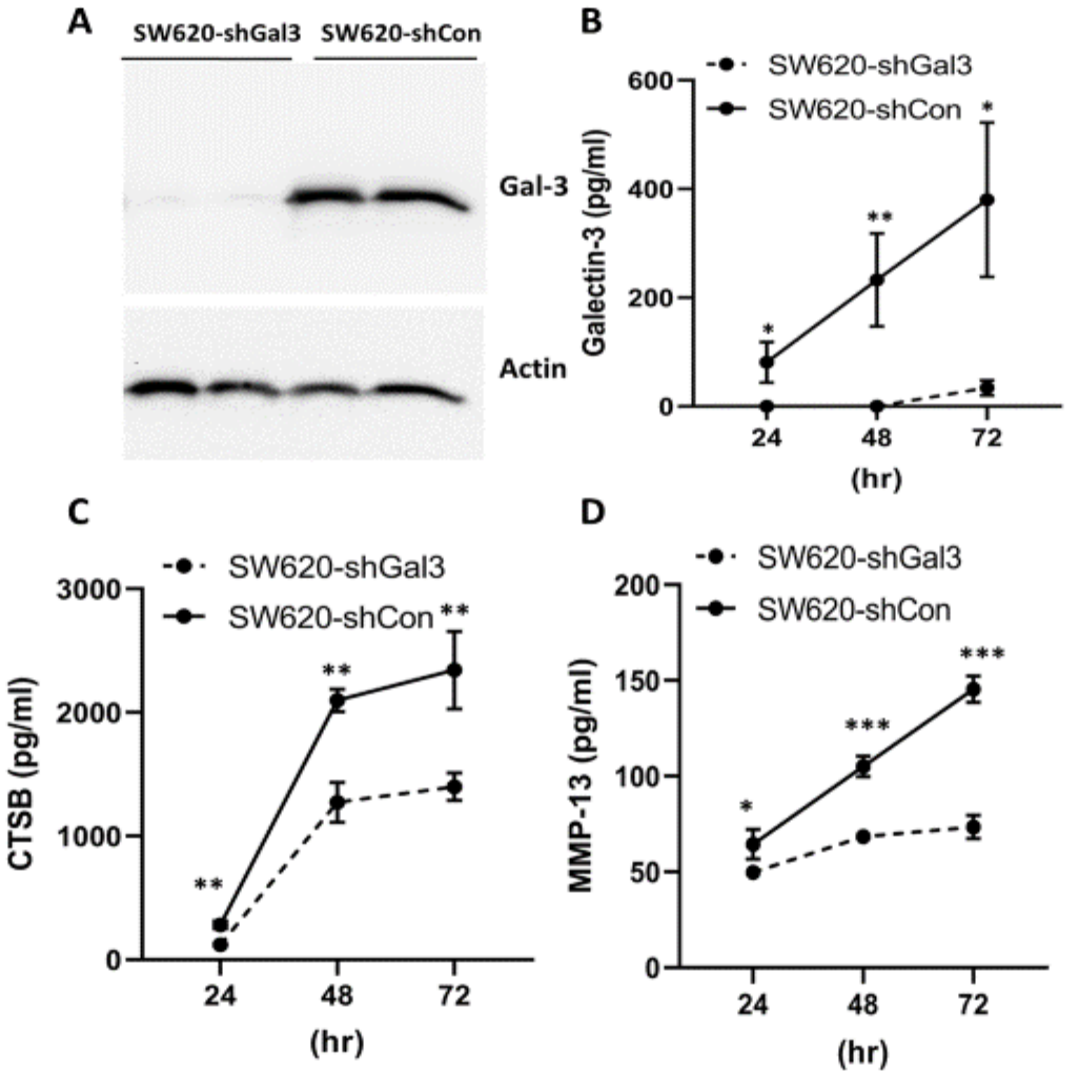

Figure 3.12. Galectin-3 expression and secretion promotes protease secretion in colon cancer cells. Galectin-3 expression and secretion in galectin-3 knockdown SW620-shGal3 and control SW620-shCon cells were assessed by immunoblotting (A) and galectin-3 ELISA (B), respectively. Galectin-3 expression (A) and secretion (B) were both substantially reduced in shRNA galectin-3 knockdown cells. SW620-shCon cells secreted higher concentrations of cathepsin B (C) and MMP-13 (D) than SW620-shGal3 cells when assessed by ELISA. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (ANOVA with Šídák post-hoc).

To further determine the relationship between galectin-3 expression and protease secretion, we separated heterogenous HT-29 colon cancer cells into invasive and none-invasive sub-populations through 24 hr incubation in trans-wells. The cells that remained in the trans-wells (non/less-invasive HT29-N) and the cells which invaded to the bottom side of the trans-wells (invasive HT29-I) were collected. These two subpopulations of HT29 cells were cultured in fresh flask for 3 days. The morphology of HT29-N cells is more epithelial-like while HT29-I cells are more fibroblasts-like (Figure 3.13A). When these two sub-populations of HT29 cells were cultured in new trans-wells, HT29-I cells showed 2.4-fold higher invasion than HT29-N cells (Fig 3.13B).

The expression and secretion of two subpopulations HT29 cells were also measured using immunoblotting and ELISA. The invasive HT29-I cells were found to express 12- and 1.55-fold, respectively, higher levels of galectin-3 than the non/less-invasive HT29-N cells in sub-confluent and fully confluent cells respectively (Fig 3.14A). Likewise, significantly higher level of galectin-3 was secreted by HT29-I cells than the HT29-N cells (Fig 3.14B). After 72 hours, a 1.9-fold higher level of galectin-3 was secreted by HT29-I than HT29-N cells.

Given the differential galectin-3 expression and secretion in two subpopulations of HT29 cells, we then assessed the protease secretion in those invasive and non-invasive HT29 cells. HT29-I and HT29-N cells were cultured at the same cell numbers for 96 hours, and secretion levels of Cathepsin B and MMP-13 were analysed by ELISA. Much higher levels of MMP-13 (Fig 3.15A) and Cathepsin-B (Fig 3.15B) were secreted by HT29-I than HT29-N cells. At 72 hr, 89% and 43% higher MMP-13 and Cathepsin-B levels, respectively, were secreted by HT29-I cells than HT29-N cells.

Taken together, the results showed that both SW620-shCon cells and HT29-I cells expressed and secreted higher levels of galectin-3 comparing to SW620-shGal3 cells and HT29-N cells, respectively, while both SW620-shCon cells and HT29-I cells had higher levels of Cathepsin B and MMP-13 secretion than SW620-shGal3 cells and HT29-N cells, respectively. These results suggest that higher endogenous galectin-3 expression and secretion enhances proteases secretion in human colon cancer cells.

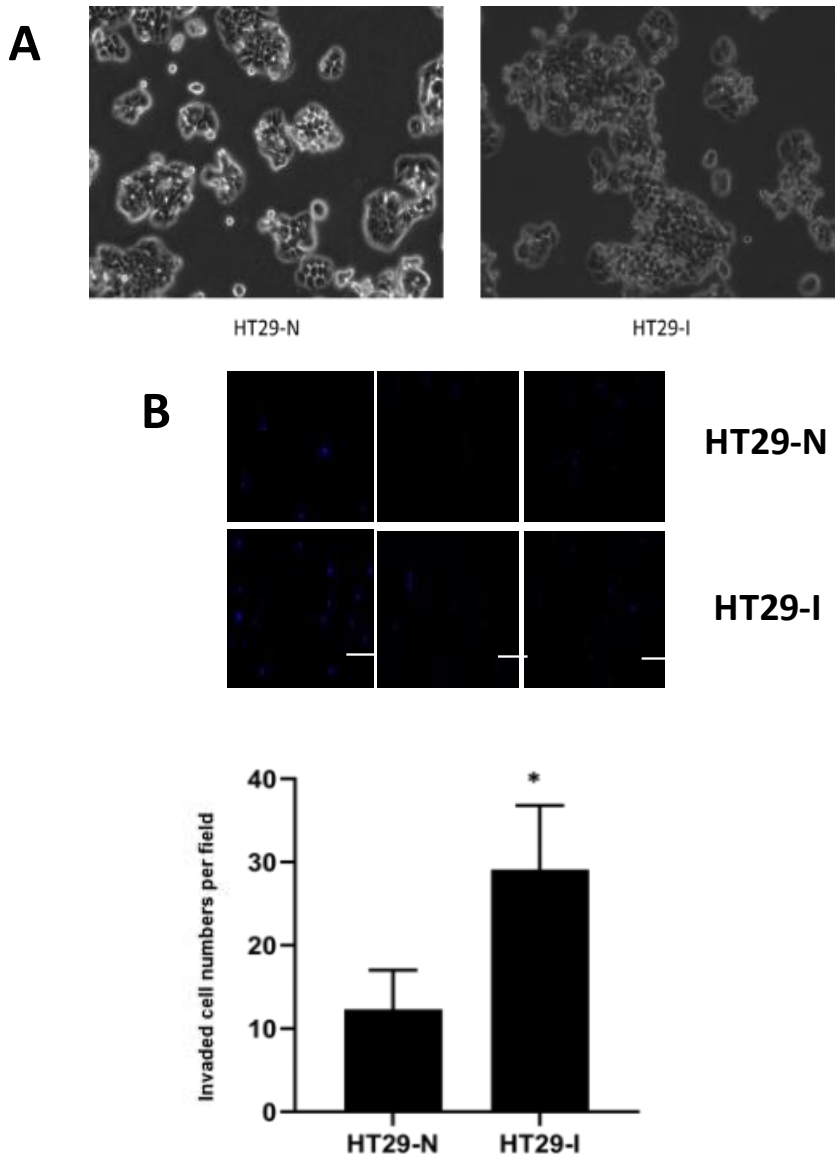

Figure 3.13. Galectin-3 expression and secretion promotes protease secretion and cell invasion of HT29 cells. (A) HT29 cells were separated into invasive (HT29-I) and less/non-invasive (HT-29N) sub-populations. Scale bar = 100 µm. (B) When both sub-populations reseeded into the Transwell with Matrigel, HT29-I cells showed significant higher invasion than HT29-N cells. Data are presented as mean ± SD from three independent experiments, each in triplicate. * $p < 0.05$ (t-test). Typical images of HT29 cells after 24-hour culture are shown in A.

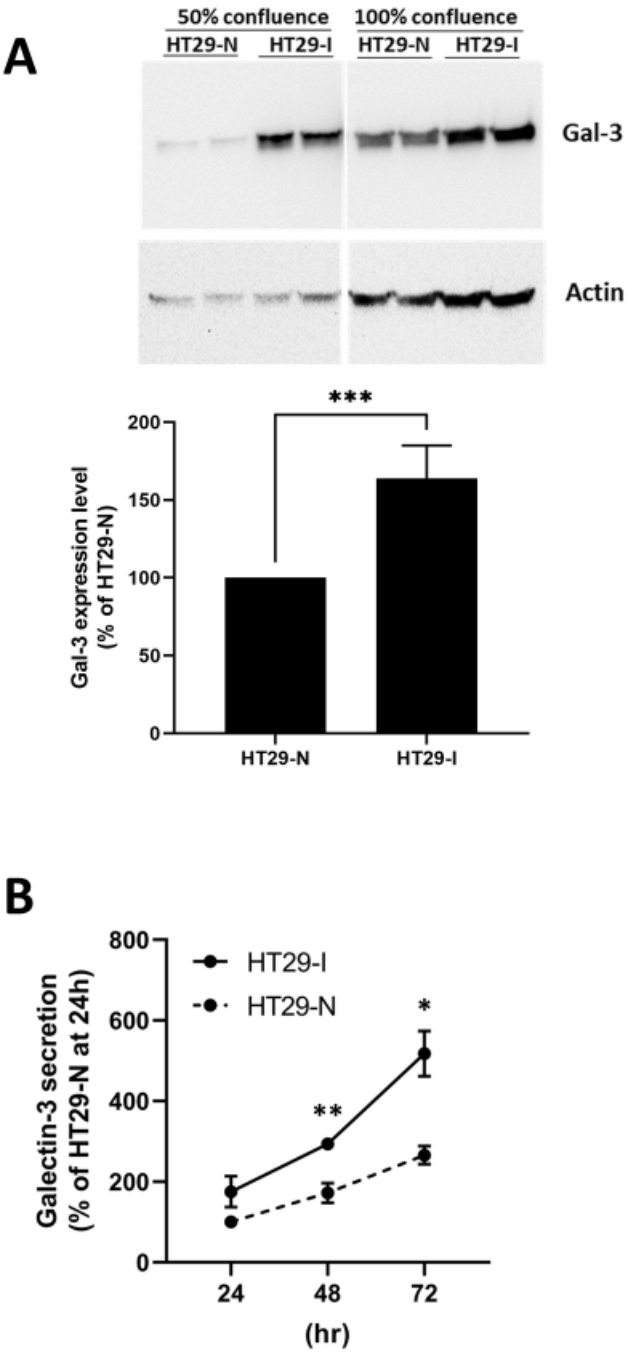

Figure 3.14. Invasive HT29 cells express and secrete higher galectin-3. HT29-I cells express (B) and secrete (C) higher amounts of galectin-3 than the non-invasive HT29-N cells when assessed by immunoblotting (A) and ELISA (B). Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (t-test and ANOVA with Šídák post-hoc).

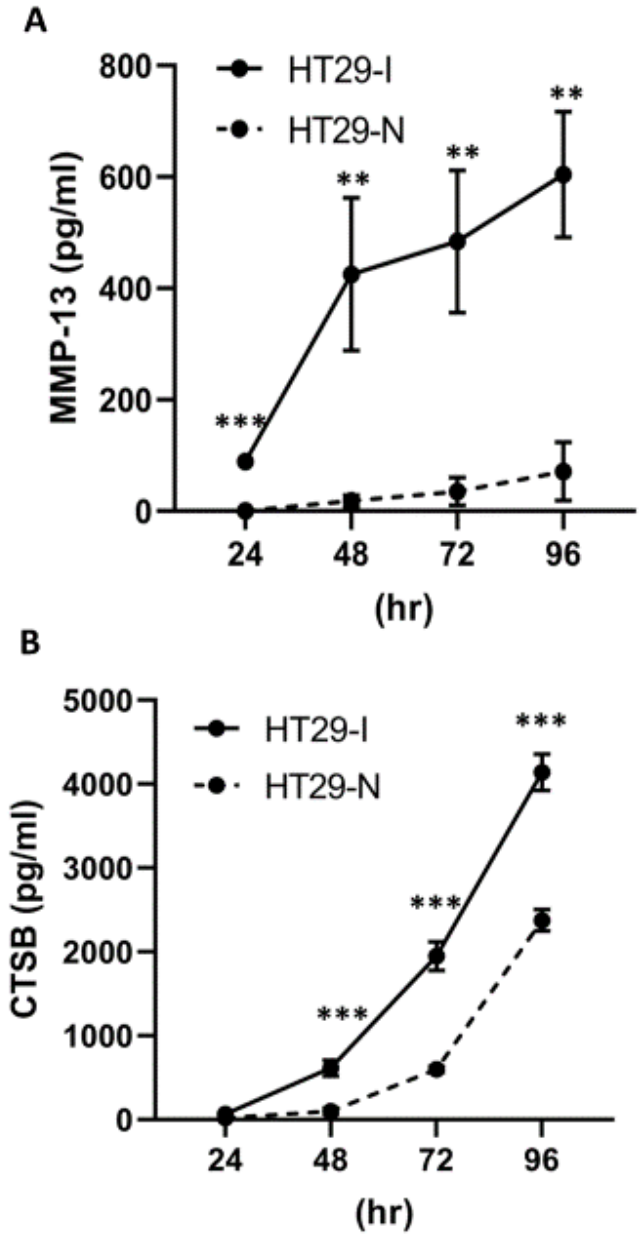

Figure 3.15. HT29-I cells secrete higher level of protease than HT29-N cells. Levels of CTSB and MMP-13 in the culture medium of HT29-N and -I cells were assessed by ELISA. HT29-I cells secreted higher concentrations of cathepsin B (A) and MMP-13 (B) than HT29-N cells. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$, (ANOVA with Šídák post-hoc).

3.4.5 Galectin-3 mediates protease secretion predominately through its extracellular action

To further investigate the effect of galectin-3 on protease secretion, multiple galectin-3 inhibitors were employed in this study. Many therapeutic strategies have recently been developed to inhibit galectin-3 actions in cancer. These include carbohydrate-based small synthetic inhibitors, anti-MUC1 antibodies, a TF antigen-mimicking vaccine, peptide-based inhibitors, truncated galectin-3 form and natural galectin-3 inhibitors such as modified citrus pectin. In this study, the classic galectin-3 inhibitors lactose, asialofetuin (ASF) as well as two new inhibitors developed in our lab modified heparin F3 and K2 (unpublished) were used [432]. Lactose and galactose are themselves natural inhibitors of galectin-3, though they have weak binding affinity. ASF is a glycoprotein, and express the Thomsen-Friedenreich-antigen [433], which is a ligand for galectin-3. Also, chemically modified, low sulfated heparin derivatives have been identified as potent galectin-3 binding inhibitors. These heparin derivatives bind to the galetin-3 carbohydrate-recognition domain and inhibit galectin-3-ligand binding and galectin-3-mediated metastasis [432]. K2 is a compound galectin-3 inhibitor recently developed in our lab (unpublished data).

The Cathepsin B secretion was assessed in the presence or absence of the galectin-3 inhibitors, in SW620-shCon and SW620-shGal3 cells (Figure 3.4.5.1). After 24 hours treatment, the level of Cathepsin B secretion was significantly reduced in SW620-shCon cells by these inhibitors, in which 18%, 16%, and 20% reduction caused by lactose, ASF and K2, respectively (Figure 3.4.5.1A). much less effect of these inhibitors on Cathepsin B secretion was seen in SW620-shGal3 cells, in which only 9%, 0.2%, and 9% reduction caused by lactose, ASF and K2, respectively (Figure 3.4.5.1B).

When the effect of those inhibitors was compared in HT29 cells, the introduction of lactose, ASF and K2 caused reduction of Cathepsin B secretions in both HT29-I and HT29-N cells in which the reduction in HT29-N cells was much less than in HT29-I cells (3.4.5.1C). In HT29-N cells, comparing to control, 6%, 9% and 10% reductions were caused by lactose, ASF and K2, respectively. Meanwhile, in HT29-I cells, 15%, 11% and 13% reductions were caused by lactose, ASF and K2, respectively (3.4.5.1D).

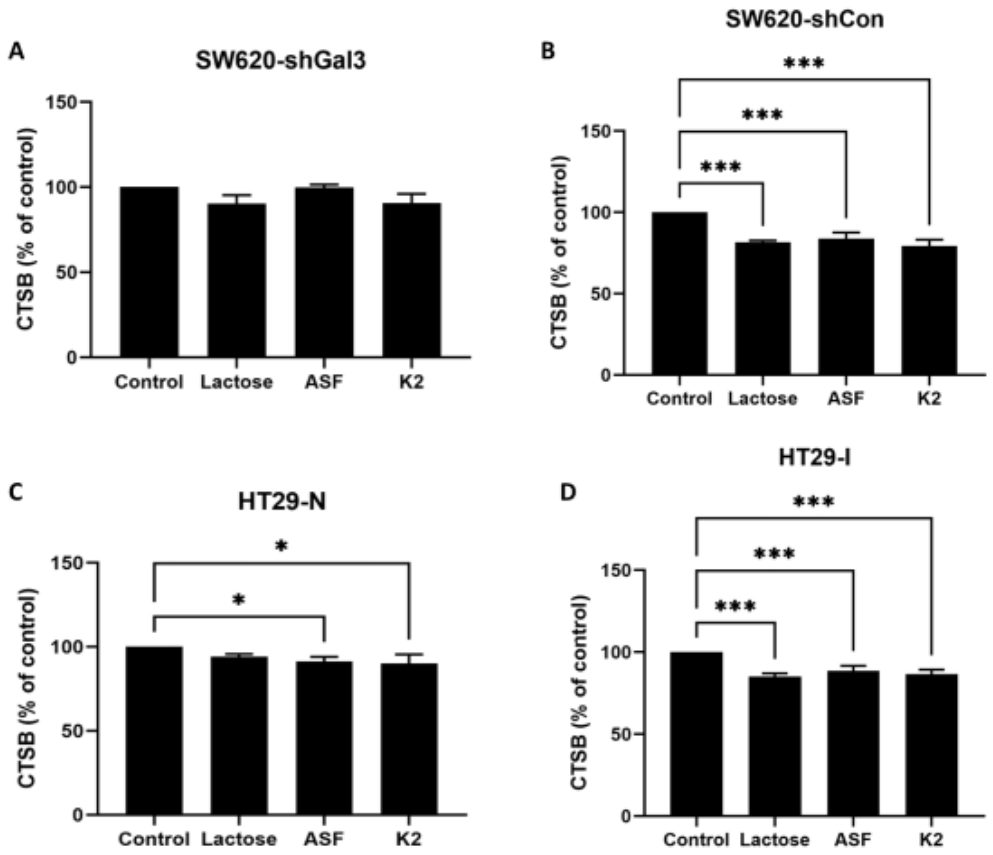

Figure 3.16. Galectin-3 inhibitors suppress Cathepsin B secretion. Each group of cells were cultured at least 6 hours prior to introduction of the inhibitors: lactose (100 µg/ml), ASF (20 µg/ml) and K2 (2 µM) for 24 hours. The galectin-3 inhibitors caused significant reduction of Cathepsin B secretion in SW620shCon (B) and HT29-I (D) cells while less so in SW620shGal3 (A) and HT29-N (C) cells. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, * $p < 0.05$ (ANOVA with Dunnett's test).

Moreover, the reduction of Cathepsin B secretion in SW620-shCon caused by galectin-3 inhibitors were dose dependent (Fig 3.17). Lactose caused 62% decrease at 400 µg/ml comparing to the control, while it only caused 51% and 59% decrease at 100 µg/ml and 200 µg/ml, respectively (Fig 3.17A). ASF caused 27% decrease at 80 µg/ml comparing to the control, while it caused 17% and 23% decrease at 20 µg/ml and 40 µg/ml, respectively (Fig 3.17C). K2 caused 27% decrease at 4 µM comparing to the control, while it only caused 14% and 23% decrease at 1 µM and 2 µM, respectively (Fig 3.17B). F3 caused 32% decrease at 40 µg/ml comparing to the control, while it caused 17% and 24% decrease at 10 µg/ml and 20 µg/ml, respectively (Fig 3.17D).

Similar dose-dependent reduction of Cathepsin B secretion caused by galectin-3 inhibitors were also found in HCT116 cells (Fig 3.18). Lactose caused 21% decrease at 400 µg/ml comparing to the control, while it only caused 8% and 17% decrease at 100 µg/ml and 200 µg/ml, respectively (Fig 3.18A). ASF caused 47% decrease at 80 µg/ml comparing to the control, while it caused 23% and 37% decrease at 20 µg/ml and 40 µg/ml, respectively (Fig 3.18C). K2 caused 21% decrease at 4 µM comparing to the control, while it only caused 6% and 13% decrease at 1 µM and 2 µM, respectively (Fig 3.18B). F3 caused 26% decrease at 40 µg/ml comparing to the control, while it caused 11% and 19% decrease at 10 µg/ml and 20 µg/ml, respectively (Fig 3.18D).

The inhibitory effect of galectin-3 inhibitors on secretion of Cathepsin B and MMP-13 in HT29 cells were also dose dependent. As shown in Figure 3.4.6.4, lactose at 400 µg/ml caused 41% and 15% reduction in Cathepsin B secretion in HT29-I and HT29-N, respectively, while lactose at 100 and 200 µg/ml, only caused 25% and 33% reduction in HT29-I cells, and 6% and 11% reduction in HT29-N cells, respectively.

Together, these results indicate that the presence of galectin-3 inhibitors inhibit Cathepsin B secretion in human colon cancer SW620-shCon, HCT116, and HT29-I cells, which secrete higher level of galectin-3, and has less effect on Cathepsin secretion in SW620-shGal3 and HT29-N cells, which secreted low level of galectin-3. These results provide further support to the notion that galectin-3 promotes protease secretion in cancer cells and this effect of galectin-3 is likely via the galectin-3 extracellular actions.

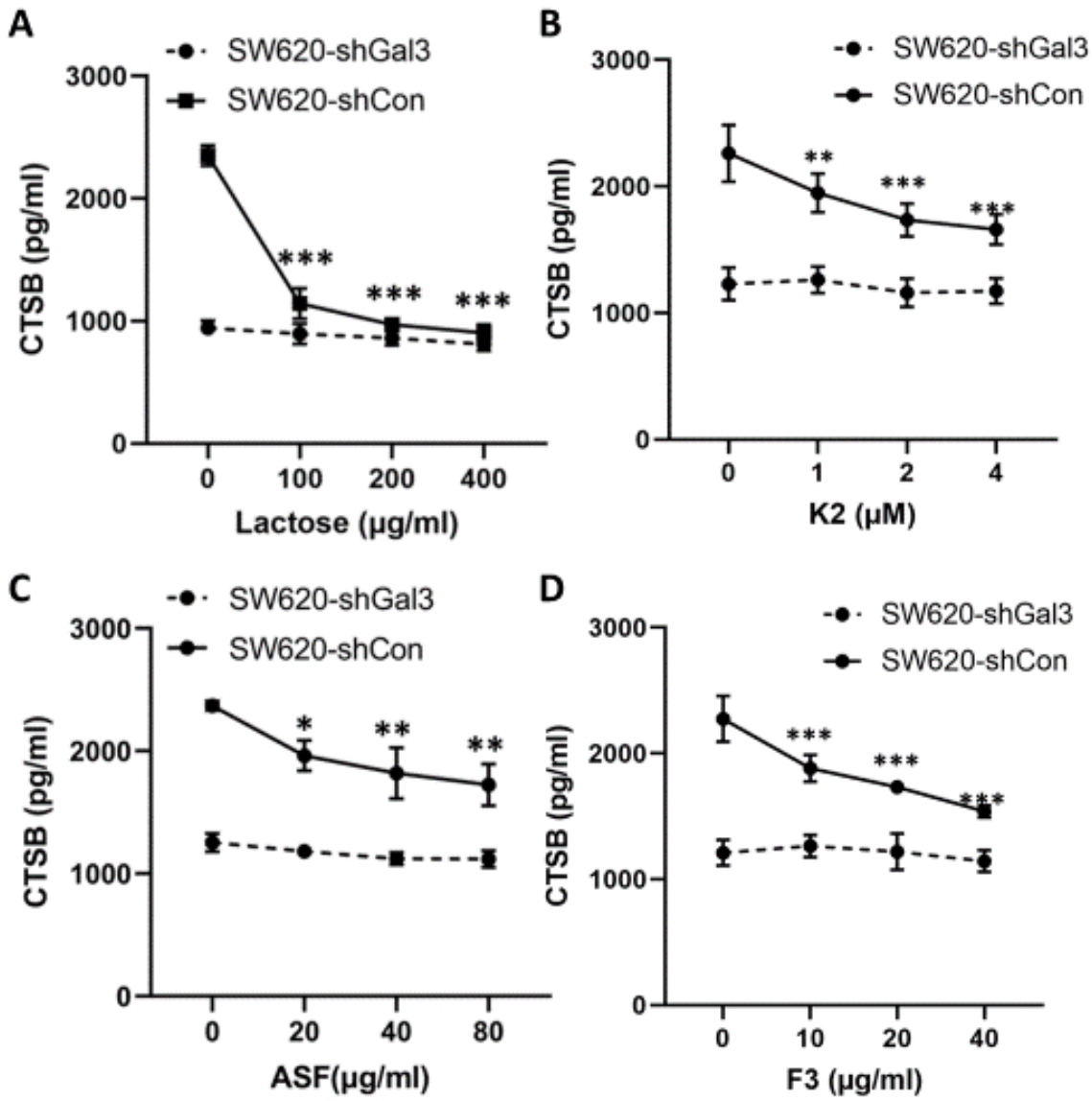

Figure 3.17. The galectin-3-mediated cathepsin-B secretion was inhibited by the presence of galectin-3 inhibitors in a dose dependent manner. SW620-shCon and SW620-shGal3 were cultured at the same cell number and incubate for at least 6 hours before introduction of lactose (A), K2 (B), ASF (C) and F3 (D) at different concentrations for 24 hours. Each of inhibitors inhibit CTSB secretion dose-dependently in SW620-shCon cells but not in SW620-shGal3 cells. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (ANOVA with Dunnett's test).

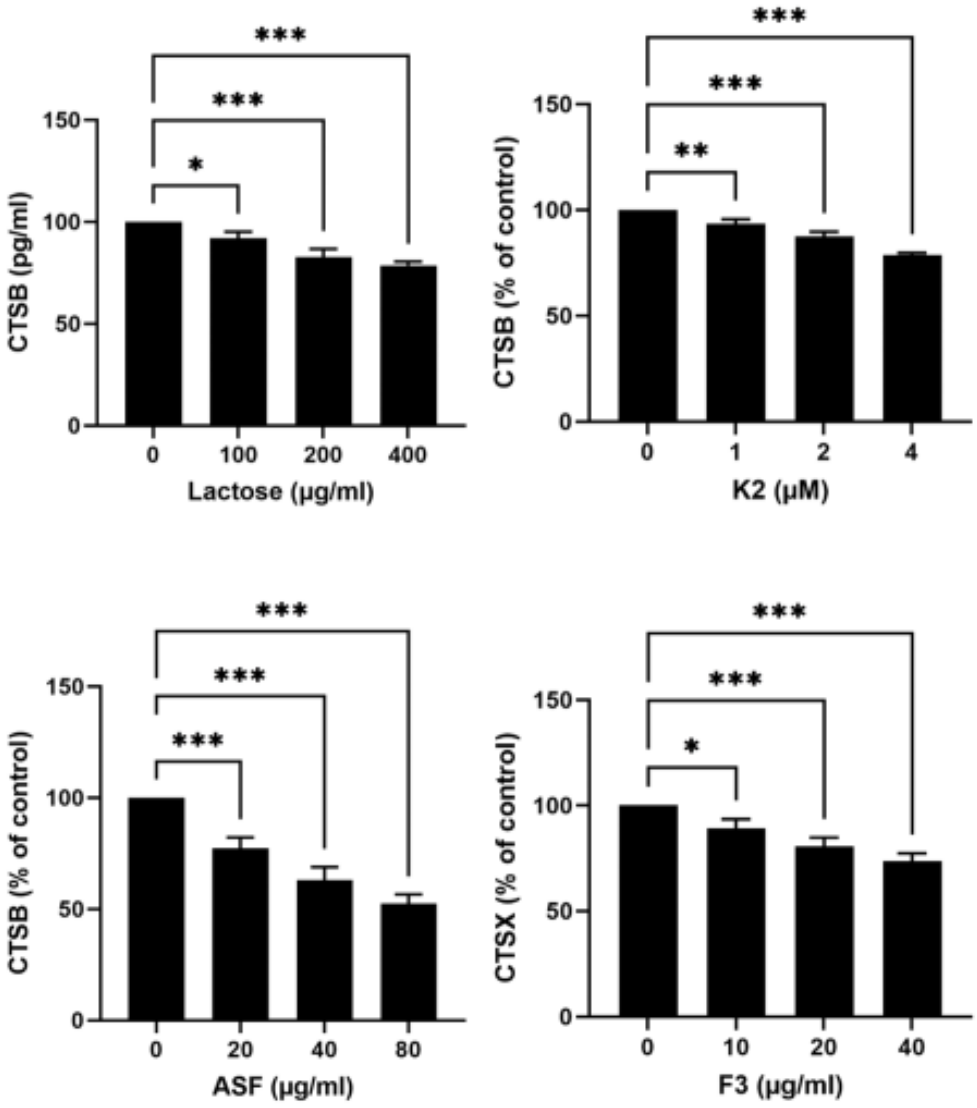

Figure 3.18. The galectin-3-mediated cathepsin-B secretion was inhibited by the presence of galectin-3 inhibitors in a dose dependent manner. HCT116 cells were cultured and incubate for at least 6 hours before the introduction of lactose (A), K2 (B), ASF (C) and F3 (D) at different concentrations for 24 hours. Each of the inhibitors inhibits CTSB secretion dose-dependently in HCT116 cells. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (ANOVA with Dunnett's test).

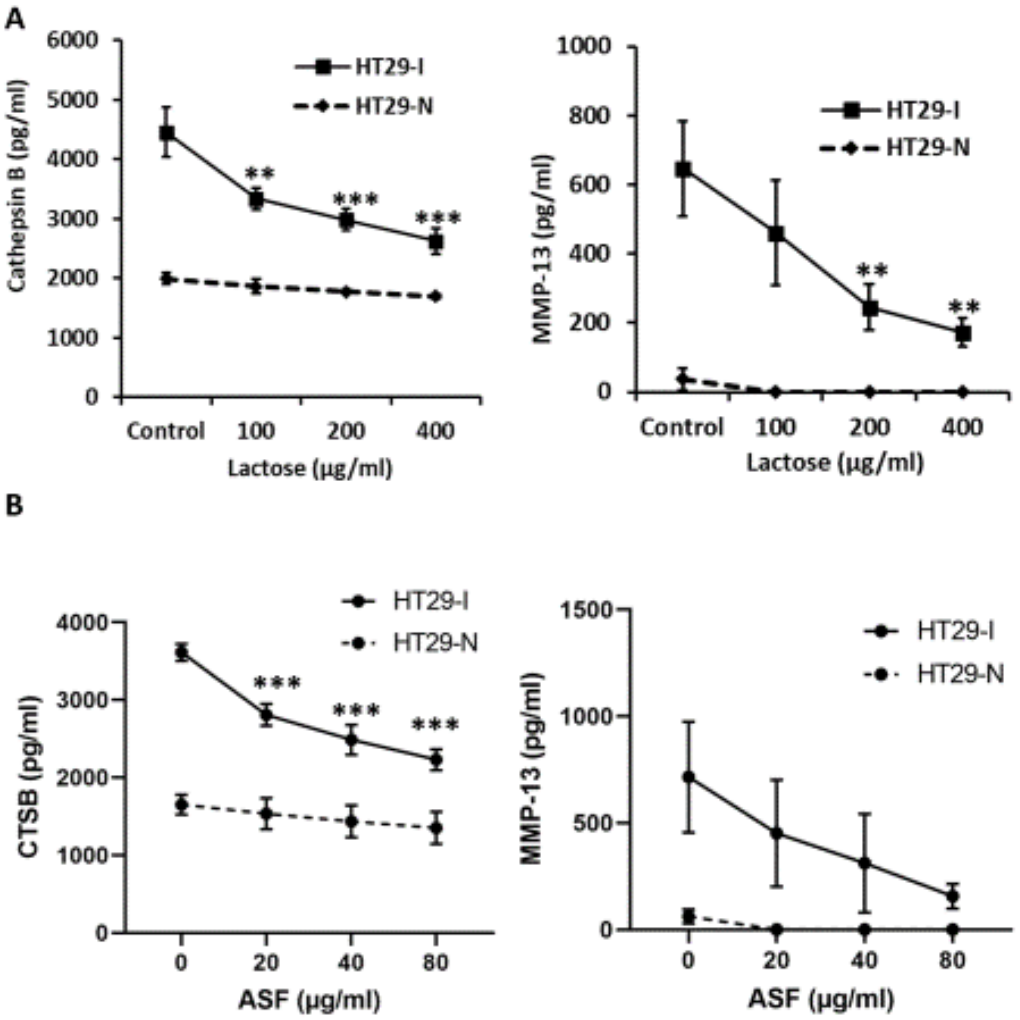

Figure 3.19. The galectin-3-mediated cathepsin-B secretion was inhibited by the presence of galectin-3 inhibitors in a dose dependent manner. HT29-N and HT29-I cells were cultured and incubated for at least 6 hours at the same cell number before the introduction of lactose or ASF at different concentrations for 24 hours. The secretion of CTSB and MMP-13 were assessed using ELISA. Lactose (A) and ASF (B) inhibit CTSB and MMP-13 secretion dose-dependently in HT29 cells. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$ (ANOVA with Dunnett's test).

We also assessed the effect of these galectin-3 inhibitors on protease secretion induced by exogenous introduction of recombinant galectin-3. In this part of the study, galectin-3 at 10 µg/ml and its inhibitors were added immediately to the plates after the medium was changed to serum-free medium. The secretion of Cathepsin B was analysed after 24 hours incubation using ELISA (Fig 3.20). The results showed that Cathepsin B secretion was increased by 18% in response to galectin-3 compared to control in SW620-shGal3 cell. While exogenous galectin-3-mediated Cathepsin B secretion was reduced by 19%, 18%, and 21% in response to lactose, ASF and K2, respectively (Fig 3.20A). In SW620-shCon cells, Cathepsin B secretion was increased by 23% in response to galectin-3, while this effect of galectin-3 on Cathepsin B secretion was reduced by 28%, 26% and 31% by lactose, ASF and K2, respectively (Fig 3.20B).

When these were tested in HT29 cells (Fig 3.20 CD), HT29-N cells treated with galectin-3 showed 15% increase of cathepsin B secretion compared to control. In the presence of galectin-3 inhibitors, cathepsin B secretion was reduced to 94%, 90% and 90% of the control by lactose, ASF and K2, respectively. HT29-I cells treated with galectin-3 showed 13% increase compared to control cells, and the presence of galectin-3 inhibitors caused exogenous galectin-3-mediated cathepsin B secretion reduced to 98%, 95% and 95% of the control by lactose, ASF and K2, respectively.

We also tested two newly developed galectin-3 inhibitors from our laboratory: compound L (CL) and compound K (CK). SW620-shCon (Fig 3.21A), HCT116 (Fig 3.21B) and SW620-shGal3 (Fig 3.21C) cells were treated with galectin-3 (10 µg/ml), compound L (10 µM) or compound K (10 µM) and the secretion of Cathepsin B was analysed by ELISA. The results showed that compound L and compound K both caused significant reduction of Cathepsin B secretion in SW620-shCon and HCT116

cells but less so in SW620-shGal3 cells. In comparison to the control, compound L and compound K at 10 μM caused 61% and 80% reduction of Cathepsin B secretion in SW620-shCon cells, respectively, while caused 70% and 84% reduction of Cathepsin B secretion in HCT116 cells, respectively. In response to exogenous galectin-3 treatment, cathepsin B secretion was increased by 35% and 49% in SW620-shCon cells and HCT116 cells, respectively, while the presence of gelactin-3 inhibitors reduced exogenous galectin-3-induced secretion of cathepsin B by 38% (compound L) and 49% (compound K) in SW620-shCon cells, and by 55% (compound L) and 64% (compound K) in HCT116 cells. Meanwhile, in SW620-shGal3 cells, compound L and compound K caused much less decrease of cathepsin B secretion compared to control: only by 10% and 15%, respectively. However, the inhibitory effect on galectin-3 inhibitors were also seen in SW620-shGal3 cells, in which the secretion of cathepsin B was increased by 98% in response to galectin-3 compared to control, and this galectin-3-induced cathepsin B secretion was reduced by 38% and 63% in the presence of compound L and compound K, respectively.

Together, these results suggest that galectin-3 promote cathepsin-B and MMP-13 secretion in an autocrine or paracrine manner, and this effect of galectin-3 is predominately mediated through its extracellular action.

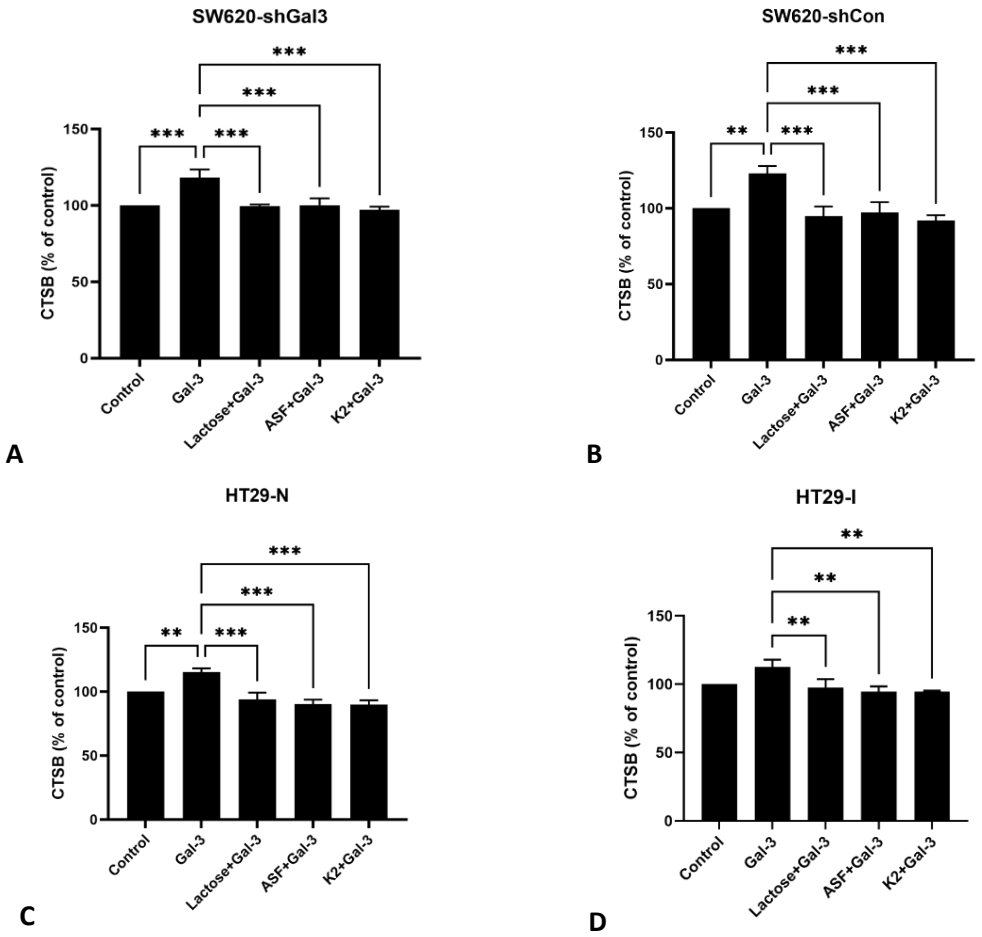

Figure 3.20. Galectin-3 inhibitors suppress galectin-3 induced Cathepsin B secretion. Cells were cultured for 24 hours followed by replacing the medium with 0.05% serum-free medium. Recombinant galectin-3 (10 ug/ml), lactose (100 µg/ml), ASF (20 µg/ml) or K2 (2 µM) were introduced to the cells at the same time and incubated for 24 hours. The culture supernatant was collected and analysed by cathepsin B ELISA. The galectin-3 inhibitors reduce galectin-3-induced Cathepsin B secretion in SW620shGal3 (A), SW620shCon (B), HT29-N (C) and HT29-I (D) cells. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$ (ANOVA with Tukey test).

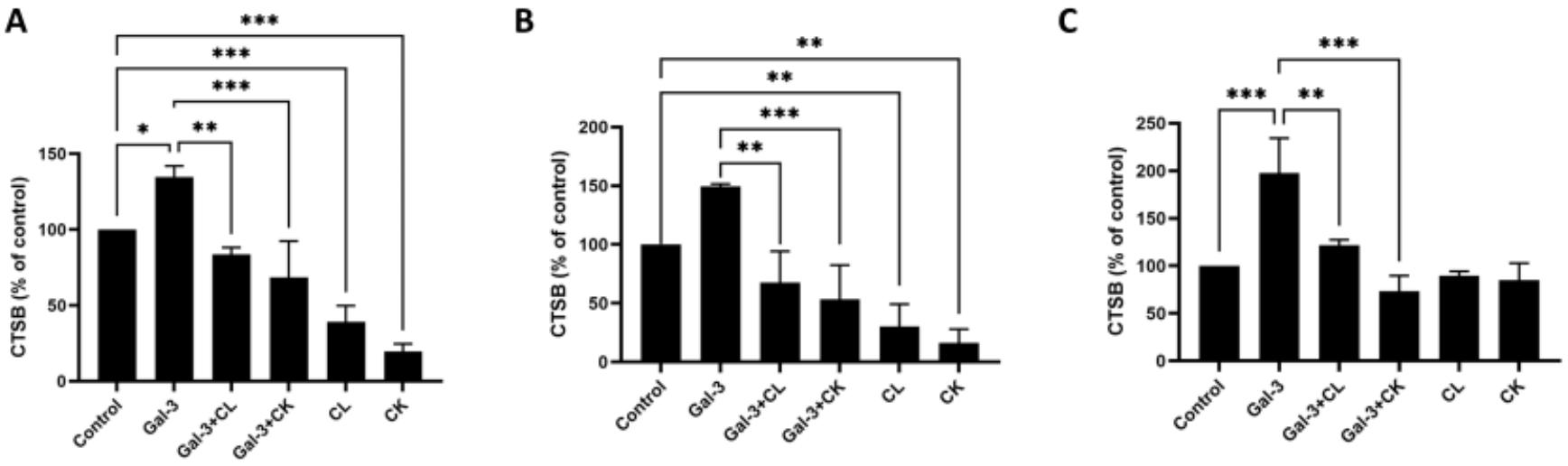

Figure 3.21. Novel galectin-3 inhibitors suppress galectin-3 induced Cathepsin B secretion. SW620 cells were cultured for 24 hours followed by replacing medium with 0.05% serum-free medium. The treatment with galectin-3 (10 ug/ml), compound L (10 μM) and compound K (10 μM) were conducted at the same time and incubated for 24 hours. Compound L and compound K reduced galectin-3-induced CTSB secretion in in SW620shCon (A) and HCT116 (B) cells while less change in SW620shGal3 (C) cells. They also significantly reduced galectin-3-induced Cathepsin B secretion in all three cells lines. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (ANOVA with Tukey test)

3.5 Summary of the results

1) Galectin-3 induces secretion of several proteases such as cathepsin A, B and X and MMP-1, 12, and 13 in colon cancer SW620 and HCT116 cells but less so in SW480 cells.

2) The promotion of protease secretion by galectin-3 is dose- and time-dependent.

3) Galectin-3 does not affect cellular expression of these proteases.

4) Higher galectin-3 expression and secretion in cells is associated with higher Cathepsin B and MMP-13 secretion.

5) Galectin-3 mediates protease secretion predominately through its extracellular action.

3.6 Discussion

This chapter of the study shows that galectin-3 induces secretion of several proteases in human colon cancer SW620 and HCT116 cells but less so in SW480 cells. This effect of galectin-3 on protease secretion is galectin-3 dose-dependent and is through its extracellular actions.

There are over 900 protease genes identified in the human genome [179]. In normal physiological conditions, protease secretion is tightly controlled and remains at a low level [434] and the proteolytic actions of these proteases contribute to the homeostasis maintenance [93]. Overexpression of proteases exists in many types of cancers [435] and facilitates tumour cell invasion and spreading [436]. It is believed that degradation of ECM by various proteases [437] is the initial step in tumour cell invasion at primary tumour sties [438]. In this part of the study, the secretion of several proteases including MMP-1, MMP-12, MMP-13, Cathepsin A, Cathepsin B and Cathepsin X by cancer cells was shown to be significantly increased in response to galectin-3.

To minimize the effect of cell proliferation on protease secretions, protease secretion was measured after 24-hour culture (the doubling time of these cells was around 20 to 26 hours).

The presence of galectin-3, proteases secretion was higher from SW620 cells than SW480 cells. SW480 and SW620 cells were derived from the same patient. SW480 cells were from the primary tumour with an epithelioid morphology while SW620 cells were from the metastasized tumour with a rounded or fibroblast-like appearance. During EMT, cells acquire migratory and invasive properties, which in line with our discovery of more proteases were secreted from SW620 cells than SW480 cells.

Cathepsins proteases are overexpressed in various cancers and display differential localization within cells. The overexpression of cathepsins proteases is often

associated with poor prognosis as well as enhanced malignancy in patients. The secretion of cathepsin proteases from cancer cells is responsible for disrupting cell-cell adhesion and inducing local invasion and angiogenesis [187]. In colon cancer, it is reported that cathepsin B is significantly upregulated in CRC tissues, and is predominantly expressed by macrophages at the leading edge of invading tumours [439] Cathepsin B is also overexpressed in various other cancers such as breast, gastric, lung, prostate cancer and gliomas [440]. It is particularly highly expressed at the invasive edge of the tumours [441, 442] and promotes cancer cell invasion [443]. Cathepsin A is a serine carboxypeptidase and involved in ECM remodelling. It was reported that Cathepsin A is associated with proliferation and invasion of lung adenocarcinoma cells [444]. Increased Cathepsin X protein levels and activity have been found in various cancers and it is associated with low patient survival rate as well poor prognosis [445-447]. In colon cancer, cathepsin X was thought to be a prognostic and predictive marker, and it was reported to act as an adhesion factor in CRC, supporting cell–cell stabilization and tumour cell anchorage to ECM [448]. Enhanced Cathepsin X expression and secretion in cancers are shown to influence cancer progression via its proteolytic activities of substrates that are involved in tumour adhesion, invasion and metastasis [449]. MMP-1, MMP-12, and MMP-13 are primarily responsible for ECM degradation in cancer progression [450]. MMP-1 and MMP-13 are both overexpressed in colorectal carcinomas and are related to poor prognosis in colorectal patients [451, 452], in which MMP-13 was associated with poor prognosis in patients with colorectal cancer metastasis to the liver [453]. They are reported to promote colon cell invasion and metastasis [455-457]. The discovery in this study that galectin-3 increases the secretion of including MMP-1, MMP-12, MMP-13, Cathepsin A, Cathepsin B and Cathepsin X by colon cancer cells suggests that increasing the

secretion of proteases is probably one of the mechanisms behind galectin-3-mediated cancer promotion reported in many previous studies [429, 458, 459].

Many studies have reported galectin-3 as a multi-functional promoter of cancer progression and metastasis [460]. Galectin-3 is found intracellularly as well as extracellularly [431]. Cytoplasmic galectin-3 interacts with Bcl-2 and prevents cell apoptosis [325]. Binding of extracellular galectin-3 to cell surface glycans on growth factors [461], death receptors [462] and adhesion molecules [463] promotes cancer cell adhesion, invasion, angiogenesis, and escape of tumour cells from immune surveillance [464].

Galectin-3 has previously been reported to increase secretion of MMP-1 and -9 in melanoma cells [465] by binding to LAMP1 [391, 466]. It has also been shown to increase MMP-1 expression and enhance cancer cell invasion of gastric cancer cells [390]. The present study shows that galectin-3 induces secretion of a number of proteases including MMP-1, MMP-12, MMP-13, Cathepsin A, Cathepsin B and Cathepsin X differentially in SW620, HCT116 and SW480 cells. This implies that the secretion of proteases induced by galectin-3 may not always the same and may depend on the expressions of particular galectin-3 binding glycans and receptors. Many cell surface glycoproteins such as growth factor receptors, adhesion and signalling proteins are known to be recognized by galectin-3 [467] and are differentially expressed by different cancer types.

Chapter 4

Investigation of Galectin-3-mediated protease secretion on cancer cell invasion, monolayer integrity and permeability

4.1 Introduction

It has been shown that the expression and secretion of proteases are involved in many processes during cancer progression such as apoptosis, proliferation, invasion and angiogenesis, in which different proteolytic activities are involved to facilitate tumorigenic promotion.

Cysteine cathepsins are a group of proteases in lysosomes that are overexpressed in many cancers. Translocation of cathepsins often occurs during cancer progression in which cathepsins are secreted to the cell surface and extracellular milieu, and thus, they can facilitate tumour-promoting functions. Cathepsins are primarily responsible for degradation of components of basement membranes and ECM, which allows tumour cells to escape the primary site. They can also activate other proteases including MMPs and uPA, through a proteolytic cascade at the cell surface, which in turn promotes tumour invasion. In addition, cathepsins are the main enzyme that catalyse cell adhesion proteins such as E-cadherin, which disrupt adherens junctions and promote tumour cell migration and invasion.

Among cysteine cathepsins, Cathepsin B is one of the most studied cathepsins and is closely associated with cancer progression. The overexpression of cathepsin B has been show in thyroid, breast and colon cancer [439], whereas silencing of cathepsin by antisense decreased the tumour migration and invasion in osteosarcoma cells [446]. In a murine mammary carcinoma model, shRNA silencing of cathepsin B also shown to reduce the type I collagen degradation *in vitro* as well as tumour metastasis to bone *in vivo* [247]. In meningioma, cathepsin B and MMP-9 knockdown caused downregulated kinase signal transduction and reduced cancer cell proliferation, invasion and angiogenesis *in vitro*, as well as decreased tumour growth *in vivo* [468],

whereas silencing of uPAR and cathepsin B decreased TGF β1-mediated signal transduction and tumour invasion [469]. These findings imply a proteolytic cascade that involves cathepsin B, MMPs and the plasminogen activator. Moreover, cathepsins overexpression was seen in mammary cancer cells which enhanced the tumour invasion *in vitro* and *in vivo* [470, 471]. It was found that the tumour growth, invasion and angiogenesis were reduced in the transgenic model for pancreatic islet cell carcinogenesis that crossed with cathepsin B null mice [261]. On the other hand, by crossing the transgenic mouse model of mammary carcinoma with cathepsin B-overexpressing mice, there were enhanced tumour growth as well as cathepsin B production in the mammary tumours in the transgenic mice [471]. It was also found the cathepsin B-induced fibroblasts activation through TGF-β1 is responsible for increased cell invasion in the esophageal epithelial cells [266]. Furthermore, cathepsins including cathepsin B have been reported to cleave E-cadherin which leads to loss of cell-cell adhesion and therefore enhance tumour invasion. Tumours from cathepsin B null mice were seen to maintain sufficient E-cadherin production, while decreased E-cadherin level was observed in invasive tumours from wild-type mice [472]. Therefore, cathepsin B plays a key role in interaction between tumour cells and other cells in the tumour microenvironment, and it is associated with loss of cell-cell contact and increased invasion and metastasis.

MMPs also play a crucial role in promoting tumour invasion and metastasis as they are the primary contributor of degradation of ECM. Degradation of the physical barrier is a key step of cancer progression, where MMPs often localize at the invadopodia, a cell surface structure that represent where active ECM degradation occurs. There are many transmembrane MMPs present in invadopodia to degrade ECM components and promote invasion, including MMP- 2, -9 and -14, as well as ADAM family [473]. It

has been shown that MMPs are overexpressed in many types of cancers including, basal cell, head and neck, lung, breast, prostate, colon, thyroid, gastric and ovarian cancer [474], and the expression is directly associated with the invasiveness and metastatic ability of the tumour. For example, the expression of MMP-2 and -3 was seen to promote vascular invasion and lymph node metastasis in squamous cell carcinoma of esophagus [475]. Overexpression of MMP-13 is also found to be related to the metastasis capacity of head and neck carcinoma [476], and expression of MMP-11 was seen to increase local tumour invasion in head and neck carcinoma [477]. Among MMPs, MMP-13 is capable of cleaving collagens I, II and III and a wide range of substrates. Laminin-5 is an ECM protein which is mostly expressed in the basement membrane structure and is responsible for static adhesion of the epidermis and dermis as well as hemidesmosome formation [478-480]. Cleavage of laminin-5 by MMP-2 and MMP-14 was reported to reveal cryptic site and allow breast epithelial cells to obtain motility [481, 482]. MMP-13 can also cleave lamininin-5 and has been shown to generate even smaller laminin fragments in comparison to that by MMP-2 cleavage and stimulates tumour cell invasion [483]. TGF-beta is a primary inducer of EMT, and active form of TGF-beta is released from TGF-beta-complex through proteolytical cleavage by MMP-28 [484]. Same activation was also reported by MMP-13 with chondrocytes in matrix vesicles where secreted MMP-13 activates latent TGF-beta in the progress of mineralization of growth plate cartilage [485]. Inhibition of MMP-13 expression in breast cancer cells at tumour bone interface is shown to significantly reduce the TGF-beta signalling, resulting in decreased tumour-induced osteolysis [486]. This suggests that MMP-13 may be an important mediator for epithelial cancer bone metastasis. Moreover, MMP-13 is also involved in activation of other MMPs such as MMP-2 and MMP-9 via cleavage of pro-MMP-2 and pro-MMP-9. It has been

reported that pro-MMP-9 is cleaved and activated by MMP-13 in osteoarthritic chondrocytes [487] as well as in the progression of chronic periodontitis [181], indicating a role of MMP-13/MMP-9 activation cascade in cancer cell invasion and metastasis.

Similar to cathepsins and MMPs, galectin-3 is also known to promote cancer cell invasion and metastasis. Galectin-3 overexpression was found to promote tumour proliferation and invasion in pancreatic cancer [488]. The upregulated galectin-3 nuclear expression was closely associated with cell differentiation and vascular invasion in esophageal squamous cell carcinoma [489]. It was also found that the upregulated galectin-3 expression caused increased cell adhesion to ECM, tumour cell migration and invasion in lung cancer [490]. In addition, it was shown that galectin-3 facilitates cell motility in gastric cancer by up-regulating PAR-1 and MMP-1.

The discovery that galectin-3-induces secretion of a number of those proteases from cancer cells led us to investigate the functional consequences on cancer cell invasion and cell-cell contact.

4.2 Hypothesis and Aims

4.2.1 Hypothesis

Galectin-3-induced protease secretion increases cancer cell invasion and interrupt cancer cell monolayer integrity.

4.2.2 Aims

The aims of this part of the study are:

1) to determine the effect of galectin-3-mediated protease secretion on colon cancer cell invasion
2) to assess whether galectin-3-induced protease secretion is associated with change of cell monolayer integrity and permeability

4.3 Methods

4.3.1 Assessment of the effect of galectin-3 on cancer cell invasion

Trans-well inserts (8 µm pore size) in 24-well plates were coated with 100 µl Matrigel matrix proteins (20 µg/ml) for 2 hr at 37°C. After a gentle wash with phosphate buffered saline, they were introduced with 150 µl cell suspension ($1x10^5$ cells/ml) containing 10 µg/ml recombinant galectin-3 or 10 µg/ml BSA (control) in 1% FCS medium. Transwell invasion assay was then performed as previously described in chapter two.

4.3.2 Assessment of the effect of galectin-3-mediated protease secretion on cancer cell invasion

HT29-I and HT29-N cells were cultured in flasks as well as in 6-well plates, at $1x10^5$ cells/ml at 37 ºC for 24 hours. The cells in the flasks were then released by trypsinization and counted. The culture medium (conditioned media) from the 6-well plates were collected and centrifuged at 1,956 g for 5 min. HT29-I cells released from the flask were then resuspended in conditioned media from HT29-N cells and HT29-N cells released from the flask were resuspended in conditioned media from HT29-I cells, to make final concentration of $1x10^5$ cells/ml. 150 µl of these cell suspensions were then introduced onto 8 µm transwell with Matrigel.

HCT116 cells were cultured in the 8 µm transwell with Matrigel with 1% FCS McCoy medium with 10 µg/ml BSA (control), 1% FCS McCoy medium with 10 µg/ml galectin-3 (Gal-3), conditioned media from SW620-shCon cells (CM-SW620-shCon) and conditioned media from SW620-shGal3 cells (CM-SW620-shGal3), respectively, to

make the final concentration of $1x10^5$ cells/ml, and total volume of 150 µl cell suspension.

SW620-shGal3 cells were cultured in the 8 µm transwell with Matrigel with 1% FCS DMEM medium with 10 µg/ml BSA (control), 1% FCS DMEM medium with 10 µg/ml galectin-3 (Gal-3), conditioned media from HT29-I cells (CM-HT29-I) or conditioned media from HT29-N cells (CM-HT29-N), respectively, to make the final concentration of $1x10^5$ cells/ml, and total volume of 150 µl cell suspension.

The subsequent steps in the invasion assay were described in chapter two.

4.3.3 Assessment of the effect of galectin-3-mediated protease secretion on cell monolayer integrity

The establishment of a cell monolayer was described in chapter two. Caco2 cells were cultured in 0.4 µm transwells and TEER was measured every 24 hours. The reading of TEER reached a plateau after approximately 14 days as the reading was capped around 3000 Ωcm^2 (Fig 4.1). On day 15, the Caco2 monolayer was cultured with conditioned medium from SW620 cells treated with 10 µg/ml BSA (CM-SW620+BSA), conditioned medium from SW620 cells treated with 10 µg/ml galectin-3 (CM-SW620+Gal3), conditioned medium from SW620 cells treated with 2 µM K2 (CM-SW620+K2), culture medium from SW620-shCon cells (CM-SW620-shCon), culture medium from SW620-shGal3 cells (CM-SW620-shGal3), culture medium from HT29-I cells (CM-HT29-I) or culture medium from HT29-N cells (CM-HT29-N), and TEER readings were measured at 0, 1, 2 and 4 hours.

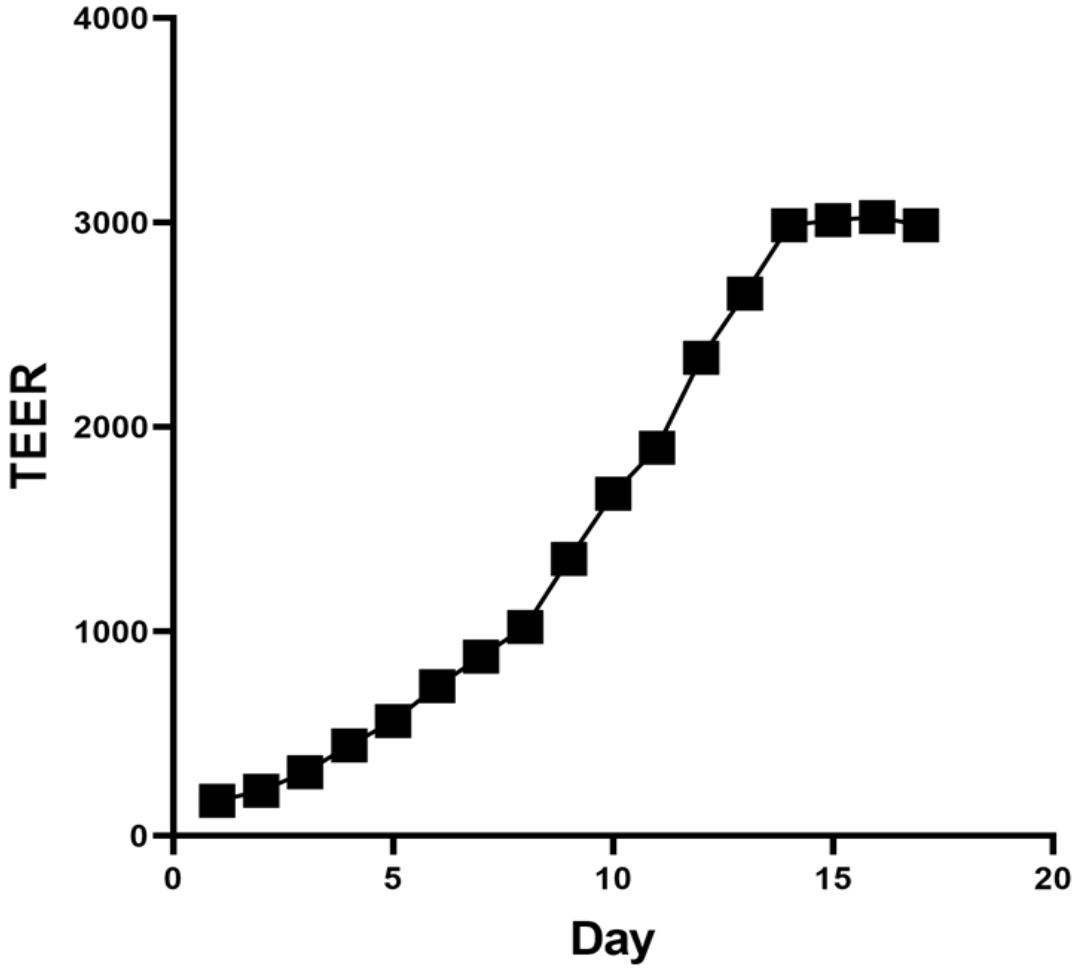

Figure 4.1 TEER of Caco2 monolayer. Caco2 cells were culture in 0.4 µm Transwell inserts, and TEER was measured every 24 hours. The reading of TEER reached plateau approximately 14 days.

4.3.4 Assessment of the effect of galectin-3-mediated protease secretion on cell leakage

For assessment of the cell monolayer permeability, 1 mg/ml FITC-dextran (20 kDa) was introduced to the tight cell monolayers (TEER, approximately 3000 Ωcm^2) in the 0.4 µm Transwell inserts as described in chapter two. The conditioned media of SW620-shGal3 cells (SW620-shGal3-CM), SW620-shCon cells (SW620-shCon-CM), HT29-I cells (HT29-I-CM) and HT29-N cells (HT29-N-CM) were introduced with FITC-dextran. The recombinant MMP-13 (1000 pg/ml) and recombinant Cathepsin B (1000 pg/ml) were introduced to the separate Transwell inserts with FITC-dextran included (as positive control). The combination of three antibodies against MMP-1 (10 µg/ml), MMP-13 (20 µg/ml) and Cathepsin B (20 µg/ml) was also introduced to the Transwell inserts with CM. The Transwell inserts were incubated for 30 min at room temperature and the culture medium in the bottom wells was collected and centrifuged at 1,956 g for 5 min and the fluorescence intensity was measured using a fluorescence microplate reader GENios Plus (TECAN, Reading, UK).

4.4 Results

4.4.1 Exogenous galectin-3 promotes human colon cancer cell invasion

It is known that the presence of proteases in the tumour microenvironment can aid primary tumour cell invasion by digestion of basement proteins [22]. To determine the impact of galectin-3-mediated protease secretion on cancer progression, we first assessed the influence of galectin-3 on tumour cell invasion through basement proteins. Cells were introduced to 8 µm Transwell for 24 hours and 48 hours in the presence of galectin-3 or control BSA. In respond to the introduction of galectin-3, SW620 cell invasion showed a significant dose-dependent increase (Fig 4.2) of cell invasion of with 1.5-fold (5 µg/ml) and 2.7-fold (10 µg/ml) higher at 24 hours and 1.4-fold (5 µg/ml) and 2.15-fold (10 µg/ml) higher at 48 hours. Introduction of exogenous galectin-3 to SW480 cells also caused an increase of SW480 cell invasion but to a less degree in comparison to SW620 cell (Fig 4.3), in which the introduction of 10 µg/ml galectin-3 caused a 1.3-fold and 1.22-fold increase at 24 hours and 48 hours, respectively. At 24 hr, HCT116 cells showed a 1.7- and 2-fold increase of cell invasion in response to 5 and 10 µg/ml exogenous galectin-3 (Fig 4.4), respectively, while 1.5- and 1.9-fold increase at 48hr. Introduction of 10 µg/ml exogenous galectin-3 to HT29 cells caused 2.5- and 2.2-fold, respectively, increases of invasion of HT29-N and HT29-I cells (Fig 4.5). These results suggest that introduction of exogenous galectin-3 promotes cancer cell invasion through basement proteins.

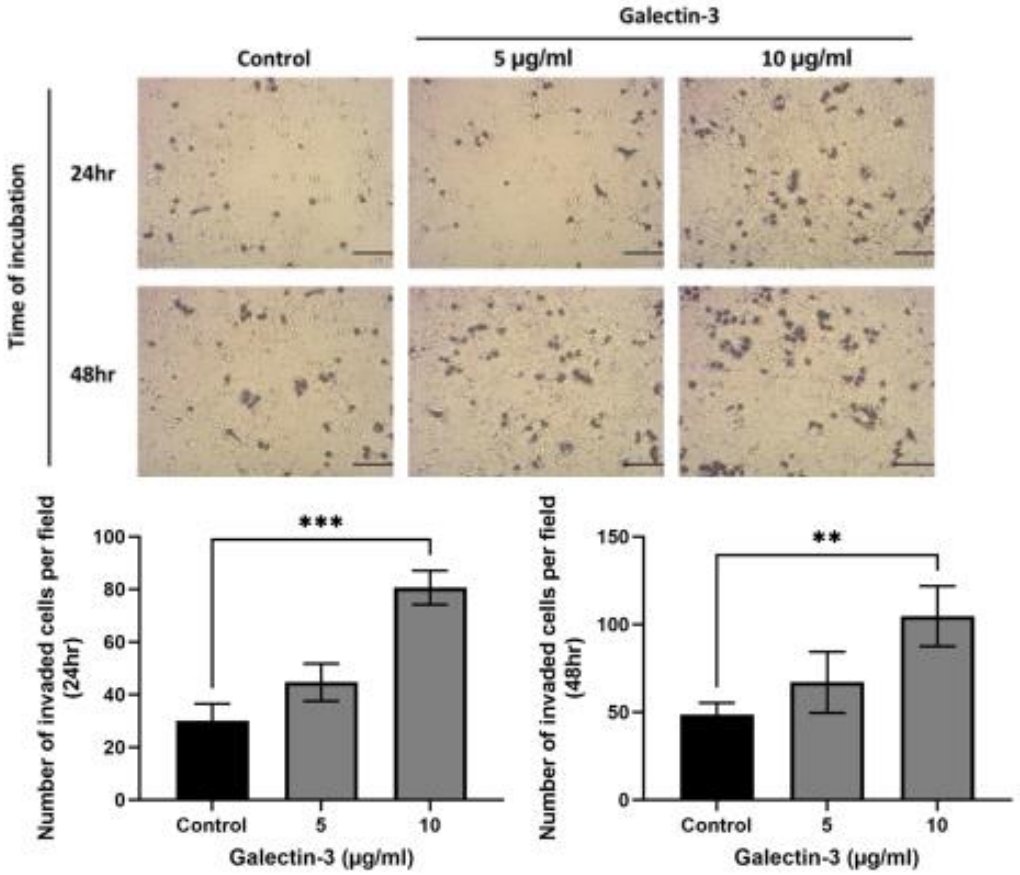

Figure 4.2 Galectin-3 increases cancer cell invasion. SW620 cells were plated onto 8 µm transwell with Matrigel for 24 and 48hr. Introduction of exogenous galectin-3 increased SW620 cell invasion through Matrigel. Scale bar = 100 µm. Data are presented as mean ± SD from three independent experiments, each in triplicate. Representative images are shown at the top. *** $p < 0.001$. ** $p < 0.01$ (ANOVA with Dunnett's test).

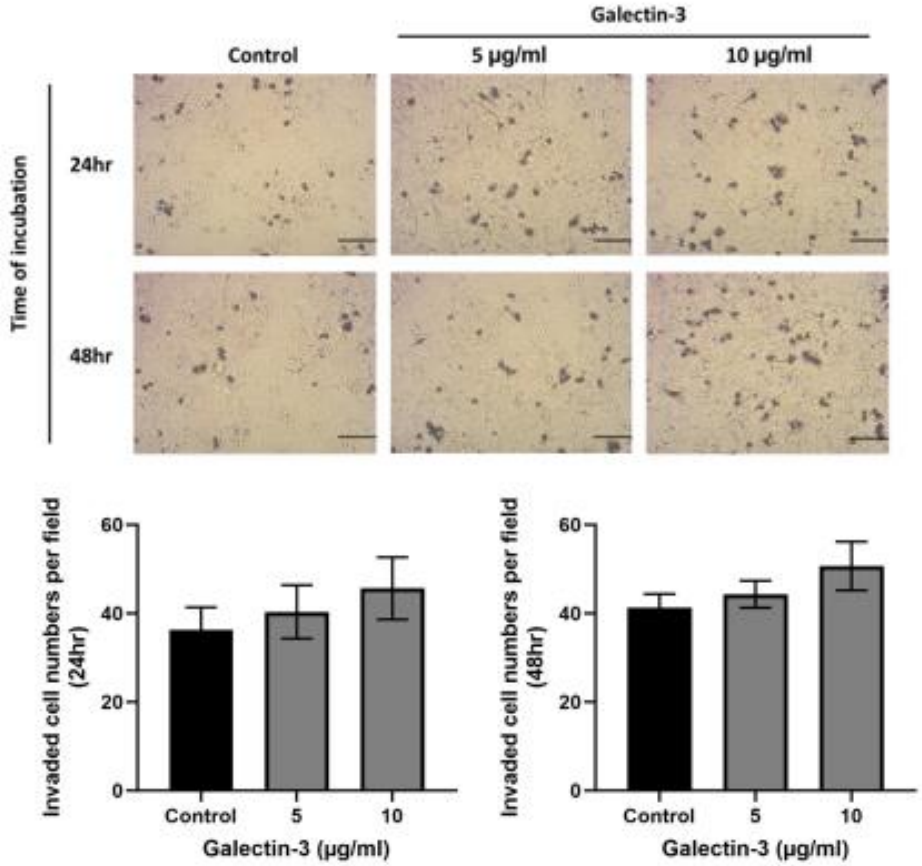

Figure 4.3 Galectin-3 increases cancer cell invasion. SW480 cells were plated onto 8 µm Transwell with Matrigel for 24 and 48hr. Introduction of exogenous galectin-3 increased SW480 cell invasion through Matrigel. Scale bar = 100 µm. Data are presented as mean ± SD from three independent experiments, each in triplicate. Representative images are shown at the top.

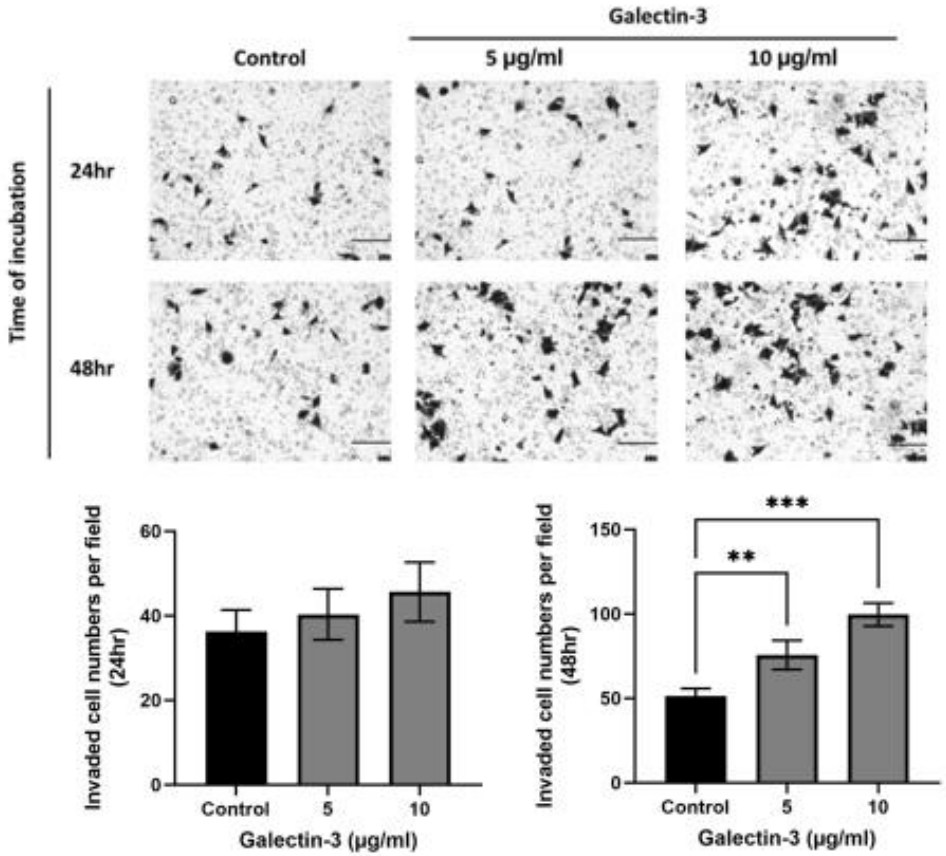

Figure 4.4 Galectin-3 increases cancer cell invasion. HCT116 cells were plated onto 8 µm Transwell with Matrigel for 24 and 48hr. Introduction of exogenous galectin-3 increased HCT116 cell invasion through Matrigel. Scale bar = 100 µm. Data are presented as mean ± SD from three independent experiments, each in triplicate. Representative images are shown at the top. *** $p < 0.001$, ** $p < 0.01$. (ANOVA with Dunnett's test).

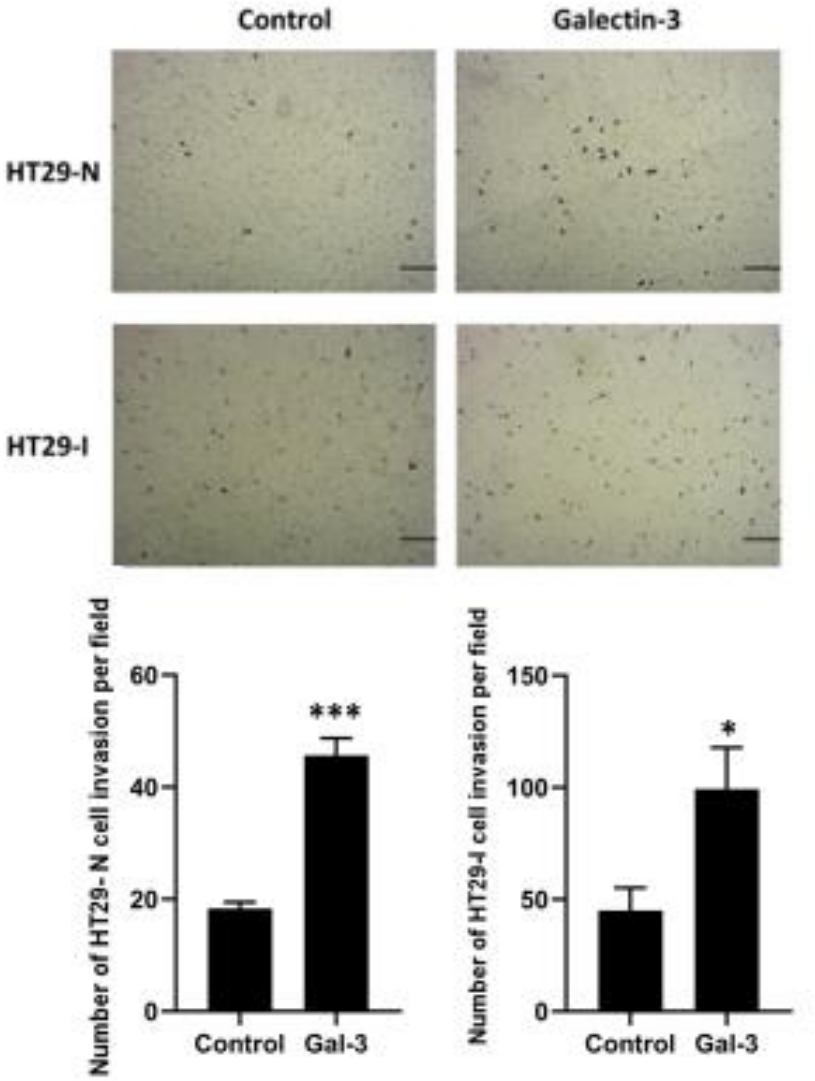

Figure 4.5 Galectin-3 increases cancer cell invasion. HT29-I and -N cells were plated onto 8 µm transwell with Matrigel for 24hr. Introduction of exogenous galectin-3 increased invasion of both HT29-I and -N cells. Scale bar = 200 µm. Data are presented as mean ± SD from three independent experiments, each in triplicate. Representative images are shown at the top. *** $p < 0.001$ * $p < 0.05$ (t-test).

4.4.2 Galectin-3-mediated protease secretion promotes cancer cell invasion

In order to dissect the relationship between galectin-3-mediated protease secretion and cell invasion, we exchanged the culture media from HT29-I and HT29-N cells (Fig 4.6). When the culture medium of HT29-N cells was replaced with conditioned medium from HT29-I cells, which contains higher levels of proteases, HT29-N cell invasion was significantly increased (162%) in comparison to the cells without medium change. On the other hand, when the culture medium of HT29-I cells was replaced with conditioned medium from HT29-N cells, which contains less proteases, HT29-I cell invasion was significantly reduced (46%) in comparison to cells without medium change. When conditioned medium from SW620-shGal3 and SW620-shCon cells, in which SW620-shCon cells contains higher level of galectin-3 and proteases than SW620-shGal3 cells, was used to assess the invasion of HCT116 cells (Fig 4.7), HCT116 cell invasion was significantly higher when cultured in the conditioned medium from SW620-shCon cells than conditioned medium from SW620-shGal3 cells. Again, introduction of exogenous galectin-3 significantly increased HCT116 cell invasion. We also assessed cell invasion of SW620-shGal3 cells which has minimum galectin-3 production in presence of different conditioned media (Fig 4.8). When the cells were cultured with conditioned media from HT29-N and HT29-I cells, in which conditioned media from HT29-I cells contains higher levels of galectin-3 and proteases than HT29-N cells, SW620-shGal3 cell invasion was seen to be 1.48-fold higher when cultured in conditioned media from HT29-I cells than condition media from HT29-N cells. The introduction of exogenous galectin-3 significantly increased SW620-shGal3 cell invasion.

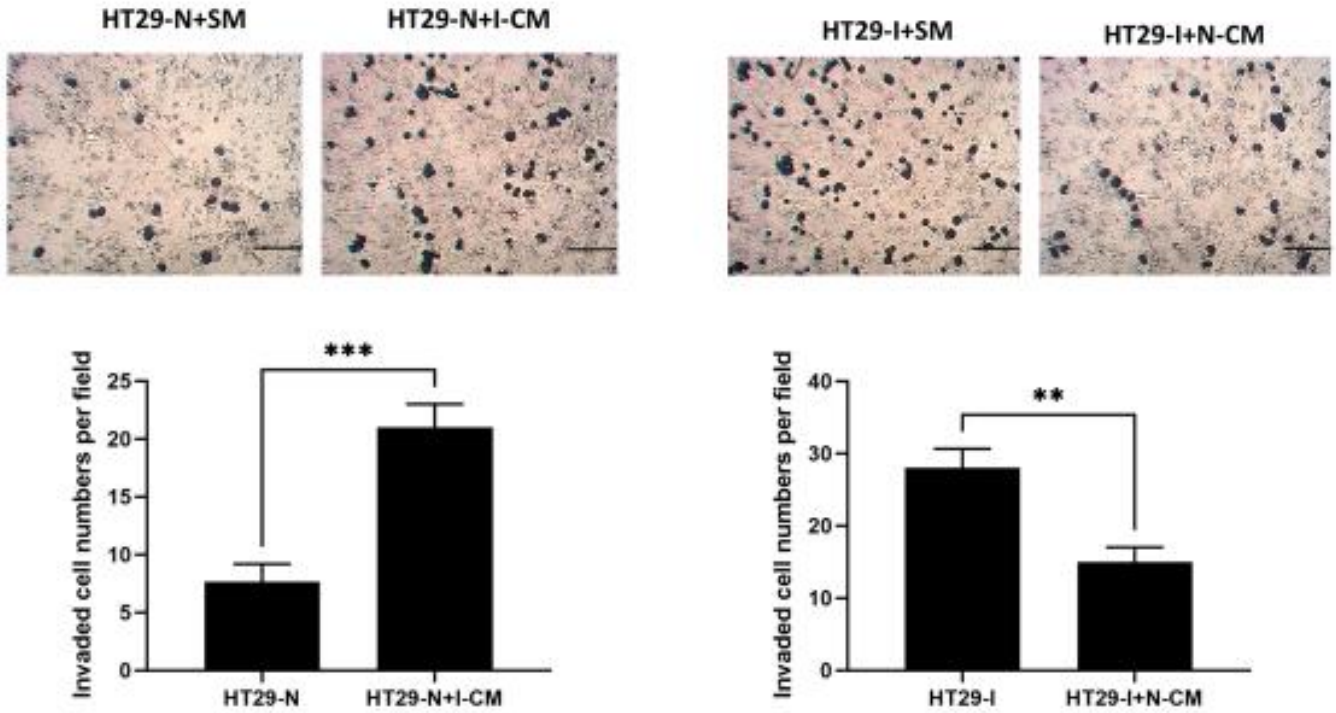

Figure 4.6. Galectin-3 increases cancer cell invasion. HT29-I and -N cells were plated onto 8 µm transwell with Matrigel for 24hr. Introduction of exogenous galectin-3 increased invasion of both HT29-I and -N cells. Scale bar = 100 µm. Data are presented as mean ± SD from three independent experiments, each in triplicate. Representative images are shown at the top. *** $p < 0.001$, $p < 0.01$ (t-test).

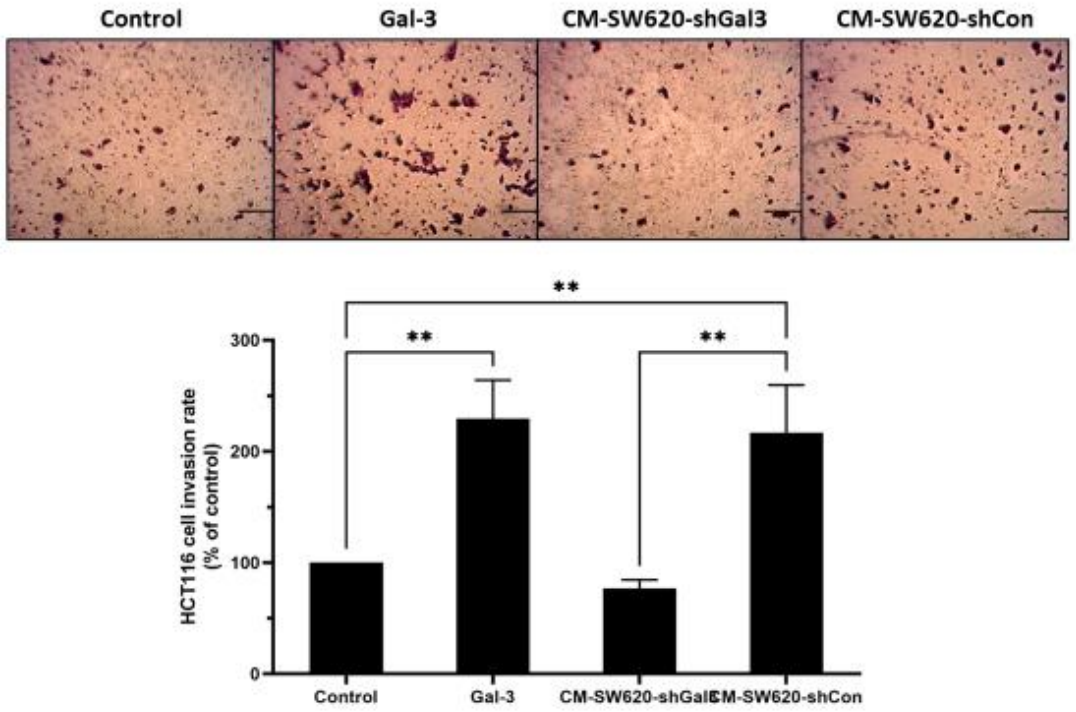

Figure 4.7 Galectin-3-mediated protease secretion increases cancer cell invasion. HCT116 cells were cultured with culture media with galectin-3 or conditioned media from SW620-shGal3 or SW620-shCon cells and plated onto 8 µm Transwell with Matrigel. Scale bar = 100 µm. Data are presented as mean ± SD from three independent experiments, each in triplicate. Representative images are shown at the top. ** $p < 0.01$, (ANOVA with Tukey test).

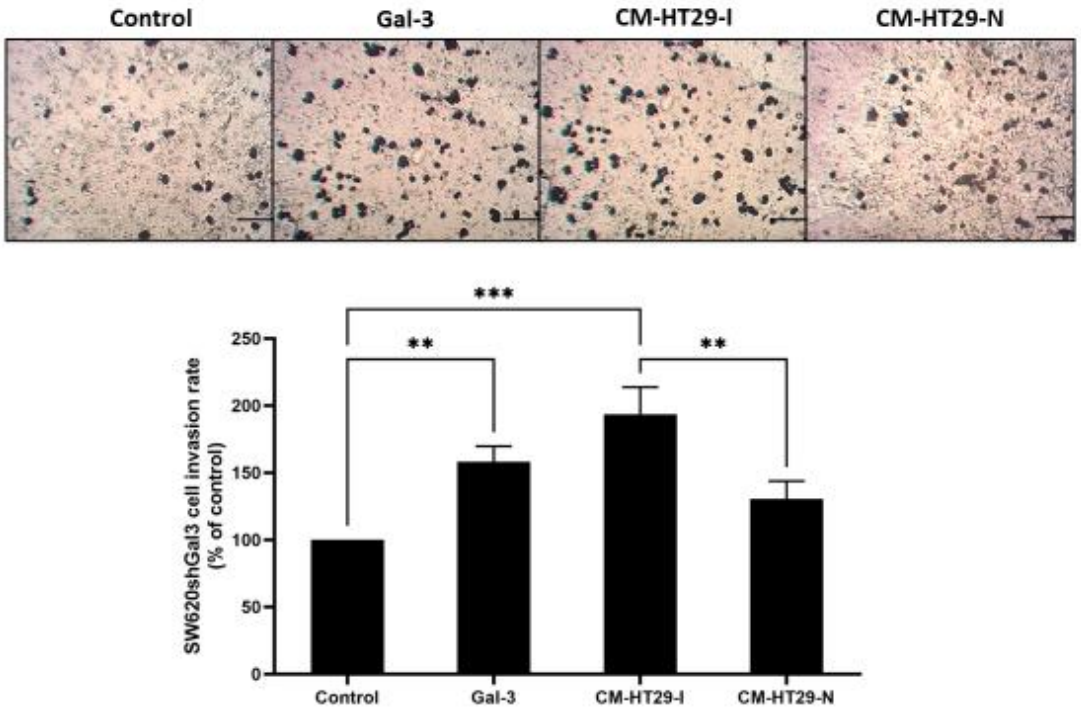

Figure 4.8 Galectin-3-mediated protease secretion increases cancer cell invasion. SW620-shGal3 cells were cultured with culture media with galectin-3 or conditioned media from HT29-I or HT29-N cells and plated onto 8 µm Transwell with Matrigel. Scale bar = 100 µm. Data are presented as mean ± SD from three independent experiments, each in triplicate. Representative images are shown at the top. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (ANOVA with Tukey test).

Moreover, the presence of the galectin-3 inhibitors was shown to reduce the cell invasion in human colon cancer cells. In comparison to control, HT29-I cells showed to have 39% decrease of cell invasion in the presence of lactose, while HT29-N cells only had 29% decrease of cell invasion. In the presence of ASF, HT29-I cells showed to have 41% decrease of cell invasion compared to control, while HT29-N cells had 39% decrease of cell invasion (Fig 4.9). In addition, in respond to galectin-3 inhibitors, cell invasion of SW620-shCon and SW620-shGal3 cells was also decreased. In the presence of lactose, SW620-shCon cell invasion was reduced by 33% compared to the control cells, while SW620-shGal3 cell invasion showed 8% decrease. Introduction of ASF caused a 23% decrease of SW620-shCon cell invasion while only caused a 5% decrease of SW620-shGal3 cell invasion (Fig 4.10). Taken together, these results suggest that galectin-3-induced protease secretion promotes tumour cell invasion through basement proteins.

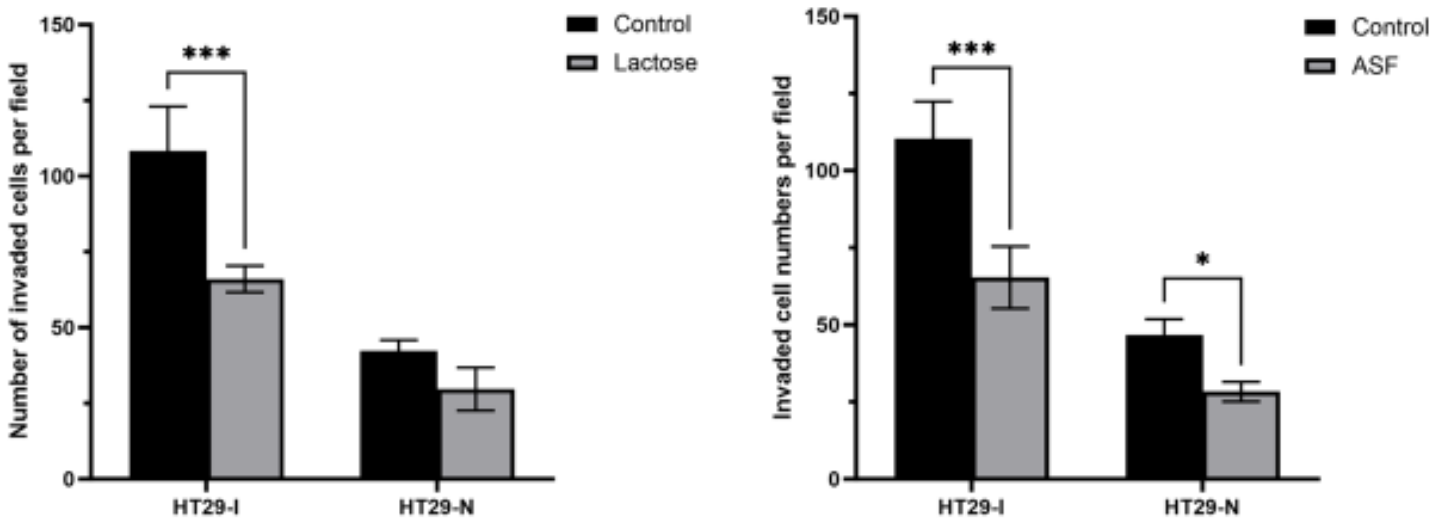

Figure 4.9 Galectin-3 binding inhibitors reduces HT29 cell invasion. HT29-I and -N cells were plated onto 8 µm transwell with Matrigel for 24hr. Introduction of lactose or ASF reduced invasion of both HT29-I and -N cells but more so in HT29-I cells. Data are presented as mean ± SD from three independent experiments, each in triplicate. *** $p < 0.001$, * $p < 0.05$ (ANOVA with Šídák post-hoc).

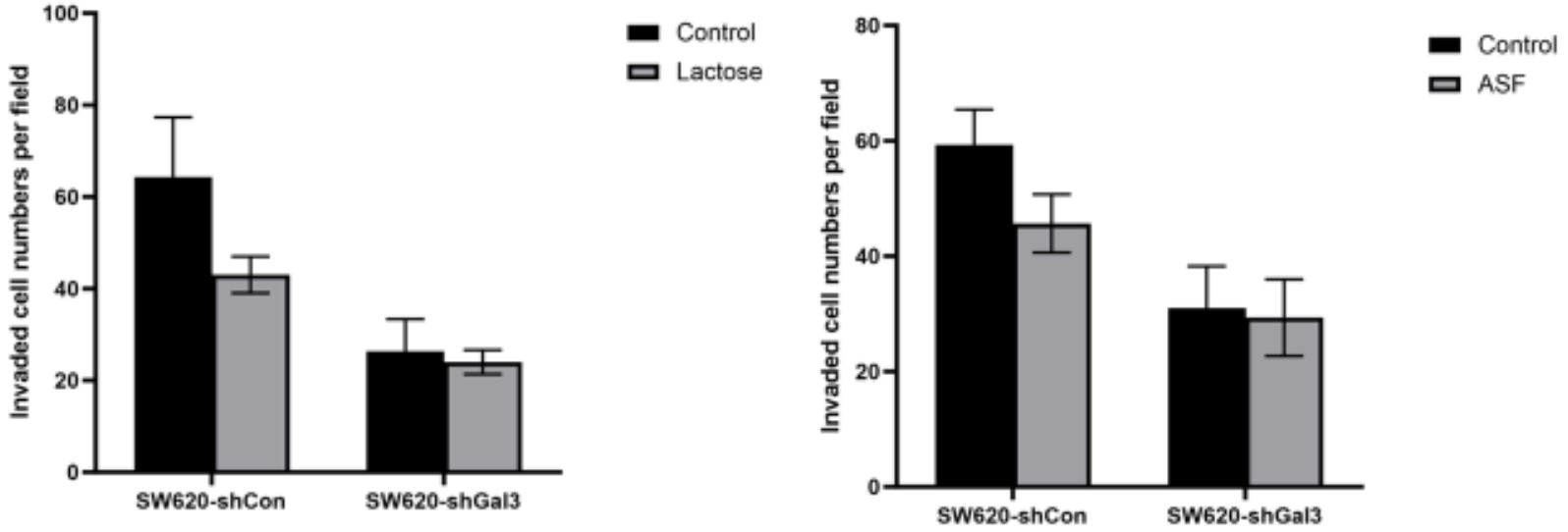

Figure 4.10 Galectin-3 inhibitor ASF reduces the SW620 cell invasion. SW620 cells were plated onto 8 μm transwell with Matrigel for 24hr. Introduction of lactose or ASF reduced invasion of both SW620-shCon and -shGal3 cells but more so in SW620-shCon cells. Data are presented as mean ± SD from three independent experiments, each in triplicate.

4.4.3 Galectin-3-mediated protease secretion disrupt cancer cell monolayer integrity

It is known that some proteases such as cathepsin-B can degrade cell surface adhesion molecules and disrupt cell adhesion [23-25]. For example, cathepsin-B can cleave E-cadherin at the cell surface and disrupt cell-cell junctions [26]. To test the influence of galectin-3-induced protease secretion on epithelial monolayer integrity, Caco2 cells, which can form tight cell-cell junctions in culture, and which are frequently used as a model to study epithelial integrity and the paracellular movement of compounds across an epithelium [27], were cultured to form a tight monolayer in trans-wells. Monolayer integrity was monitored after introduction of conditioned media. SW620 cells were cultured with 10 µg/ml galectin-3 or BSA, and the conditioned media were introduced onto the monolayer. It was found that conditioned medium from SW620 cells treated with galectin-3 caused a higher reduction in the transepithelial electric resistance (TEER) in comparison to that from control BSA (Fig 4.11). At 4 hr, conditioned media from SW620 cells treated with galectin-3 caused 50.57% reduction while conditioned medium from SW620 cells with control BSA only caused 36% reduction, in which CM-SW620 treated with galectin-3 caused 1.38-fold higher reduction of TEER than CM-SW620 with control BSA. SW620 cells were cultured with 2 µM galectin-3 inhibitor K2 or BSA, and the conditioned media were introduced onto the monolayer. At 4 hr, it was found that conditioned medium from SW620 cells treated with K2 caused a 16% reduction in the TEER while conditioned medium from SW620 cells without K2 treatment caused 43% reduction, which is 2.59-fold higher (Fig 4.12).

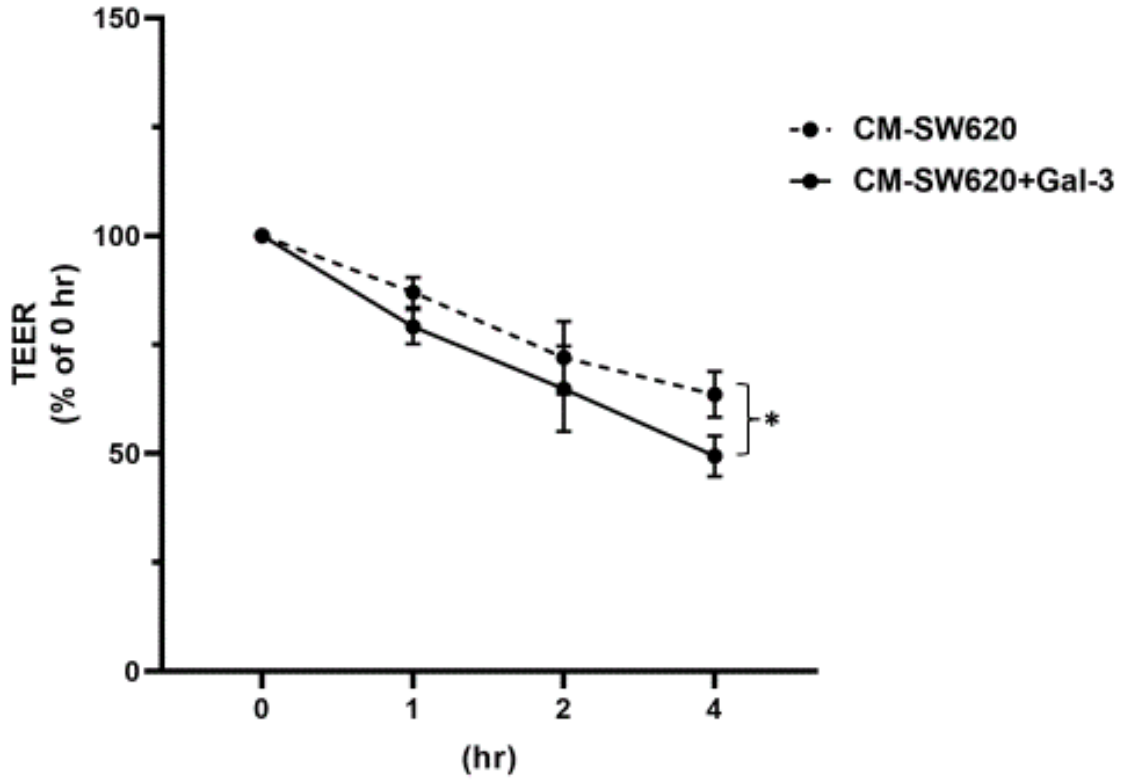

Figure 4.11. Galectin-3-induced protease secretion disrupts cell monolayer integrity of Caco2 cells. Caco-2 cells were cultured in trans-wells to confluence and the medium was replaced with conditioned media from SW620 cells in presence of galectin-3 or BSA, and TEER readings were measured. Data are presented as mean ± SD of three independent experiments, each in triplicate. * $p < 0.05$ (ANOVA with Šídák post-hoc).

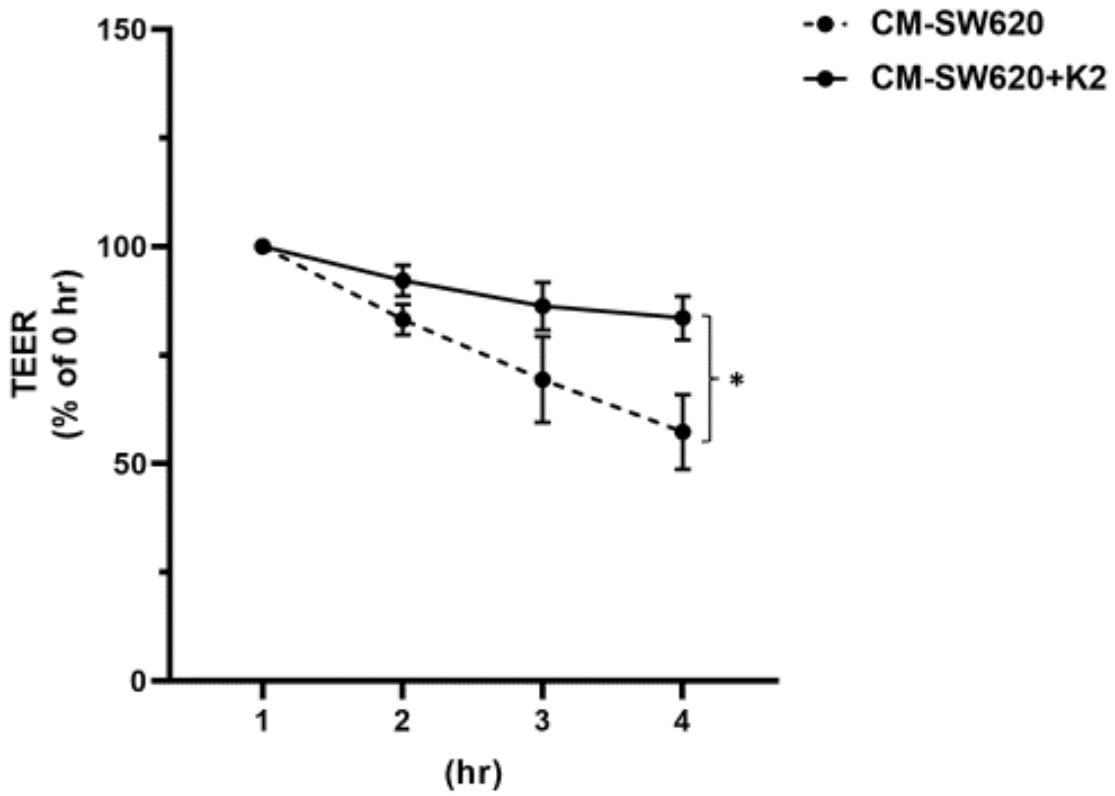

Figure 4.12. Galectin-3-induced protease secretion disrupts cell monolayer integrity of Caco2 cells. Caco-2 cells were cultured in trans-wells to confluence and the medium was replaced with conditioned media from SW620 cells in presence or absence of K2, and TEER readings were measured. Data are presented as mean ± SD of three independent experiments, each in triplicate* $p < 0.05$ (ANOVA with Šídák post-hoc).

Next, SW620-shCon and SW620-shGal3 cells were cultured for 24 hours and the culture media were collected and used as conditioned medium in Caco-2 monolayer integrity assessment. It was found that introduction of the conditioned medium from SW620-shCon cells caused a significantly higher reduction in the transepithelial electric resistance in comparison to that from SW620-shGal3 cells (Fig 4.13). At 4 hr, the reduction of TEER caused by condition media from SW620-shCon cells is 2-fold higher than the reduction caused by conditioned media from SW620-shGal3 cells. HT29-I and HT29-N cells were also cultured for 24 hours, and culture media were introduced onto Caco-2 monolayer. Introduction of conditioned medium from HT29-I cells also resulted in more reduction of cell TEER in comparison to that from HT29-N cells (Fig 4.14). At 4 hr, the reduction of TEER caused by conditioned media from HT29-I cells is 1.5-fold higher than the reduction caused by conditioned media from HT29-N cells.

Taken together, these results showed that galectin-3-mediated protease secretion disrupts tumour cell monolayer integrity.

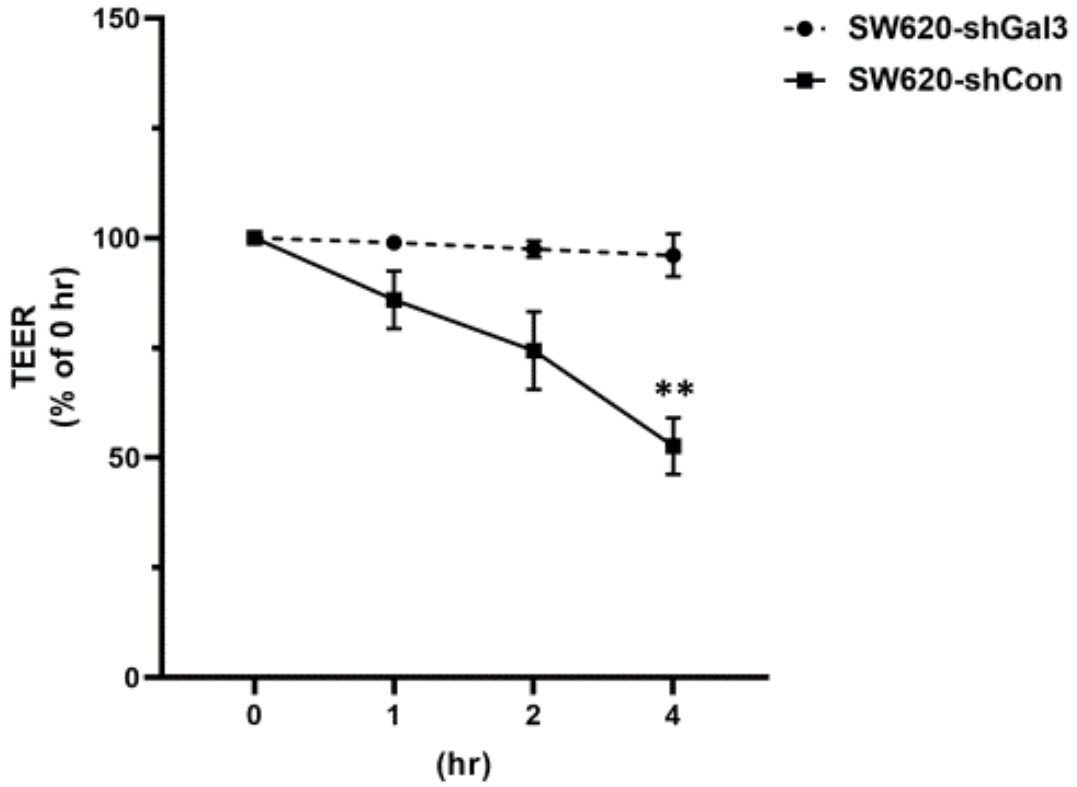

Figure 4.13. Galectin-3-induced protease secretion disrupts cell monolayer integrity of Caco2 cells. Caco-2 cells were cultured in trans-wells to confluence and the medium was replaced with conditioned media from SW620-shCon and SW620-shGal3 cells, and TEER readings were measured. Data are presented as mean ± SD of three independent experiments, each in triplicate, ** $p < 0.01$, (ANOVA with Šídák post-hoc).

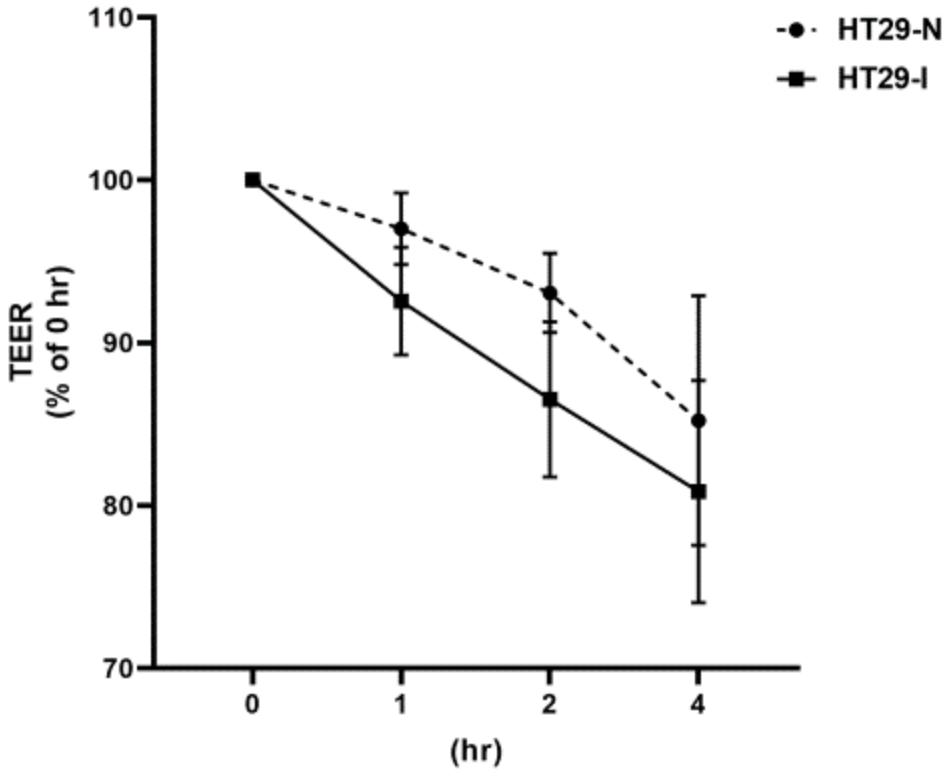

Figure 4.14. Galectin-3-induced protease secretion disrupts cell monolayer integrity of Caco2 cells. Caco-2 cells were cultured in trans-wells to confluence and the medium was replaced with conditioned media from HT29-I and HT29-N cells, and TEER readings were measured. Data are presented as mean ± SD of three independent experiments, each in triplicate.

4.4.4 Galectin-3-mediated protease secretion causes cell monolayer leakage

To further assess the impact of galectin-3-mediated protease secretion on epithelial cancer cell monolayer integrity, FITC-dextran was introduced as a transport marker in the assessment. Penetration of FITC-dextran through the Caco-2 monolayer to the bottom of the trans-wells was seen to be 2-fold higher when the cells were cultured in conditioned medium from SW620-shCon cells than in that from SW620-shGal3 cells (Fig 4.15). The penetration of FITC-dextran through the Caco-2 monolayer was also seen to be 1.5-fold higher when the cells were cultured in conditioned medium from HT29-I cells than from HT29-N cells (Fig 4.16).

In order to confirm the cell leakage was caused by related proteases, we also included active recombinant Cathepsin B and MMP-13 in the experiments. In comparison to the cells cultured in normal medium, introduction of 1000 pg/ml exogenous cathepsin-B or MMP-13 significantly increased FITC-dextran penetration through the Caco-2 monolayer (Fig 4.15 and 4.16). Moreover, introduction of a combination of antibodies against MMP-1, MMP-13 and cathepsin-B significantly reduced FITC-dextran penetration induced by culture in conditioned media from SW620-shCon but had minimal effect on that cultured in conditioned media from SW620-shGal3 cells (Fig 4.17). Likewise, the introduction of antibodies significantly reduces FITC-dextran penetration induced by culture in conditioned media from HT29-I cells but to a less degree on that cultured in conditioned media from HT29-N cells (Fig 4.18). Given that HT29-I and SW620-shCon cells secrete higher level of these proteases than HT29-N and SW620-shGal3 cells, respectively, these results indicate that galectin-3-induced protease secretion causes significant increases cell monolayer permeability and leakage.

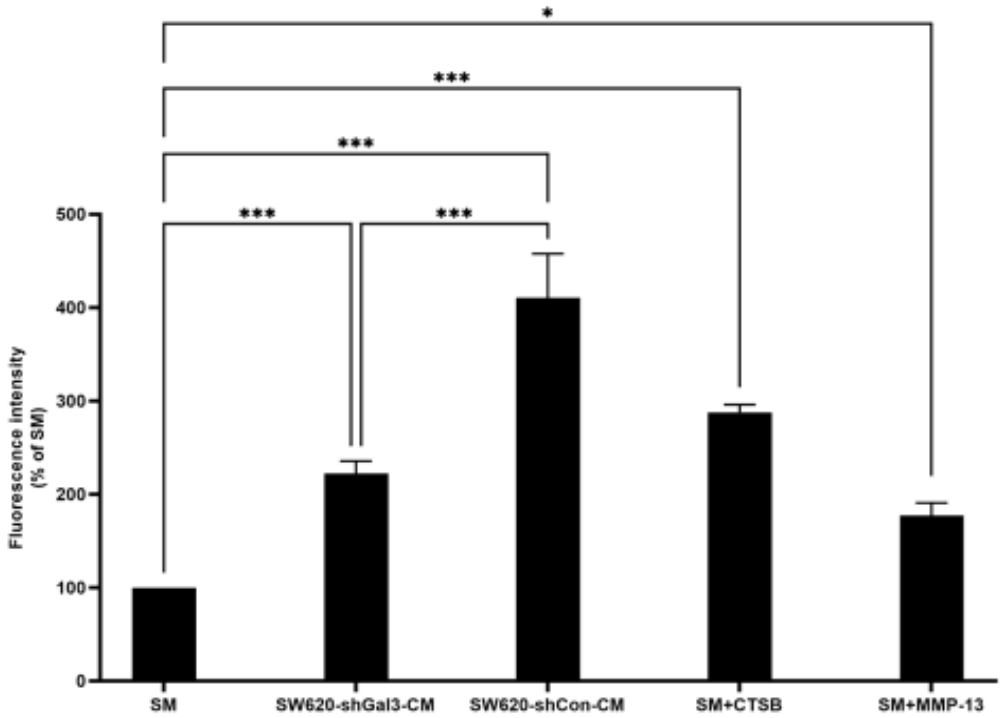

Figure 4.15. Galectin-3-induced protease secretion causes cell monolayer leakage. Caco-2 cells were cultured in trans-wells to confluence and the media was either replaced with fresh culture medium (SM) or conditioned culture medium from SW620-shGal3 (SW620-shGal3-CM) and SW620-shCon (SW620-shCon-CM) cells with addition of 1mg/ml FITC-dextran for 0.5 hr and the fluorescence intensities in the medium of the bottom wells measured. Data are presented as mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$, * $p < 0.05$ (ANOVA with Tukey test).

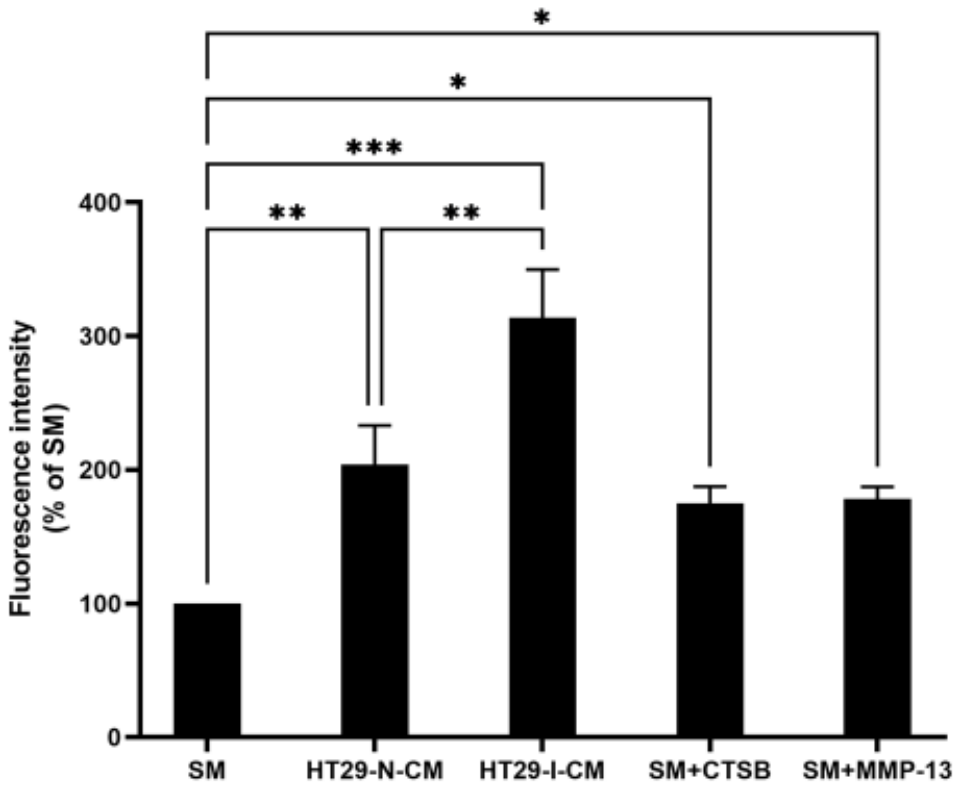

Figure 4.16. Galectin-3-induced protease secretion causes cell monolayer leakage. Caco-2 cells were cultured in trans-wells to confluence and the media was either replaced with fresh culture medium (SM) or culture medium from HT29-I (HT29-I-CM) and HT29-N (HT29-N-CM) cells with addition of 1mg/ml FITC-dextran for 0.5 hr and the fluorescence intensities in the medium of the bottom wells measured. Data are presented as mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (ANOVA with Tukey test).

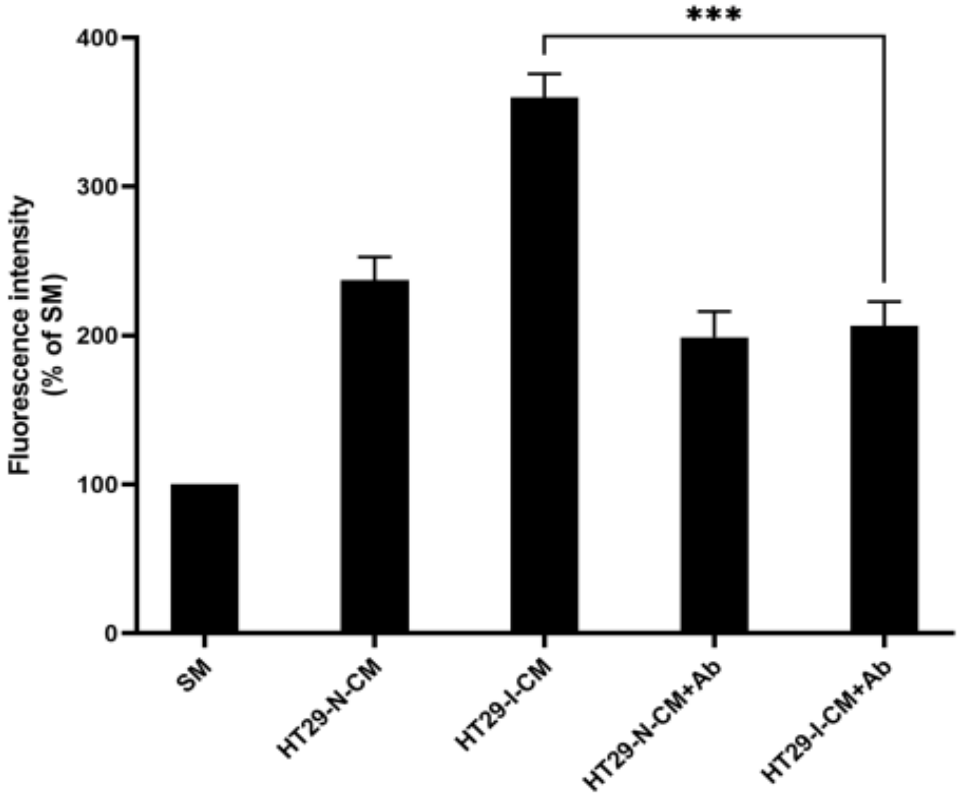

Figure 4.17. Galectin-3-induced protease secretion cause cell monolayer leakage. Caco-2 cells were cultured in trans-wells to confluence and the medium was replaced with conditioned media from HT29-N and HT29-I cells, and a combination of antibodies against MMP-1, MMP-13 and CTSB were added into the conditioned media of HT29-N and HT29-I cells before they were introduced to Caco-2 monolayers and subsequent analysis of the fluorescence intensity in the bottom wells. Data are presented as mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$, (ANOVA with Tukey test).

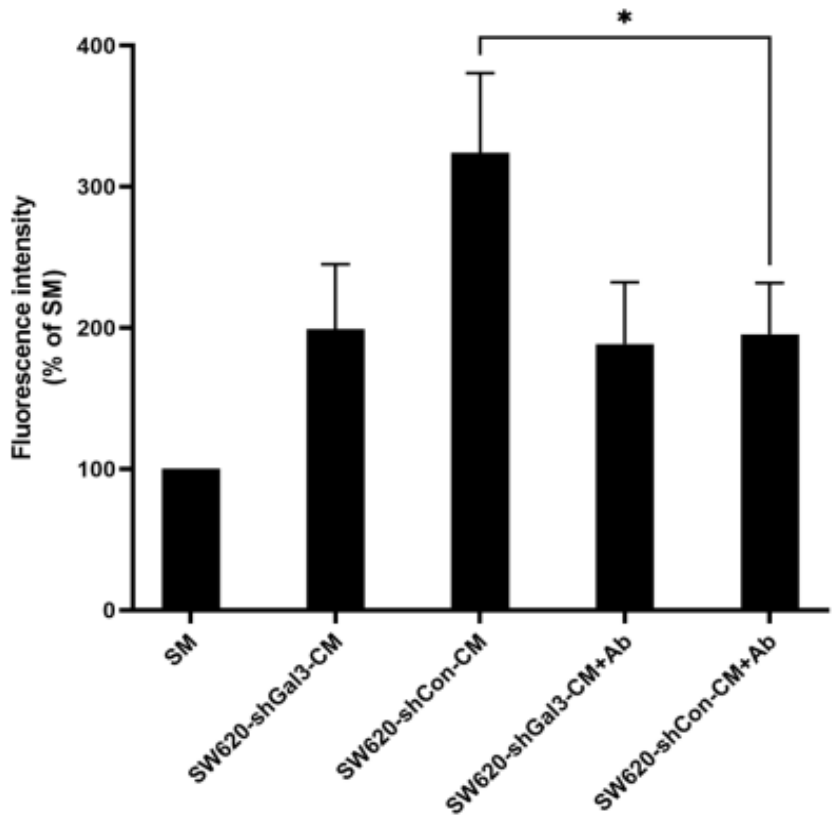

Figure 4.18. Galectin-3-induced protease secretion increases permeability of Caco2 monolayer. Caco-2 cells were cultured in trans-wells to confluence and the medium was replaced with conditioned media from SW620-shGal3 and SW620-shCon cells, and a combination of antibodies against MMP-1, MMP-13 and CTSB were added into the conditioned media of SW620-shGal3 and SW620-shCon cells before they were introduced to Caco-2 monolayers and subsequent analysis of the fluorescence intensity in the bottom wells. Data are presented as mean ± SD of three independent experiments, each in triplicate. * $p < 0.05$ (ANOVA with Tukey test).

4.5 Summary of results

1) Galectin-3-mediated protease secretion induces human colon cancer cell invasion through basement proteins
2) Galectin-3-mediated protease secretion disrupts cancer cell monolayer integrity
3) Galectin-3-mediated protease secretion increases cell monolayer permeability and leakage

4.6 Discussion

The results from this part of study show that galectin-3 mediated protease secretion increases cancer cell invasion through matrix proteins, and it also increases cell monolayer integrity by disrupting cell-cell contact.

Galectin-3 has been previously reported to promote cancer cell invasion and metastasis. For example, upregulation of galectin-3 was associated with pancreatic cancer proliferation and invasion [491]. Galectin-3 overexpression was shown to enhance cell adhesion to ECM components, cell migration and invasion in lung cancer [490]. In colon cancer, galectin-3 overexpression has been shown to be associated with cancer metastasis [492, 493]. It is reported that galectin-3 promotes colon cancer cell migration through its interaction with EGFR and promoting metastasis [378]. In this study, the introduction of galectin-3 caused increased cell invasion in four colon cancer cell lines: SW620, SW480, HCT116 and HT29 cells. The double time of SW620 and SW480 cells is 20 to 26 hours while the doubling time of HCT116 cells is 18 hours in standard culture medium with 10% FCS. To minimize possible impact of cell proliferation on protease secretion, the cells were cultured in 1% FBS or serum-free medium in the upper chamber of the trans-well to slow down cell proliferation. Moreover, the invaded cells shown in the photomicrographs displayed mostly as single cells, which indicates they were most likely not yet divided after invasion.

Subsequently, it was found that the promotion on cell invasion by galectin-3 is facilitated through protease secretion induced by galectin-3. The basement proteins underneath the epithelium are rich in protease substrates and can be catalytically digested by proteases including cathepsin B [494], MMP-1 [495] and MMP-13 [437]. Cathepsin-B has been shown to cleave laminin, fibronectin, type IV collagen and

tenascin-C in matrix proteins to aid tumour cell invasion [496]. MMP-1 and MMP-13 can also directly degrade type I and III collagens enriched in ECM [175]. The increased secretion of those proteases by galectin-3 in the tumour microenvironment therefore supports tumour cell invasion through the basement in tumour cell spreading.

Epithelium monolayer integrity is crucial in maintaining tissue homeostasis [497]. Disruption of epithelial integrity is a critical step in primary tumour cell invasion [498]. Briefly, normal epithelial cells are well-organized in monolayer or multilayer sheets with cells connected with neighbouring cells by cell-cell adhesion structures. However, in most human cancer, cells gradually lose epithelial morphology and acquire a mesenchymal-like phenotype through EMT. These changes provide cancer cells with ability to escape from the primary tumour and to migrate to distant region and is essential for cancer metastasis.

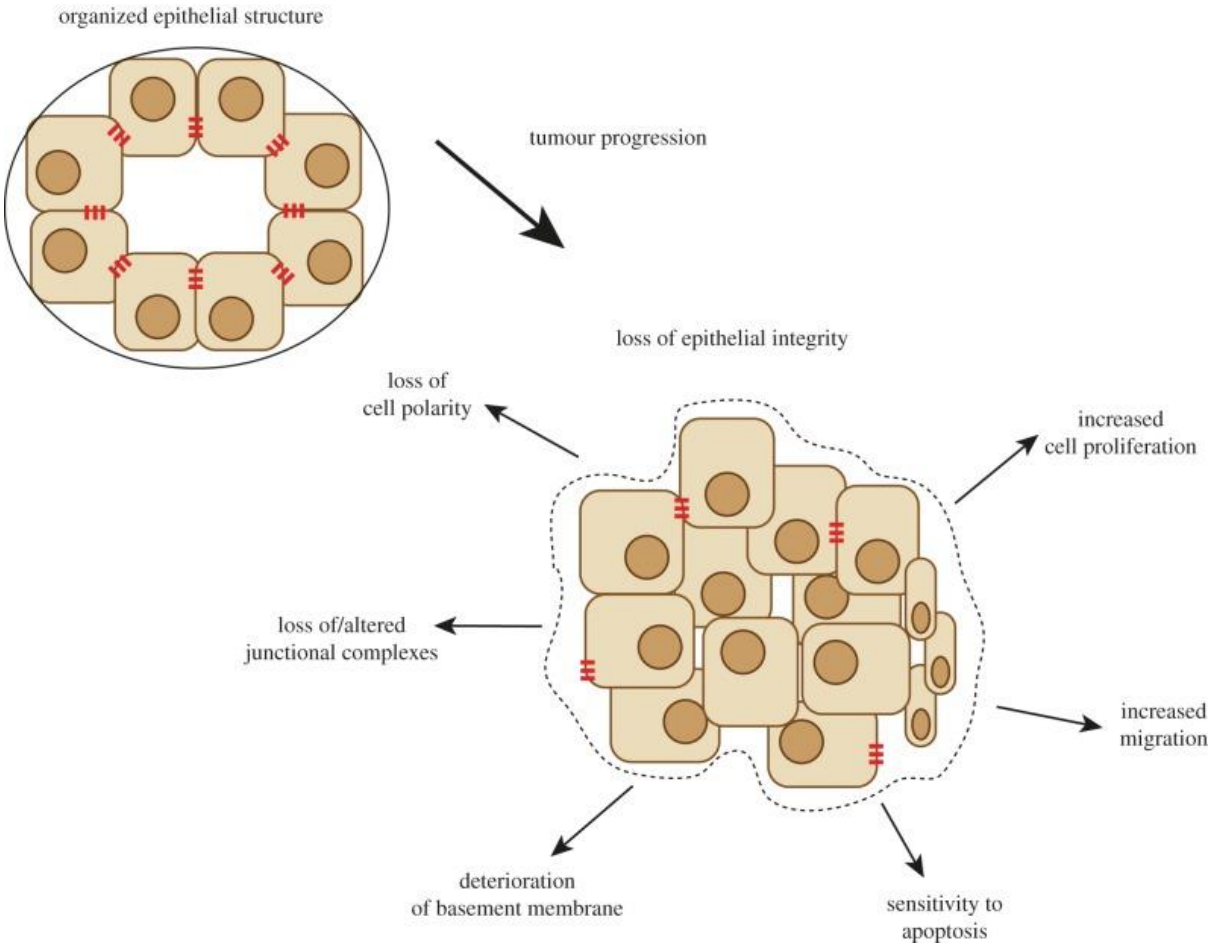

Figure 4.6.1 Disruption of epithelial integrity in cancer. A schematic presentation of cellular level changes during tumour progression including loss of cell polarity, loss of junctional complexes and deterioration of basement membrane. These changes results in increased cell proliferation and migration, and decreased sensitivity to apoptosis. Image is sourced from [498].

There are several characteristics of loss of epithelial integrity including loss of cell polarity, degradation of basement membrane and disruption of organized epithelial structure (Fig 4.6.1). These characteristics favours transformation of benign tumours into malignant tumours in many ways. For example, degradation of basement membrane is considered as the diagnostic marker that define the in-situ type of the carcinoma from invasive carcinoma [499]. The disruption of cell-cell junctions is closely associated with the EMT in cancer progression, in which junctional complexes are distorted, resulting in increased migratory focal adhesions [500]. Moreover, unstable

alignment of mitotic spindle leads to cell mass growing in multiple direction, therefore causing hyperplasia, as well as increasing aneuploidy [501, 502]. Disruption of cell adhesion and ECM signal transduction allows cells to migrate and invade [503]. During loss of epithelial integrity, lipid asymmetry is also disrupted which results in deregulated PI3K signalling, and loss of basal polarity causes loss of basement membrane, thus promotes invasion and metastasis [503, 504].

In this study, the loss of epithelial integrity is likely to be facilitated by galectin-3 induced proteases secretion mainly through disrupting cell-cell adhesions. E-cadherin is a key cell adhesion molecule for maintaining tight cell-cell junctions in the epithelium and reported to be proteolytically cleaved by cathepsins [472]. Cathepsin B can also bind to the annexin II heterotetramer (AIIt) and activates other proteases such as matrix metalloproteinases and urokinase plasminogen activator (uPA) and indirectly disrupts epithelial cell-cell contact and monolayer integrity [505-507]. In addition, cathepsins and MMPs are both demonstrated to degrade components of basement membrane [494, 508], which contributes to loss of epithelial integrity. The increased cancer cell secretion of cathepsin-B by galectin-3 can therefore decrease epithelium integrity and aids tumour cell break up at primary tumour sites.

Chapter 5

Investigating cell signalling in Galectin-3-mediated protease secretion

5.1 Introduction

It has been shown that the secretion of galectin-3 is driven by nonclassical pathways, and the extracellular function of galectin-3 is facilitated in a paracrine or autocrine manner. The oligomerization of galectin-3 allows the protein to interact with ECM and cell surface glycans, therefore influencing cellular functions. On the other hand, cytoplasmic and nuclear galectin-3 is able to bind to other proteins in the cytosol and nucleus to affect more cellular functions. To this notion, galectin-3 is associated with cell proliferation, differentiation and death through its ability to initiate, enhance or attenuate intracellular signalling pathways.

Structurally, galectin-3 contains a short amino terminal motif known as collagen α-like domain which is responsible for its shuttling between nucleus and cytosol. This motif can be catalysed by MMPs and prostate specific antigen, which results in the lattice formation and oligomerization of the protein. The CBD domain of galectin-3 contains the anti-death motif of the Bcl2 protein family, which accountable for the association with several extracellular and intracellular protein-carbohydrate or protein-protein interactions, in turn, regulating different signalling pathways that involved in cancer progression.

Galectin-3 is known to bind to β1,6-GlcNAc-branched N-glycans and glycoproteins on the cell surface or in the ECM, which accountable for regulating downstream cell signal transduction and influencing the distribution of glycoproteins [311]. For example, it has been shown that the anti-galectin-3 antibody inhibited the PI3K reactivation that mediated by β4-integrin N-glycans [509]. It was also found that the RAS-induced PI3K/AKT activation requires galectin-3 to response to growth factor stimulation [510]. In addition, the β-catenin expression in the nucleus is increased by galectin-3 which

results in the accumulation of β-catenin and further enhances Wnt signalling in human colon cancer cells via regulating GSK-3β phosphorylation [319]. It has also been shown that IL-8 expression and secretion is increased by galectin-3 via NF-κB signalling in pancreatic stellate cells, and the galectin-3-mediated IL-8 expression and secretion are completely inhibited by NF-κB inhibitor and integrin-linked kinase [511].

In TME, galectin-3 overexpression is associated with cancer cell migration, invasion and metastasis [512]. The Wnt/β-catenin pathway participates in the regulation of cell migration through MMPs [513]. Interaction of galectin-3 and β-catenin/TCF complex was found in the nucleus where galectin-3 and β-catenin co-localized, results in increased transcriptional activity of transcription factor 4 (TCF4) in breast cancer cells [318]. Moreover, galectin-3 also regulates TCF4 activity and β-catenin expression by activating GSK-3β phosphorylation via the PI3K/AKT pathway in colon cancer cells. In addition, inhibition on galectin-3 expression resulted in decreased phosphorylated-GSK-3β and phosphorylated-AKT expression, thus mediating the phosphorylation of β-catenin [514]. Taken together, previous findings suggest that galectin-3 is involved in multiple signalling pathways in cancer progression including EGFR/FGFR pathway, Hedgehog pathway, Notch pathway, TGF-β pathway and Wnt pathway (Table 5.1).

Table 5.1. Roles of galectin-3 in key signalling pathways involved in cancer

Pathway	Primary factors	Role of galectin-3
EGFR/FGFR	Receptor tyrosine kinase, PI3K/Akt and MAPK pathways	Stabilizes EGFR, activates EGFR-mediated activation of K-Ras, PI3K and Akt, further regulates β-catenin transcriptional activity [510, 515]
Hedgehog	FAK/Akt	Enhances Akt activity [516-519]
Notch	E-cadherin, Slug, Snail	Induces Notch transcription of its target proteins [520-522] and regulates E-cadherin [523]
TGF-β	TGF-β, SMADs, MAP kinase pathways, Rho-like GTPase signalling pathways, PI3K/Akt pathways	Stabilizes TGF-β receptor and participates in the FAK and PI3K/Akt pathways [524]
Wnt	β-catenin, GSK-3β, TCF/LEF	Activates GSK-3β, binds to β-catenin and modulates its nuclear export and transcriptional activity [318, 319, 525, 526]

The expression and secretion of cathepsin B in cancer cells has been reported to involve several signalling pathways. It was found that Hedgehog signalling promotes pancreatic cancer invasion through upregulating cathepsin B expression [527]. It was also found that CD147 promoted cathepsin B transcription by activating β-catenin signalling as a result of reduced GSK-3β expression in hepatocellular carcinoma [528]. However, the regulation of cathepsin B secretion remains widely unclarified. It has been shown that catalytic activity of cathepsin B is pH-dependent [529, 530]. In addition to that, the secretion of active cathepsin B also depends on extracellular pH. In particular, low pH conditions in B16 melanoma cells induce the vesicles that contains cathepsin B to migrate to the periphery of cells, therefore increaseing the secretion of the protein [531]. Interestingly, galectins like galectin-1 have been found to activate carbonic anhydrase IX which is involved in extracellular adaptation to acidosis, to promote glioma growth and invasiveness [532]. In this part of study, we aimed to investigate the signalling pathway that was involved in galectin-3-mediated protease secretion.

5.2 Hypothesis and Aims

5.2.1 Hypothesis

Galectin-3-mediated promotion of protease secretion in colon cancer cells involves activation of cell signalling.

5.2.2 Aims

To identify the cell signalling in galectin-3-mediated protease secretion in colon cancer cells

5.3 Methods

5.3.1 Assessment of kinase phosphorylation in cells response to galectin-3 using Human Protein kinase profile array

SW620 cells were seeded at 1x10^6 cells/ml into 6-well plate and cultured to 80% confluence. The cells were then introduced with serum-free medium containing 1% BSA with 10 µg/ml galectin-3 or 10 µg/ml BSA for 30 min at 37℃. The phosphorylation levels of the 37 most common kinases in cell signalling were analysed by the Proteome Profiler Human Phospho-Kinase Array according to the manufacturer's protocols.

Subsequently, pixel densities on developed blots were quantified using Imagelab and data output was calculated as follow, value *P* stands for the phosphorylation level of the kinase of interest:

$$P = \frac{\overline{A} - \overline{N}}{\overline{R} - \overline{N}.}$$

$\overline{A}$ is the mean value of pixel densities of corresponding duplicate spots. $\overline{R}$ is the mean value of the pixel intensities of the reference spots. $\overline{N}$ is the averaged background value.

5.3.2 Immunoblotting

In order to determine the activation of galectin-3-induced kinases phosphorylation, SW620 cells were cultured onto 24-well plates at 1 x 10^6 cells/ml for 24 hr at 37°C. The culture media was then replaced by 0.5% BSA serum free DMEM medium and treated with 10 µg/ml galectin-3 after 0, 30, 60, 90 and 120 minutes. The cell lysates were collected and analysed by immunoblotting.

In order to examine the effect of PKY2 inhibitor or GSK3α/β inhibitor on galectin-3-induced kinases phosphorylation, SW620 cells were cultured onto 24-well plates at 1 x 10^6 cells/ml for 24 hr at 37°C. The culture media was then replaced by 0.5% BSA serum free DMEM medium and treated with 10 µg/ml galectin-3 or 10 µM SB2 or 10 µM PF4 for 30 minutes. The cell lysates were collected and analysed by immunoblotting.

SDS-PAGE was conducted as described in chapter two. Cellular proteins (cell lysate) separated by SDS-PAGE were electro-transferred to 0.2 µm nitrocellulose membrane. The membranes were first incubated with specific primary antibodies against phospho-PYK2 (0.5 µg/mL), phospho-GSK3α/β (0.2 µg/mL), phospho-STAT1 (0.5 µg/mL), phospho-STAT3 (0.5 µg/mL). The blots were washed 3 times with 0.05% Tween-20 in TBS before incubated with peroxidase-conjugated secondary antibody (1:5000 HRP-conjugated anti-mouse IgG secondary antibody used for PKY2 and STAT3, 1: 5000 HRP-conjugated anti-rabbit IgG secondary antibody used for STAT1 and GSK3α/β) for 1 hour. After 6 washes with 0.05% Tween-20 in TBS, the protein bands were developed using chemiluminescence Super Signal kit and visualized with Molecular Imager® Gel Doc™ XR System (Biorad). The blots were stripped by stripping buffer (Tris-HCl 62.5mM, Mercaptoethanol 100mM and SDS 2%) and re-probed with antibodies against PYK2 (1 µg/mL), GSK3α/β (0.1 µg/mL), STAT1 (1 µg/mL), or STAT3 (0.1 µg/mL). In the case of re-probed blots were unable to be seen, the same cell lysates to assess phosphorylated proteins were assessed again by immunoblotting using antibodies against PYK2 (1 µg/mL), GSK3α/β (0.1 µg/mL) and STAT1 (1 µg/mL). The density of the protein bands was quantified using Imagelab version 3.0.1.

5.3.3 ELISA

To test the effect of PKY2 inhibitor and GSK3α/β inhibitor on protease secretion, SW620 and HCT116 cells were cultured at 1 x 10^6 overnight at 37°C. Cells were then treated with SB 216763 (0.5, 1, 2.5, 5 and 10 μM) or PF 431396 (1, 2.5, 5, 10 and 25 μM) or 10 μM DMSO for 24 hours. The supernatants were centrifuged at 1,956 g for 5 minutes and analysed by ELISA as previously described.

To test the effect of PYK2 inhibitor and GSK3α/β inhibitor on galectin-3-induced protease secretion, SW620 and HCT116 cells were cultured at 1 x 10^6 for overnight at 37°C. The culture media was replaced by 0.5% BSA serum free DMEM medium, and cells were treated with 10 μg/ml galectin-3 or BSA without or with SB 216763, PF 431396 or DMSO overnight. The concentrations of cathepsin-B in the supernatants were analysed by ELISA.

5.4 Results

5.4.1 Assessment of kinase phosphorylation in response to galectin-3

To gain insight into the molecular mechanism of galectin-3-mediated protease secretion, the phosphorylation profile of 37 key signalling proteins in SW620 cells in cell response to galectin-3 was analysed using a Proteome Profiler Human Phospho-Kinase Array (Fig 5.4.1.1). After 30 min treatment, galectin-3 treatment led to changes in the phosphorylation of signalling proteins shown in Table 5.2 and Fig 5.2. Among 37 kinases, the five most increased kinase phosphorylation in respond to galectin-3 are: PYK2 (1.8-fold), GSK3α/β (1.5-fold), STAT1 (1.5-fold), P70 S6 Kinase (1.4-fold), and p53 (1.3-fold) while the five most decreased kinase phosphorylation are STAT3 (1.6-fold), Lck (1.5-fold), STAT5α/ß (1.3-fold), Akt1/2/3 (1.3-fold) and Src (1.3-fold). Four kinases, namely Protein tyrosine kinase 2 (PYK2), Glycogen synthase kinase-3 (GSK3α/β), Signal transducer and activator of transcription 1 (STAT1) and Signal transducer and activator of transcription 3 (STAT3) showed over 50% changes in response to galectin-3.

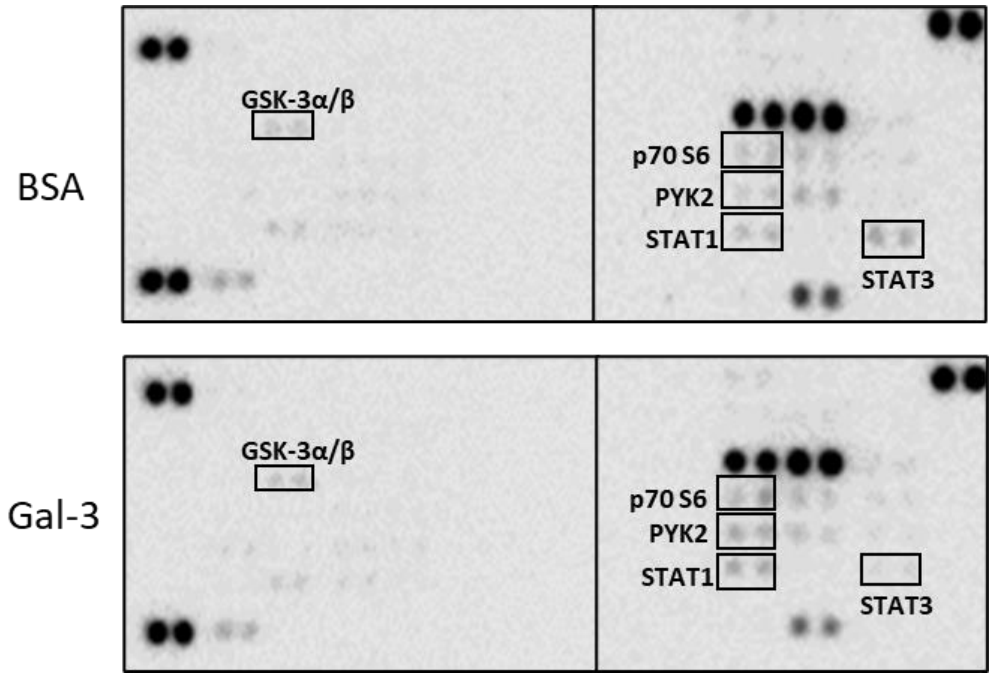

Figure 5.1. Phosphorylation of 37 signalling proteins in SW620 cells assessed by Proteome Profiler Human Phospho-Kinase Array. SW620 cells were treated with 10 µg/ml galectin-3 or BSA for 0.5 hr before analysed by Proteome Profiler Human Phospho-Kinase Array. The kinases with over 50% changes were highlighted with black rectangle.

Table 5.2 Levels of phosphorylation of 37 signalling proteins in SW620 cells in response to galectin-3 and BSA

			Changes in response to Galectin-3
	BSA	**Galectin-3**	**(% of BSA)**
PYK2	6.08	11.10	82.45
GSK-3α/ß	2.06	3.13	52.10
STAT1	6.54	9.90	51.44
P70 S6 K	9.49	13.62	43.54
p53	3.79	4.97	31.31
STAT2	2.58	3.27	26.45
c-Jun	3.24	4.03	24.51
CREB	1.97	2.29	16.47
GSK-3ß	1.97	2.29	16.28
p53	91.82	104.72	14.05
HSP27	1.92	2.16	12.64
RSK1/2/3	3.04	3.33	9.52
Akt1/2/3	4.03	4.34	7.56
PRAS40	3.79	3.98	5.21
p53	71.41	74.30	4.04
Fgr	1.87	1.92	2.57
EGFR	1.95	1.93	-0.69
ERK1/2	2.28	2.25	-1.30
p38α	3.21	3.14	-2.11
P70 S6 K	10.94	10.61	-3.01
Chk-2	3.39	3.25	-4.10
ß-catenin	15.45	13.81	-10.59
STAT3	3.08	2.72	-11.80

Yes	2.27	1.95	-14.01
WNK1	3.90	3.25	-16.75
MSK1/2	1.88	1.55	-17.85
eNOS	2.51	2.06	-17.99
Lyn	2.83	2.27	-19.60
PDGF Rß	2.13	1.67	-21.48
STAT6	2.78	2.10	-24.53
HSP60	31.80	22.87	-28.08
PLC-y1	3.67	2.61	-28.91
JNK 1/2/3	2.83	1.99	-29.77
RSK1/2	10.10	6.87	-31.98
Src	2.67	1.82	-32.11
Akt1/2/3	3.37	2.26	-32.83
STAT5α/ß	5.89	3.87	-34.25
Lck	6.83	3.70	-45.90
STAT3	9.81	3.97	-59.55

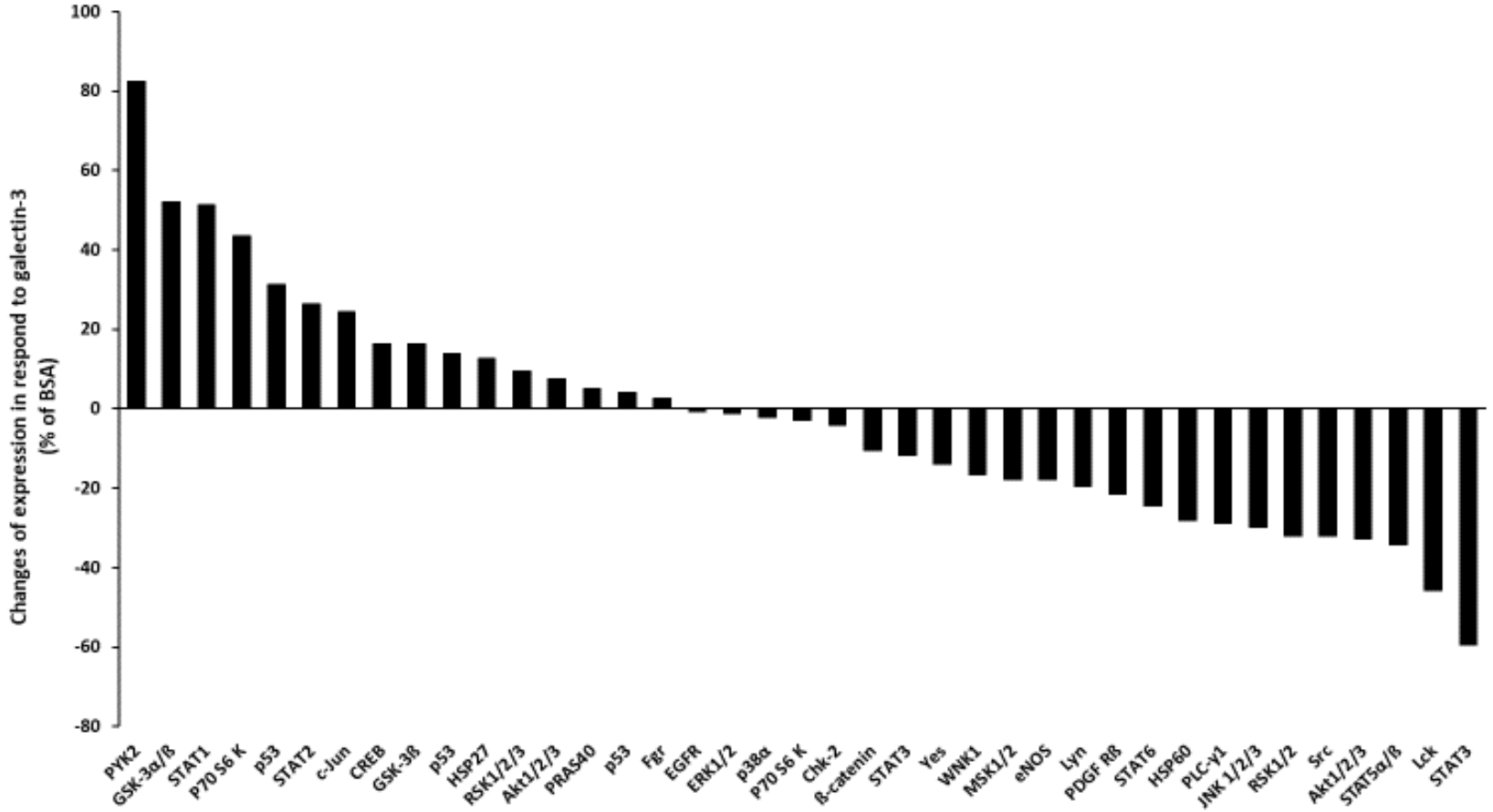

Figure 5.2. Changes of phosphorylation of 37 signalling proteins in SW620 cells in response to galectin-3. Pixel densities on array were collected and analysed using Imagelab and data output was calculated as described previously. Ratio of P_{gal3}/P_{BSA} was presented from highest to lowest (left to right). The experiment was performed once.

5.4.2 Assessment of the effects of galectin-3 on phosphorylation of STAT1, STAT3, PYK2 and GSK3

To confirm the effects of galectin-3 on activation of these kinases, SW620 cells were treated with galectin-3 for various times and the phosphorylation status of each of these four kinases was measured using immunoblotting. The introduction of galectin-3 activates phosphorylation of PYK2 after 10 minutes with a 1.5-fold increase in phosphorylation and the level of phosphorylation returned to normal level after approximately 30 minutes (Fig 5.3). The presence of galectin-3 activates STAT1 phosphorylation after 10 minutes with a 1.66-fold increase shown, and the level of phosphorylation returned to normal level after 60 minutes (Fig 5.4). The phosphorylation of GSK3α/β was activated by galectin-3 after 10 minutes with 1.5-fold and 1.5-fold increases of GSK3α and GSK3β phosphorylation, respectively, and the level of phosphorylation returned to normal level after 60 minutes (Fig 5.5). On the other hand, the phosphorylation of STAT3 was shown not to be affected by galectin-3 at any time point (Fig 5.6). These results suggest that the presence of galectin-3 caused time-dependent increases in the activation of PYK2, STAT1 and GSK3α/β, while phosphorylation of STAT3 was unaffected.

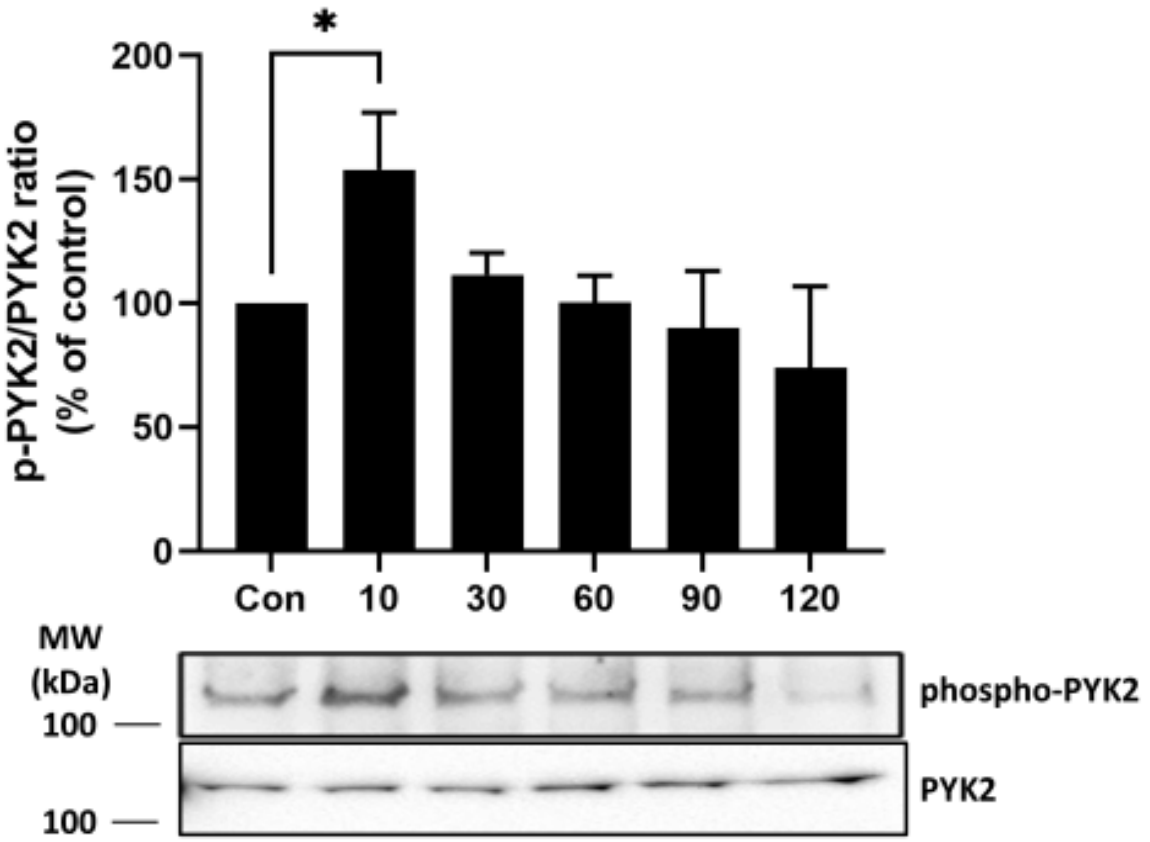

Figure 5.3. The presence of galectin-3 increases the phosphorylation of PYK2. SW620 cells treated with 10 µg/ml galectin-3 for 10, 30, 60, 90 and 120 minutes were assessed by immunoblotting using antibodies against p-PYK2. The same cell lysates were then assessed by immunoblotting using antibodies against PYK2. The band density was quantified and expressed as percentages of phospho-PYK2/PYK2 ratio. Data are presented as mean ± SD of three independent experiments. * $p < 0.05$ (ANOVA). ANOVA with Dunnett's test was performed on raw data.

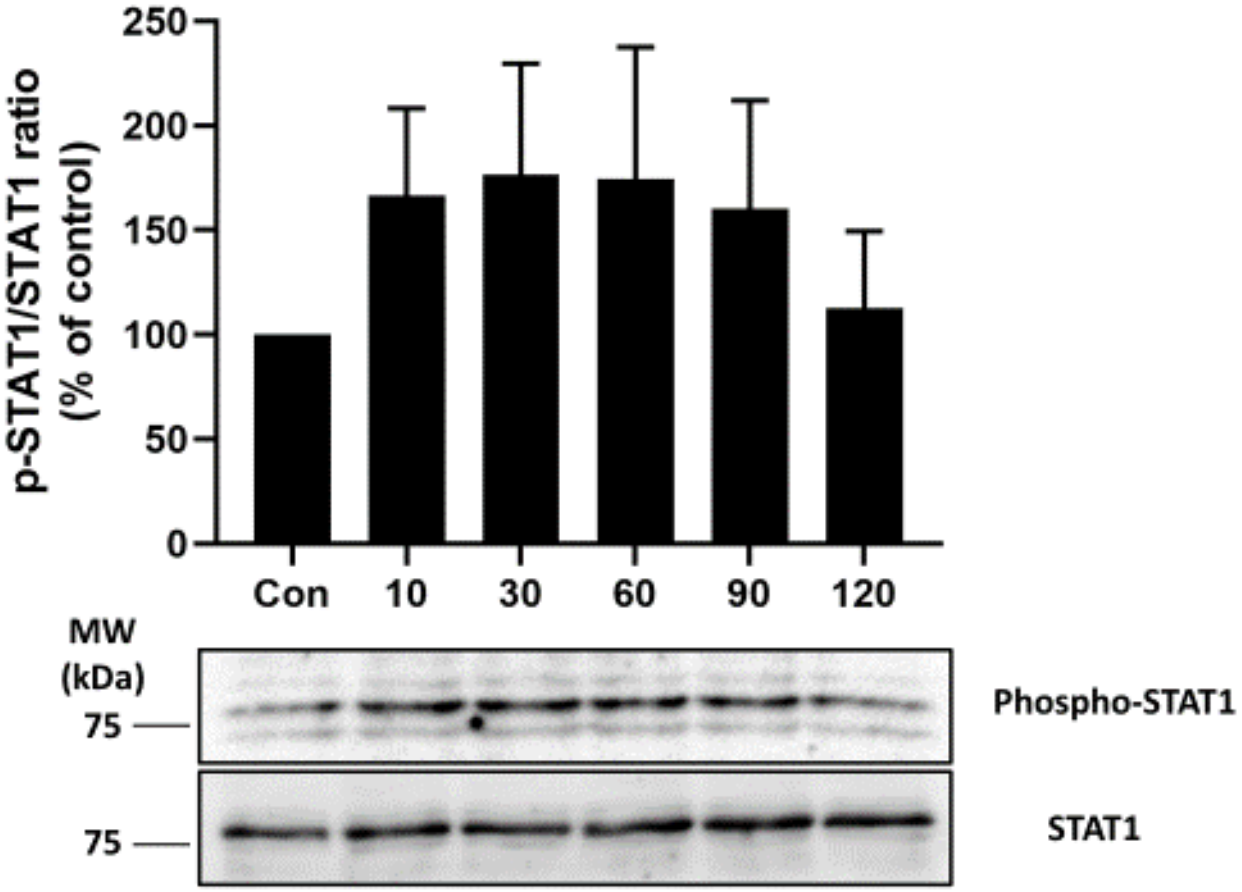

Figure 5.4. The presence of galectin-3 increases the phosphorylation of STAT1. SW620 cells treated with 10 µg/ml galectin-3 for different times were assessed by immunoblotting using antibodies against phosphor-STAT1. The blot was then stripped re-probed with antibodies against STAT1. The band density was quantified and expressed as percentages of phospho-STAT1/STAT1 ratio. Data are presented as mean ± SD of three independent experiments. ANOVA with Dunnett's test was performed on raw data.

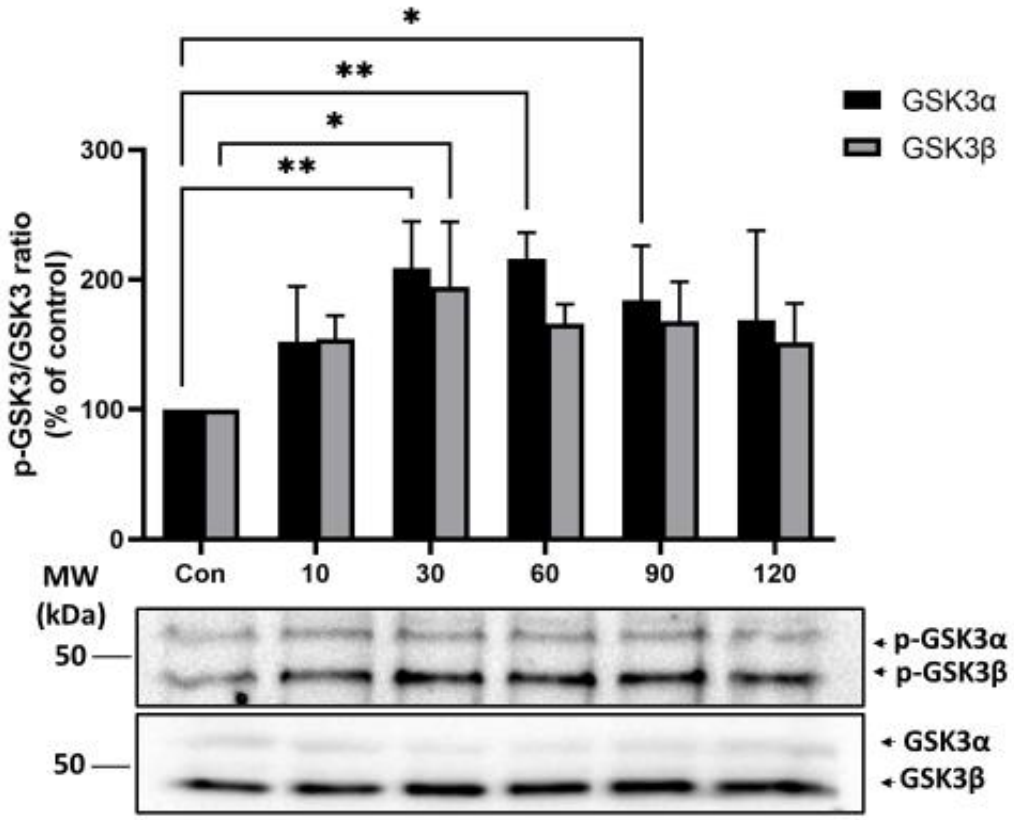

Figure 5.5. The presence of galectin-3 increases the phosphorylation of GSK3α/β. SW620 cells treated with 10 µg/ml galectin-3 for different times were assessed by immunoblotting using antibodies against phosphor-GSK3α/β. The same cell lysates were also assessed by immunoblotting using antibodies against GSK3α/β. The band density was quantified and expressed as percentages of phosphor-GSK3α/β /GSK3α/β ratio. Data are presented as mean ± SD of three independent experiments. ** $p < 0.01$, * $p < 0.05$ (ANOVA). ANOVA with Dunnett's test was performed on raw data.

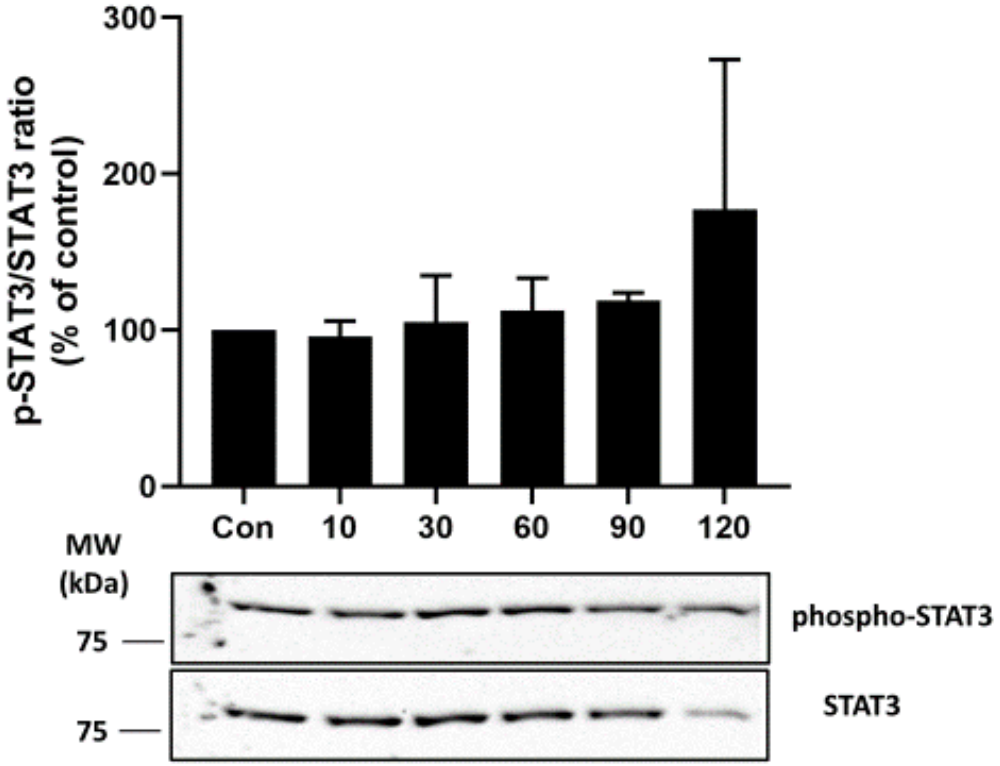

Figure 5.6. The presence of galectin-3 has no effect on the phosphorylation of STAT3. SW620 cells treated with 10 µg/ml galectin-3 for different times were assessed by immunoblotting using antibodies against phosphor-STAT3. The blots were then stripped and re-probed with antibodies against STAT3. The band density was quantified and expressed as percentages of phosphor-STAT3/STAT3 ratio. Data are presented as mean ± SD of three independent experiments. ANOVA with Dunnett's test was performed on raw data.

5.4.3 PYK2 is the upstream regulator of GSK3α/β in response to galectin-3

In order to determine the possible activation pathway of PYK2 and GSK3α/β in galectin-3-mediated effect, PYK2 inhibitor PF431396 (PF4) and GSK3α/β inhibitor SB216763 (SB2) were used to assess the phosphorylation of these kinases. SW620 cells were cultured with galectin-3 or SB2 or PF4 for 24 hours and the levels of phospho- and non-phospho- kinases were assessed using immunoblotting. After 24 hours treatment, the phosphorylation of PYK2 was decreased by 27% by introduction of PF4 (10 μM) as well as the galectin-3-induced increase in PYK2 phosphorylation (decreased by 52%), while introduction of SB2 had little effect on either PYK2 phosphorylation or galectin-3-induced increase of PYK2 phosphorylation (Fig 5.7). On the other hand, the phosphorylation of GSK3α was decreased by 70% and 85.72% by introduction of SB2 and PF4, respectively, while the phosphorylation of GSK3β was decreased by 62% and 67% by introduction of SB2 and PF4, respectively. It was also shown that galectin-3-induced increase in GSK3α phosphorylation was decreased by 79% and 93% by the introduction of SB2 and PF4, respectively, while the galectin-3-induced increase of GSK3β phosphorylation was decreased by 79% and 87% by the introduction of SB2 and PF4, respectively. (Fig 5.9). These results showed that PYK2 inhibitor PF4 was able to suppress both PYK2 and GSK3α/β phosphorylation while GSK3α/β inhibitor SB2 can only suppress GSK3α/β phosphorylation, indicating PYK2 is probably an upstream mediator of GSK3α/β in signalling transduction. Furthermore, introduction of SB2 had little effect on galectin-3-associated phosphorylation of STAT1 (Fig 5.8) and STAT3 (Fig 5.10), while the presence of PF4 also caused no/little effect on STAT1 (Fig 5.8) and STAT3 (Fig 5.5.10) phosphorylation, suggesting either STAT1

or STAT3 is involved in activation of PYK2 or GSK3α/β, and vice versa, or at least not downstream of PYK2 or GSK3α/β.

These results suggest that activation of GSK3α/β and PYK2 are both involved in the cell response to galectin-3 and PYK2 is a possible upstream mediator of GSK3α/β in galectin-3-mediated signalling transduction.

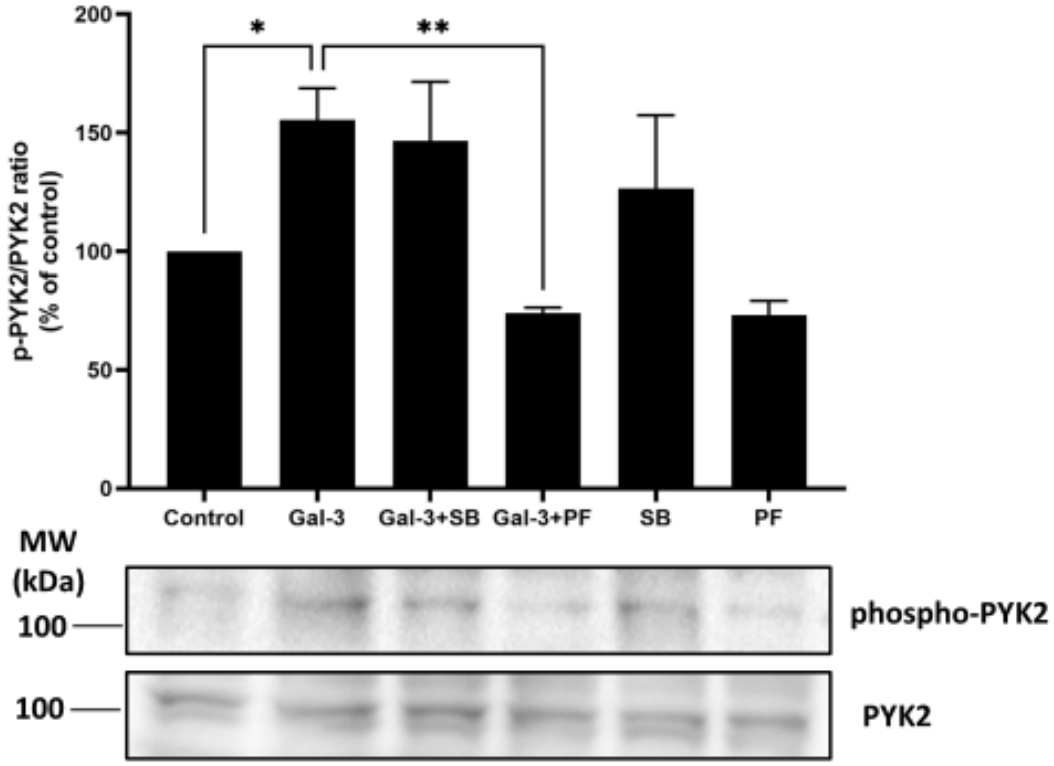

Figure 5.7. PYK2 phosphorylation was suppressed by PYK2 inhibitor but not GSK3α/β inhibitor. SW620 cells were treated with 10 µg/ml galectin-3 or BSA followed by introduction of GSK3α/β inhibitor SB 216763 (SB) or PKY2 inhibitor PF 431396 (PF) for 15 min and the levels of phosphorylated PYK2 were analysed by immunoblotting using antibodies against phosphor-PYK2. The blots were then stripped and re-probed with antibodies PYK2. The band density was quantified and expressed as percentages of phosphor-PYK2/PYK2 ratio. Data are presented as mean ± SD of three independent experiments. ** $p < 0.01$, * $p < 0.05$ (ANOVA). ANOVA with Tukey test was performed on raw data.

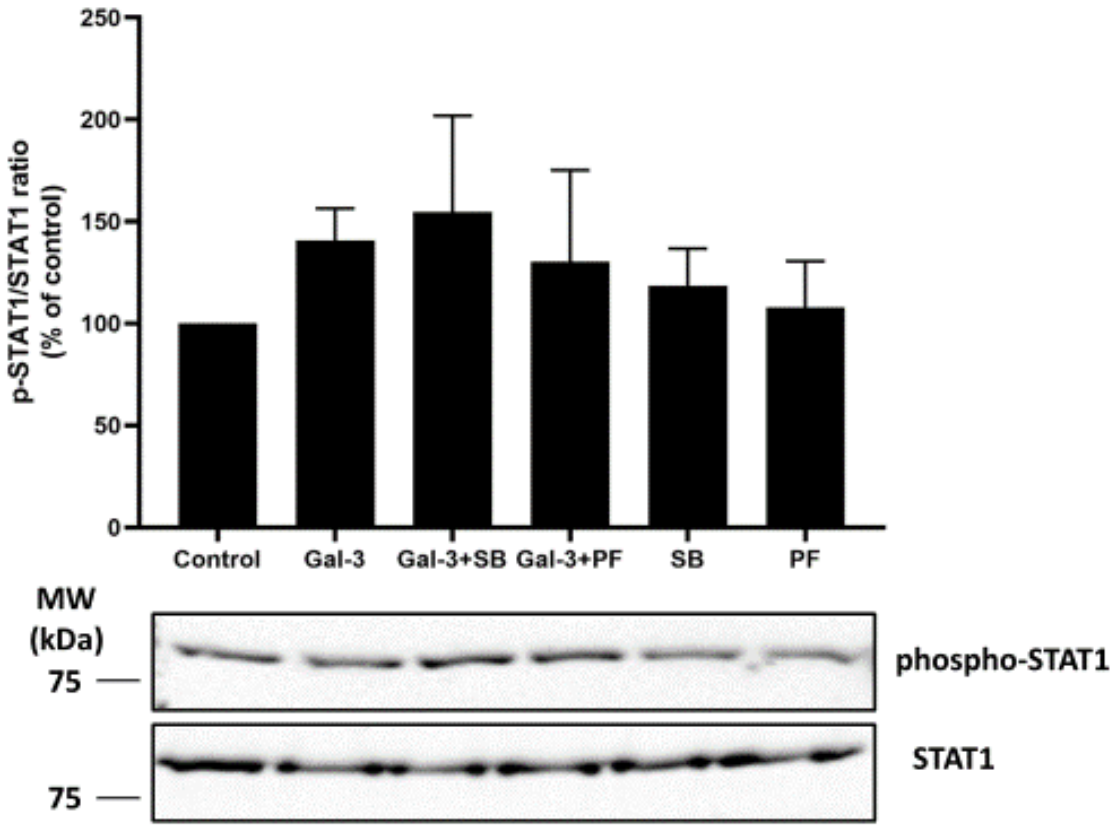

Figure 5.8. STAT1 expression was not affected by PYK2 inhibitor or GSK3α/β inhibitor. SW620 cells were treated with 10 µg/ml galectin-3 or BSA followed by introduction of GSK3α/β inhibitor SB 216763 (SB) or PKY2 inhibitor PF 431396 (PF) for 15 min and the levels of phosphorylated STAT1 were analysed by immunoblotting using antibodies against phosphor-STAT1. The same cell lysates were also assessed by immunoblotting using antibodies against STAT1. The band density was quantified and expressed as percentages of phosphor-STAT1/STAT1 ratio. Data are presented as mean ± SD of three independent experiments. ANOVA with Tukey test was performed on raw data.

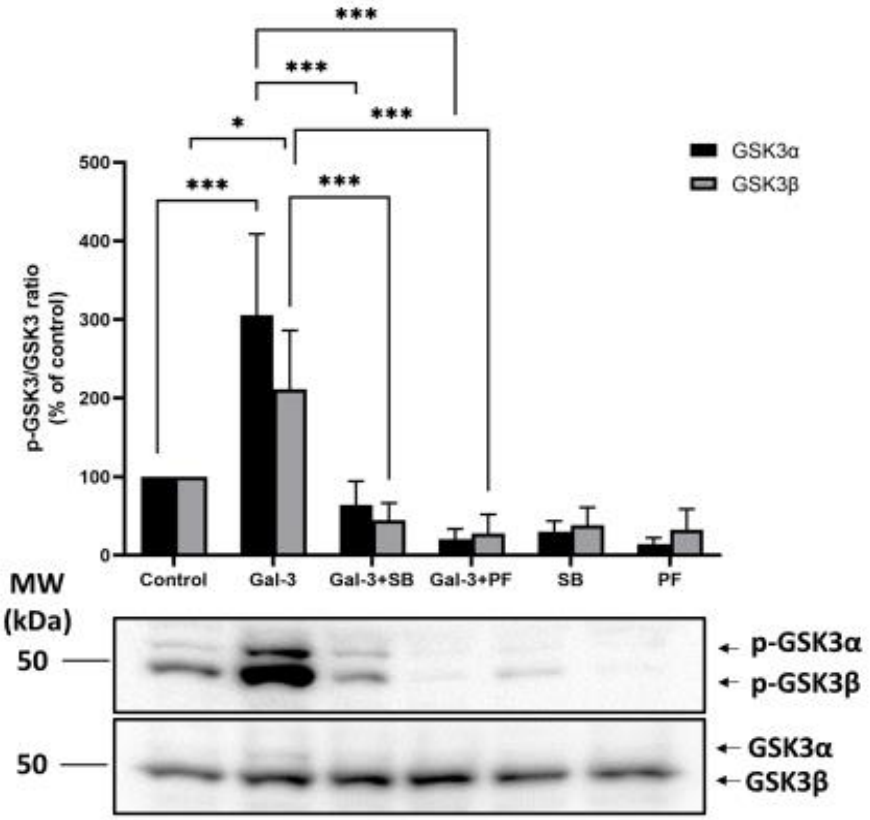

Figure 5.9. GSK3α/β expression was suppressed by PYK2 inhibitor and by GSK3α/β inhibitor. SW620 cells were treated with 10 µg/ml galectin-3 or BSA followed by introduction of GSK3α/β inhibitor SB 216763 (SB) or PKY2 inhibitor PF 431396 (PF) for 15 min and the levels of phosphorylated GSK3α/β were analysed by immunoblotting using antibodies against phosphor-GSK3α/β. The blots were the stripped and re-probed using antibodies against GSK3α/β. The band density was quantified and expressed as percentages of phosphor-GSK3α/β/ GSK3α/β ratio. Data are presented as mean ± SD of three independent experiments. *** $p < 0.001$, * $p < 0.05$ (ANOVA). ANOVA with Tukey test was performed on raw data.

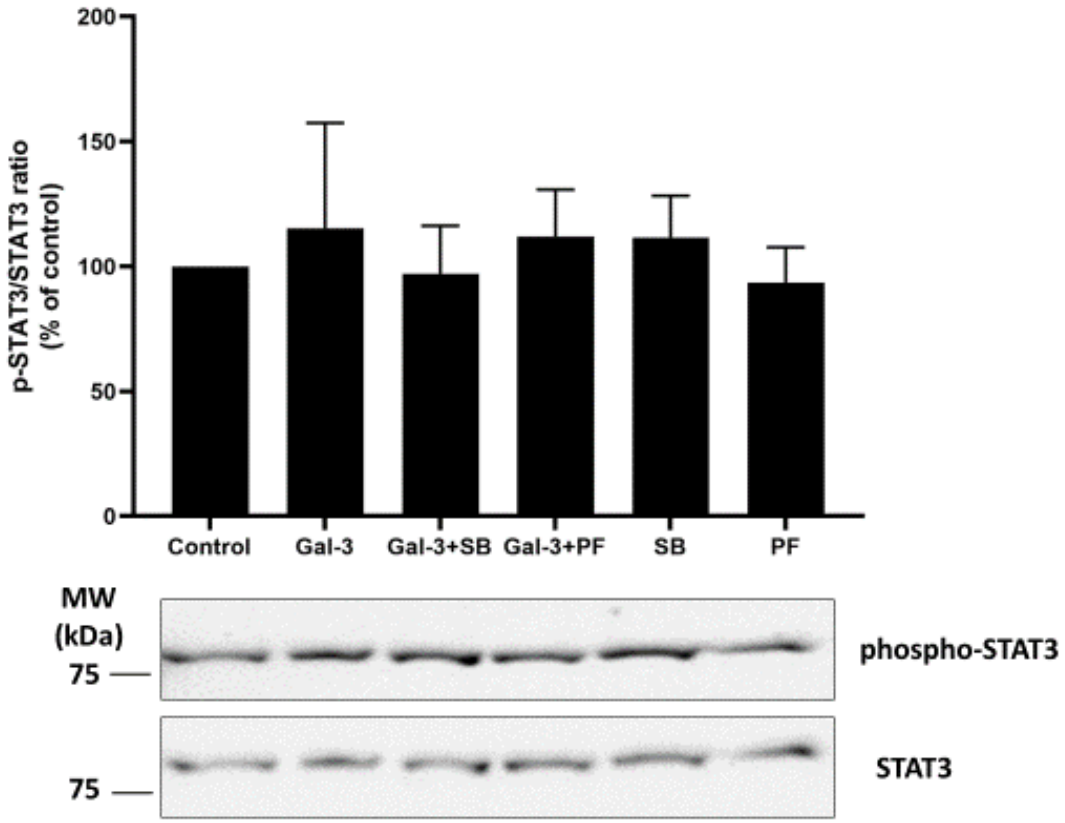

Figure 5.10. STAT3 expression was not affected by PYK2 inhibitor or GSK3α/β inhibitor. SW620 cells were treated with 10 µg/ml galectin-3 or BSA followed by introduction of GSK3α/β inhibitor SB 216763 (SB) or PKY2 inhibitor PF 431396 (PF) for 15 min and the levels of phosphorylated STAT3 were analysed by immunoblotting. The blots were the stripped and re-probed using antibodies against STAT3. The band density was quantified and expressed as percentages of phosphor-STAT3/STAT3 ratio. Data are presented as mean ± SD of three independent experiments. ANOVA with Tukey test was performed on raw data.

5.4.4 Galectin-3 mediates protease secretion through PYK2 and GSK3α/β signalling

To investigate whether phosphorylation of PYK2 and GSK3α/β were involved in galectin-3-mediated protease secretion, cathepsin B secretion in SW620 and HCT116 cells were analysed in the presence of the PYK2 inhibitor SB216763 or the GSK3α/β inhibitor PF431396, at different concentrations. The introduction of SB2 caused a dose-dependent decrease of cathepsin B secretion in SW620 cells (Fig 5.11), in which in comparison to the control, 6%, 11%, 15%, 24%, 42% and 42% reductions were seen in presence of 0.5, 1, 2.5, 5, 10 and 25 μM of SB2, respectively. The introduction of PF4 also caused a dose-dependent decrease in cathepsin B secretion in SW620 cells (Fig 5.11), where 8%, 13%, 19%, 25%, 41% and 44% of reductions were seen in the presence of 0.5, 1, 2.5, 5, 10 and 25 μM of PF4, respectively. In addition, the presence of SB2 or PF4 also led to a dose-dependent reduction of Cathepsin B secretion in HCT116 (Fig 5.12), where 0.5, 1, 2.5, 5,10 and 25 μM of SB2 caused 2%, 10%,14 %, 16%, 27% and 29% decreases of cathepsin B secretion, respectively, and 0.5, 1, 2.5, 5, 10 and 25 μM of PF4 caused 2%, 7%, 13%, 21%, 36% and 37% decreases of cathepsin B secretion, respectively.

We also analysed the secretion of MMP-13 in HCT116 cells in respond to SB2 or PF4 using ELISA (Fig 5.13). It was found that the secretion of MMP-13 was inhibited in the presence of 2.5, 5, 10 and 25 μM SB2 by 12%, 15% 20% and 21% and in the presence of 5, 10 and 25 μM PF4 by 14%, 19% and 21% respectively.

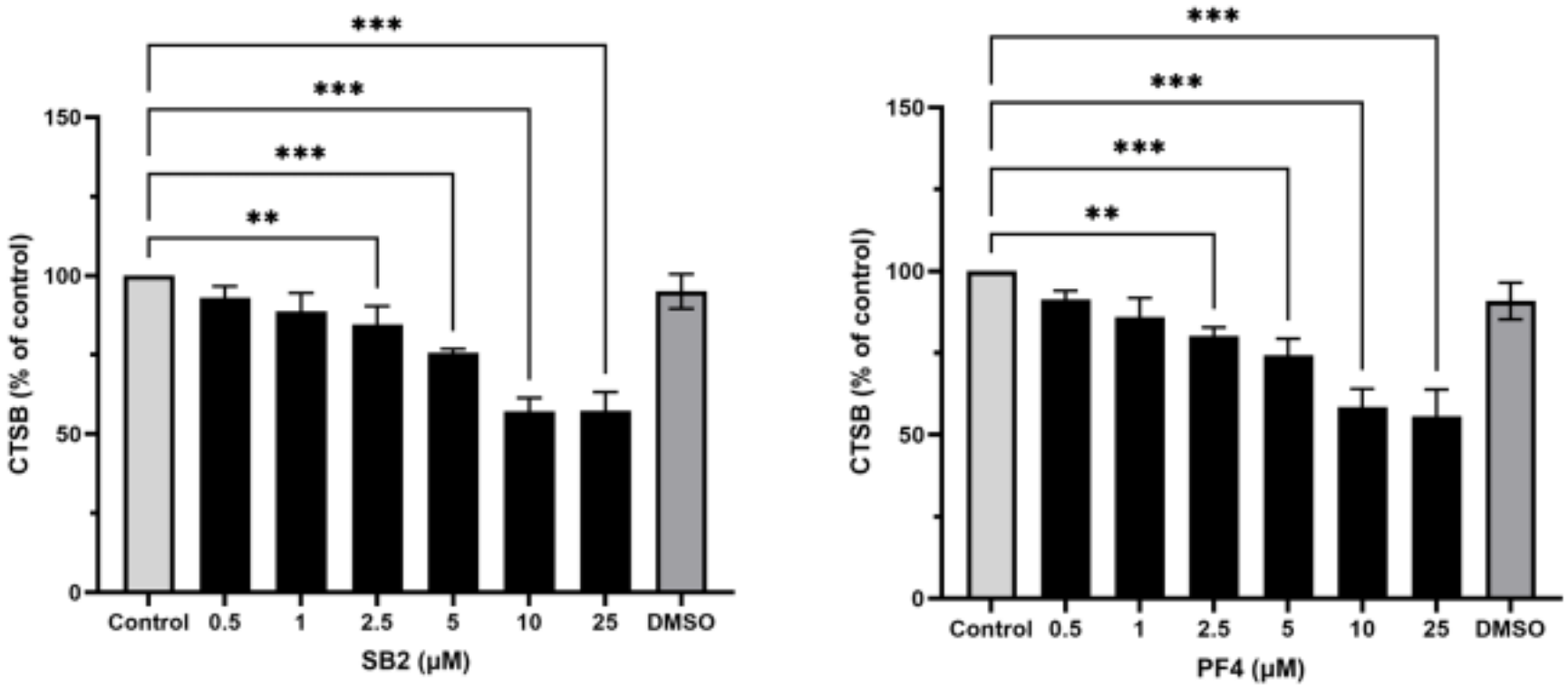

Figure 5.11 CTSB secretion was suppressed by SB2 and PF4 in SW620 cells in a dose dependent manner. SW620 cells were cultured in presence of SB2 or PF4 at 0.5, 1, 2.5, 5, 10 and 25 μM for 24 hours. The CTSB secretion was analysed by ELISA. Data are presented as mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$, ** $p < 0.01$ (ANOVA). ANOVA with Dunnett's test was performed on raw data.

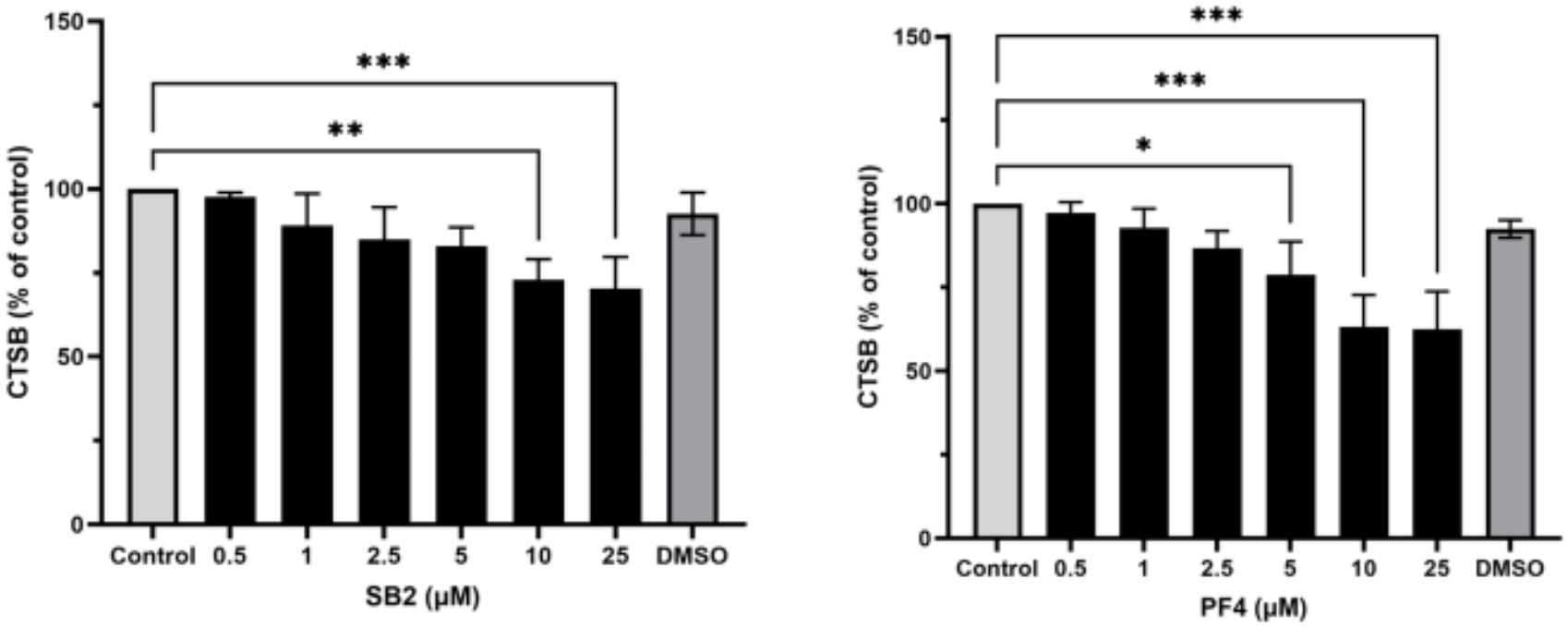

Figure 5.12. CTSB secretion was suppressed by SB2 and PF4 in HCT116 cells in a dose dependent manner. HCT116 cells were cultured in presence of SB2 or PF4 at 0.5, 1, 2.5, 5, 10 and 25 μM for 24 hours. The CTSB secretion was analysed by ELISA. Data are presented as mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$, * $p < 0.05$ (ANOVA). ANOVA with Dunnett's test was performed on raw data.

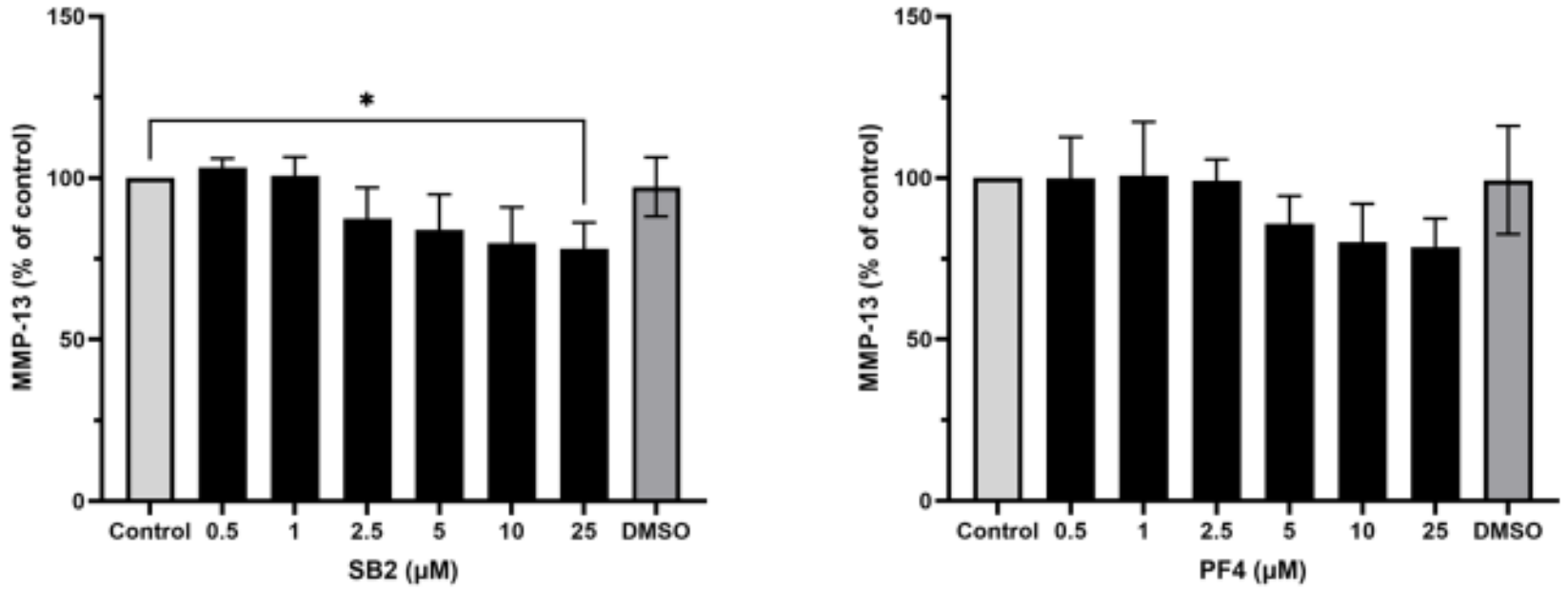

Figure 5.13. MMP-13 secretion was suppressed by SB2 and PF4 in HCT116 cells. HCT116 cells were cultured in presence of SB2 or PF4 at 0.5, 1, 2.5, 5, 10 and 25 μM for 24 hours. The MMP-13 secretion was analysed by ELISA. Data are presented as mean ± SD of three independent experiments, each in triplicate. * $p < 0.05$ (ANOVA). ANOVA with Dunnett's test was performed on raw data.

Subsequently, we investigated the effect of SB216763 or PF431396 on galectin-3-induced secretion of cathepsin. SW620 and HCT116 cells were analysed in the presence or absence of galectin-3, the GSK3α/β inhibitor SB216763 (10 μM) and the PYK2 inhibitor PF431396 (10 μM). The result showed galectin-3-induced secretion of cathepsin-B was inhibited up to 55% in the presence of SB2 and 57% in presence of PF4 in SW620 cells (Fig 5.14). Also, the presence of SB2 caused 18% reduction of galectin-3-induced secretion of cathepsin B while the presence of PF4 caused 45% reduction of galectin-3-induced secretion of cathepsin B in HCT116 cells (Fig 5.15). In addition, the secretion of MMP-13 induced by galectin-3 was also seen to be reduced by the presence of SB2 and PF4 in HCT116 cells, in which 10% and 12% of the galectin-3-induced increases were eliminated by SB2 and PF4, respectively (Fig 55.16).

These results suggest that PYK2-GSK3α/β activation is critically involved in galectin-3-mediated secretion of proteases, at least for cathepsin-B in those cells.

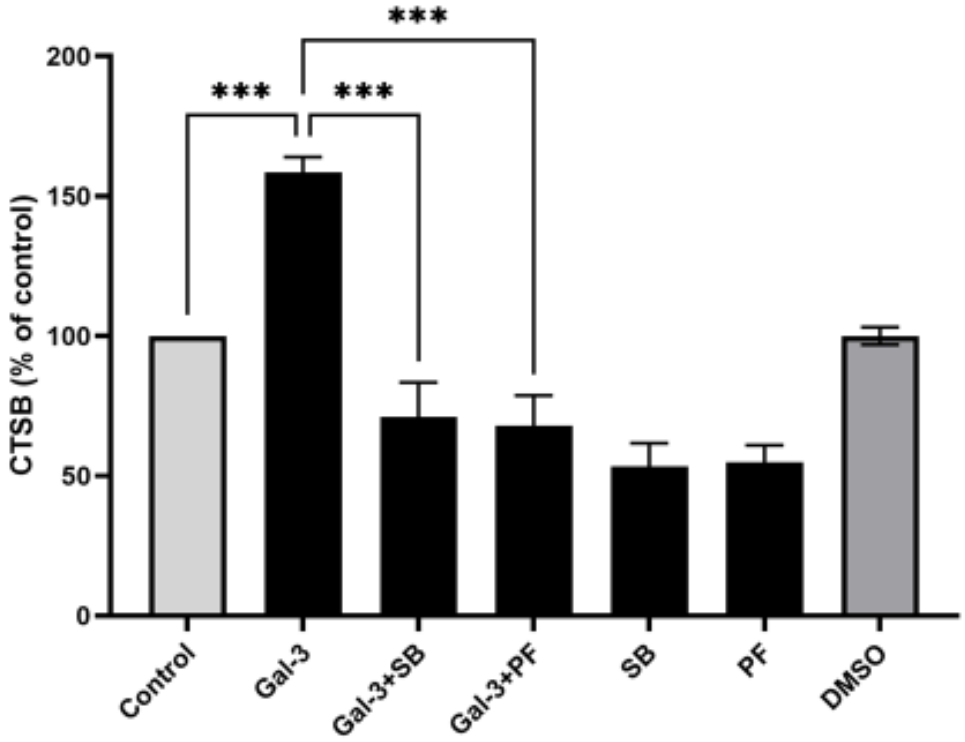

Figure 5.14. Galectin-3-induced cathepsin B secretion was inhibited by SB2 or PF4. SW620 cells were cultured in presence or absence of galectin-3, or SB2, or PF4 for overnight. The CTSB secretion was analysed by ELISA. Data are presented as mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$, (ANOVA). ANOVA with Tukey test was performed on raw data.

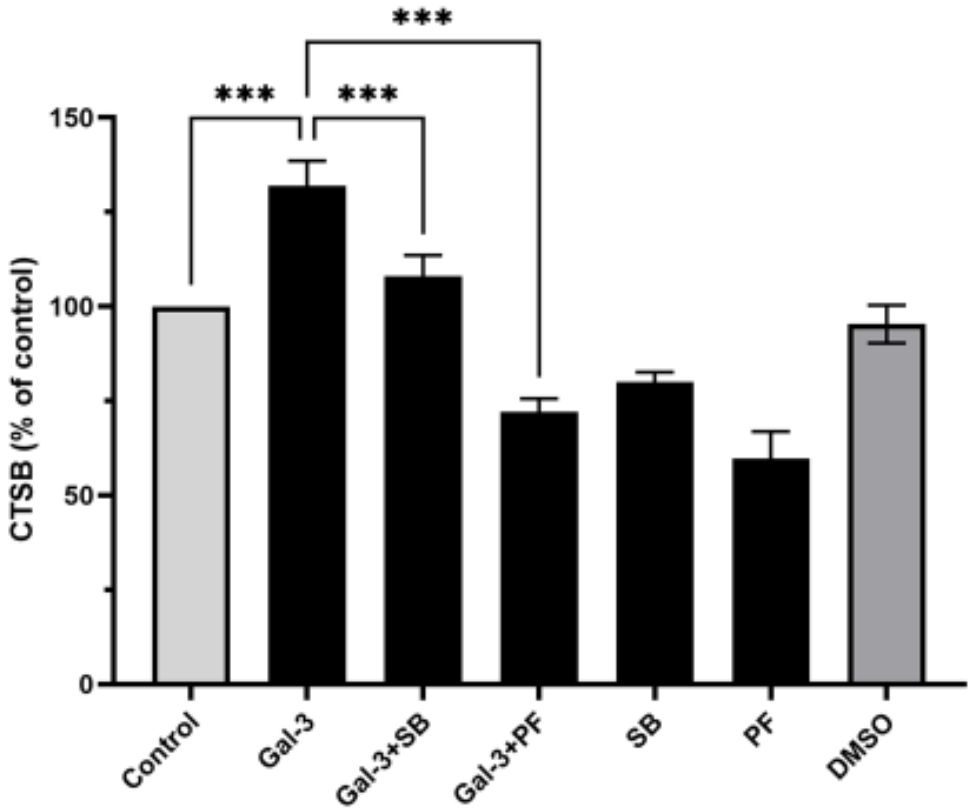

Figure 5.15. Galectin-3-induced cathepsin B secretion was inhibited by SB2 or PF4 in HCT116 cells. HCT116 cells were cultured in presence or absence of galectin-3, or SB2, or PF4 for overnight. The CTSB secretion was analysed by ELISA. Data are presented as mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$, (ANOVA). ANOVA with Tukey test was performed on raw data.

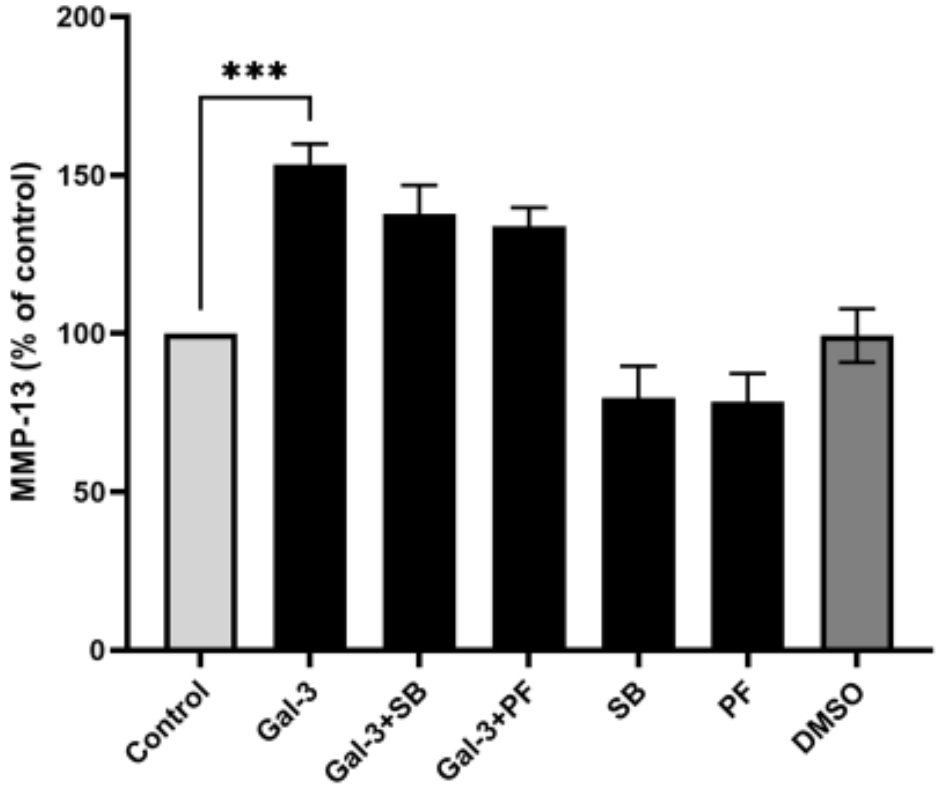

Figure 5.16. Galectin-3-induced MMP-13 secretion was inhibited by SB2 or PF4 in HCT116 cells. HCT116 cells were cultured in presence or absence of galectin-3, or SB2, or PF4 for overnight. The MMP-13 secretion was analysed by ELISA. Data are presented as mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$, (ANOVA). ANOVA with Tukey test was performed on raw data.

5.5 Summary of the results

1) Galectin-3 induced phosphorylation of several kinases in human colon cancer SW620 cells in which PYK2, GSK3, STAT1 and STAT3 showed greater than 50% changes

2) Galectin-3 activated PYK2, GSK3 and STAT1 in a time-dependent way

3) PYK2 was likely to be upstream of GSK3 in galectin-3-induced signalling pathways

4) Galectin-3 mediated cathepsin B secretion through PYK2 and GSK3α/β signalling

5.6 Discussion

Phosphorylation of several signalling molecules including PYK2 and GSK3α/β has been shown in this study to be involved in galectin-3-induced protease secretion.

The presence of galectin-3 showed to increase the phosphorylation of PYK2, GSK3α/β and STAT1. Early studies have shown that PYK2 activation can phosphorylate GSK3α/β during lysophosphatidic acid-induced neurite retraction in neuroblastoma cell line [533, 534], which suggest that PYK2 is an upstream regulator of GSK3α/β signalling. Also, Gao et al reported that PYK2 can interact directly with GSK3β in SW480 cells in GSK3 phosphorylation [535]. These findings are in line with our discovery of increased phosphorylation of PYK2 and GSK3α/β by galectin-3 in SW620 cells. There is also evidence in the literature showing phosphorylation of PYK2 activates STAT1 in mouse embryonic fibroblasts [536], which is also in line with our finding of increased phosphorylation of both PYK2 and STAT1 in response to galectin-3. The use of SB 216763 and PF-431396 in this project was aimed to determine the involvement of PYK2 and GSK3α/β in galectin-3-mediated cell signalling and protease secretion. These two kinase inhibitors are not exclusively specific to PYK2 and GSK3α/β and their presence at >10 μM concentrations has been reported to inhibit other serine/threonine kinases. In our experiment, the presence of SB 216763 and PF 431396 at 10 μM led to 62% and 27% reduction of GSK3 and PYK2 phosphorylation, respectively, suggesting the IC50 of SB216763 on GSK3α/β phosphorylation in SW620 cell line is below 10 μM, and the IC50 of PF 431396 on PYK2 phosphorylation in SW620 cell line is probably higher than 10 μM. At 10 μM, PF 431396 and SB216763 showed to inhibit PYK2 and GSK3α/β phosphorylation induced by galectin-3, respectively, and subsequent secretion of proteases. Although it cannot be exclude some of the inhibitors of these inhibitors may act through inhibition of other kinases,

their effect on reduction of galectin-3-mediated protein secretion supports the involvements of PYK2 and GSK3 in their actions.

The proposed signalling of galectin-3-induced cathepsin B secretion through phosphorylation of PYK2 and GSK3 is shown in Fig 5.17.

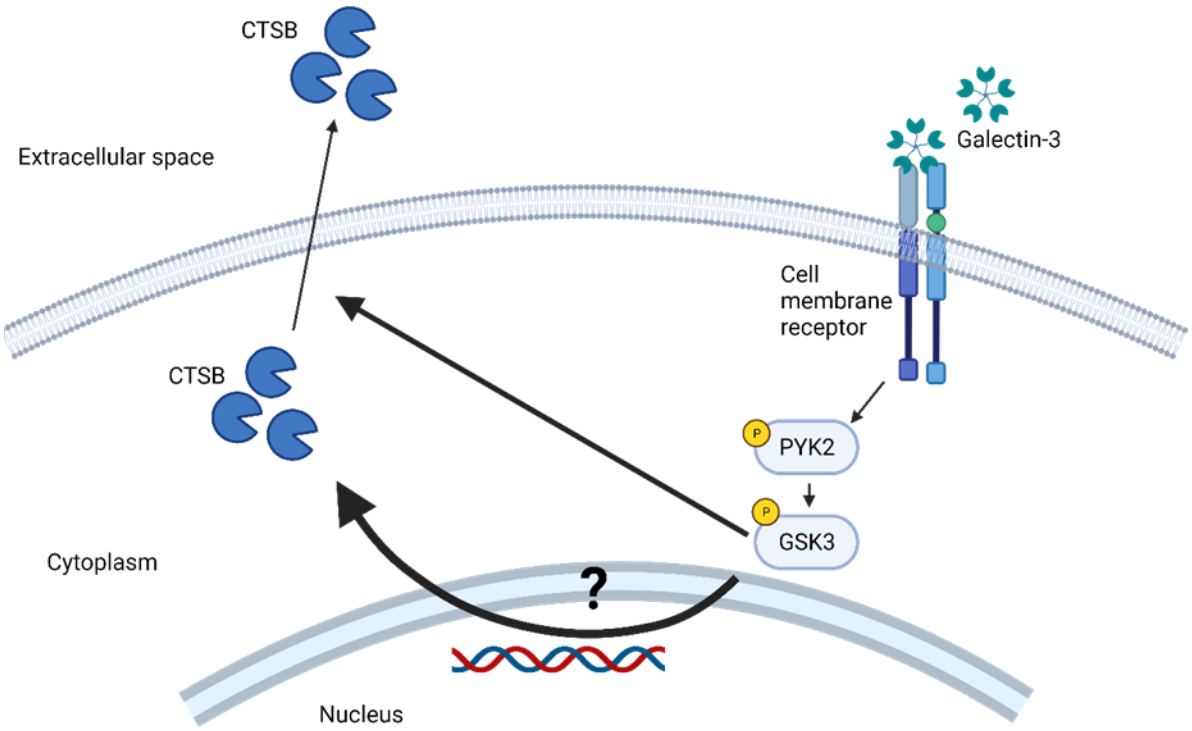

Fig 5.17. Proposed action of galectin-3 in cell signalling in protease secretion in colon cancer cells. The extracellular galectin-3 binds to its receptor on the cell surface and induces the phosphorylation of PYK2, and subsequent GSK3 phosphorylation. The phosphorylation of GSK3 leads to the increased secretion of Cathepsin B (CTSB), either directly or indirectly through activation of transcription factors which lead to expression of Cathepsin B and its secretion.

PYK2 and its close relative FAK are non-receptor tyrosine kinases that mediate cell adhesion signalling and involved in cell proliferation, migration and survival [537], and they are closely associated with cancer progression, especially tumour invasion [538].

Activation of PYK2 has been reported to be associated with cancer metastasis in cancers, including breast, liver, colon cancer, and glioma [535, 539-541]. It was reported that non-metastatic hepatocellular carcinoma with upregulated PYK2 expression increased EMT characteristics compared to control, which involved increased formation of membrane ruffle and the downregulation of E-cadherin and cytokeratin [542]. On the other hand, PYK2 knockdown induced breast cancer cells to transform into epithelial-like cells with increased E-cadherin expression [543]. The link between PYK2 activation and E-cadherin downregulation in those studies is probably in keeping with our discovery in this study, in which PYK2 activation can induce downstream cathepsin B secretion that cleaves E-cadherin. PYK2 activation has also been reported previously to regulate cathepsin B secretion from human primary macrophages during the immune response [544].

PYK2 has been reported to be upregulated in colon adenocarcinoma compared with normal colon tissues at both RNA and protein levels, and promote tumour proliferation and invasion [545]. Upregulation of PYK2 has been shown to promote the migration of ovarian cancer, glioma cells and breast cancer cells [546]. Chemokine ligand 18 (CCL18), a breast cancer cell migration stimulatory factor, has been suggested to participate in the activation of PYK2 in ovarian cancer cells [547]. Subsequent study showed that CCL18-induced increases in breast cancer cell migration were critically involved in the activation of PYK2, whereas the increase was inhibited by siRNA-PYK2 [547]. Activation of PYK2 in breast cancer cells was shown to increase PYK2 complex formation with p190 RhoGAP (p190), RasGAP, ErbB-2, and Src, leading to activation of the MAPK signalling and increase of cancer cell invasion. Inhibition of PYK2 expression by siRNA decreased the invasion of A549 lung cancer cells, and the downregulation of tropomyosin-related kinase B reduced PYK2 phosphorylation and

subsequently decreased A549 cell migration [543]. The knockdown of PYK2 decreased breast cancer cell migration through the down-regulation of MMP-9 [543]. The expression level and roles of PYK2 in human cancer are shown in table 5.3.

Table 5.3. The expression and roles of Pyk2 in human cancer.

Cancer type	Incidence	Expression in tissues	Downstream targets	Reference
Colon cancer	57.6% of colon cancer tissue samples shows PYK2 overexpression	Overexpressed	AKT, GSK3	[535, 545]
Breast cancer	79.3% of breast cancer tissue samples shows PYK2 overexpression	Overexpressed	β-catenin, β1 integrin, E-cadherin, ERK, STAT3, CD44, fibronectin, MMP, EGFR, Akt, S6K, p130 Cas, AMAP1, c-Met, NDRG1, Snail-1,2, Twist-1,2, Zeb-1,2, HER3, ZO-1, Arg, Src	[538, 548-554]
Glioma	77.4% of Astrocytomas and 84.1% glioblastomas show high grade PYK2 expression	Overexpressed	ERK, c-Met, Rac1	[555-559]
Lung cancer	54.7% of NSCLC tissues sample shows high grade PYK2 expression	Overexpressed	ALDH1a1, ERK, ABCG2, Src, Bmi-1	[560-563]
Leukaemia	81% of AML shows PYK2 expression	Overexpressed	No data	[564]
Multiple myeloma	No data	Overexpressed	β-catenin, STAT3, Akt, Paxillin, Src	[565, 566]
Intestinal cancer	No data	Overexpressed	GSK3β	[535]
Pancreatic cancer	No data	Expressed	No data	[567, 568]
Prostate cancer	32.4% of prostate cancer tissues shows PYK2 overexpression	Expressed	MAPK, FAK, ERK, S6K, Akt	[569-573]
Ovarian cancer	82.1% of the later staged ovarian cancer shows expression of p-PYK2	Overexpressed	ERK	[547, 574, 575]
Liver cancer	59% of hepatocellular carcinoma shows high grade PYK2 expression	Overexpressed	E-cadherin, fibronectin, N-cadherin, ERK, STAT5b, cytokeratin, Hic-5, MEK1/2, Src, Akt	[540, 576-579]

GSK3 is known to either act as a tumour suppressor or a tumour promoter. The phosphorylation of GSK3 at serine 9/21 has been reported to inactivate GSK3 [580]. It was reported that levels of GSK-3 expression are higher in colorectal cancer cells, compared to normal cell lines in *vitro* [581]. Activation of WNT signalling is an early event in nearly all colon cancers [582] and inhibition of GSK-3β activities can promote WNT pathway activation [583]. Furthermore, activation of WNT pathway in CRC is often through mutation of the tumour suppressor APC [584] and mutated APC has been shown previously to increase transcription of cathepsin B [443]. These findings are in line with our discovery of galectin-3-induced phosphorylation of GSK3 in association with increased cathepsin B secretion. GSK-3 is overexpressed in various other cancers such as pancreatic, ovarian, and liver cancer [585]. GSK-3β downregulation restrained the tumour growth and reduced angiogenesis and VEGF expression in pancreatic cancer [586]. GSK-3 is seen to increase production of Pax3 transcription factor, thus increase human melanoma cell survival and associated tumour growth [587]. GSK-3β was also found to regulate the crosstalk between WNT/β-catenin pathways and PI3K/AKT, which subsequently mediates tumour growth in cholangiocarcinoma [588]. In addition, serine 21/9 phosphorylation of GSK-3 α/β was seen to be highly activated in cholangiocarcinoma tissues in comparison to normal biliary tissues. GSK-3 mediated prostaglandin E2 (PGE2) expression is found to promote cholangiocarcinoma cell invasion and proliferation [589]. Moreover, GSK3 activation is reported to increase proliferation and survival of ovarian cancer cells [590] and administration of GSK3β inhibitors suppressed cell proliferation and prevented tumour formation in mice [591, 592].

It has been shown that silencing of galectin-3 in human osteosarcoma cells caused decreased activation and expression of FAK, which results in repressed osteosarcoma

cell migration and invasion [524, 593]. It was also found that galectin-3 binds to Mgat5-modified N-glycans and functions together with phosphorylated caveolin-1 (pY14Cav1) to stabilize FAK within focal adhesions (FA), and therefore promotes FA turnover and disassembly [593], in which pY14Cav1 alone is not sufficient for the task. Activation of PYK2 is usually following the integrin-mediated adhesion to ECM [594], and galectin-3 plays an important role in cancer cell adhesion to ECM [463]. Moreover, the phosphorylation of GSK3 is closely related to activation of PI3K and Akt pathways in cancer progression [595], whose activities are reported to be enhanced by galectin-3.

Taken together, activation of PYK2/GSK3 signalling in cancer cells by galectin-3 may itself influence cancer progression in addition to its induction of proteases.

Chapter 6

General discussion and future research

6.1 Key findings

- Galectin-3 induced protease secretion in colon cancer cells in both time- and dose- dependent manners
- Galectin-3 expression and secretion were associated with proteases secretion in cancer cells
- Galectin-3 mediated protease secretion predominately through its extracellular action
- Galectin-3-mediated protease secretion promotes cancer cell invasion
- Galectin-3-mediated protease secretion disrupts cancer monolayer integrity and cause cell monolayer leakage
- Galectin-3 mediates protease secretion through PYK2 and GSK3α/β signalling

6.2 Discussion

This study found that galectin-3 induces secretion of a number of proteases in colon cancer cells in an autocrine and paracrine way. Higher galectin-3 expression and secretion by cancer cells is associated with higher protease secretion. It was also found that this galectin-3-induced secretion of proteases directly promotes colon cancer cell invasion, disrupts epithelial integrity and causes leakage of the epithelial monolayer. The underlying mechanism of galectin-3 inducing protease secretion is likely via activation of PYK2/GSK3 signalling, at least for cathepsin B in this study.

It was already known that both galectin-3 and proteases are closely associated with cancer progression especially assisting with tumour escape from the primary site, in which galectin-3 participates in many signal transductions of cell-cell adhesion and cell-matrix interaction, while proteases primarily are responsible for ECM degradation and disrupting epithelium integrity. Most importantly, previous studies have shown galectin-3 is overexpressed in colon cancer and is correlated with the colorectal cancer development and poor prognosis [363]. Some of the proteases such as cathepsins were also reported to be upregulated in intestinal mucosa in CRC patients compared to normal individuals and is associated with overall mortality in CRC patients [596] In our study, galectin-3 induced secretion of several cathepsins (cathepsin A, B and X) and MMPs (MMP-1, -12 and -13) in human colon cancer cells.

Cathepsin B is reportedly upregulated in CRC tissue compared to normal tissues and is significantly associated with higher risk of colon cancer-specific and overall mortality in CRC patients [597]. Also, the mRNA level, antigen level, protein expression and proteolytic activity of cathepsin B are all higher in colorectal

carcinoma (CRC) tissues in comparison to control colorectal mucosa [598]. Epithelial cells in normal colorectal mucosa show negative immunohistochemical staining for cathepsins B [599]. Higher cathepsin B concentrations were observed during the transition from adenoma to adenocarcinoma, implying that cathepsins may be a marker of colorectal carcinoma. Interestingly, it was found that cathepsin B localization is cytoplasmic and perinuclear in the normal colonic epithelium, whereas the localization of cathepsin B shifts to the periphery (to the basal surface of plasma membrane next to the basement membrane) during the transition from adenoma to early carcinoma [598, 600], suggesting that increased secretion occurs at the later stages of colorectal carcinogenesis. Concordantly, it was found that cathepsin B is highly expressed at the invasive front of colorectal carcinoma [441, 442]. In addition, cathepsin B overexpression is observably associated with increased vascular density in colorectal tumours, suggesting that it plays a role in neoangiogenesis [601, 602]. Cathepsin B was also prominent extracellularly at the interface between the invading tumour and normal tissue [603].

The expression and secretion of MMPs are also seen to be elevated in colorectal cancer.MMP-1 and MMP-13 are overexpressed in colorectal carcinomas and are related to poor prognosis in colorectal patients [451, 452], in which MMP-13 has been associated with poor prognosis in patients with colorectal cancer metastasis to the liver [453]. It has been reported that the expression of MMP-1 was associated with later stages of colon cancer and poor prognosis [605], and it was also correlated to tumour invasiveness, lymph node involvement and metastasis [606]. It was also shown that MMP-1 expression is higher in primary cancers with lymph node involvement than metachronous metastases [607]. Moreover, MMP-13 is considered as a potent marker for colorectal carcinoma, as patients with MMP-13 expression had

a nearly 8-fold increased risk of post-operative relapse compared to those without MMP-13 expression [452].

It is well known that proteases play a crucial role in promoting tumour invasion and metastasis due to their ability to digest the physical barriers that keep cells from invading and spreading. In the present study, galectin-3-induced protease secretion resulted in promotion on colorectal cancer cell invasion. Cathepsins and MMPs are both responsible for degradation of ECM, thus promoting cancer cell invasion. In CRC, extracellular galectin-3 was also reported to increase colon cancer cell migration via activation of EGFR [378]. Some other studies have showed that the galectin-3 overexpression leads to activation of the K-Ras-Raf-Erk1/2 pathway, thus promoting the migration of colon cancer cells [379]. Cathepsin B silencing by RNAi in human colon cancer cells decreased their invasion capacity, tumoral expansion, and metastatic spread in immunodeficient mice [443]. Upregulation of MMP-13 expression by CXCR5 was shown to promote the invasion of colon cancer cells via PI3K/AKT pathway [455]. The increased secretion of these proteases by galectin-3 in the tumour microenvironment therefore supports tumour cell invasion through the basement in tumour cell spreading.

In the process of cancer cell spread to distant organ site, disrupting epithelium integrity is critical for cancer cells to acquire mesenchymal morphology. Normal epithelial cells are laid in an organized sheet to form a monolayer or multilayer in which cells are connected by adhesion molecules and junctional structures. During tumour development, EMT occurs, which results in cells gradually losing epithelial morphology and the monolayer or multilayer being disrupted. There are many factors contributing to this transition including proteases such as cathepsins and MMP-13, which are responsible for cleaving adhesion molecules like E-cadherin and degrading

components of basement membranes. In this study, primary tumour-derived SW480 cells secreted much less cathepsins and MMPs than the metastasis-derived SW620 cells. E-cadherin is a key cell adhesion molecule for maintaining tight cell-cell junctions in the epithelium and has been reported to be proteolytically cleaved by cathepsins [472]. Cathepsin B can bind to the annexin II heterotetramer (AIIt) and activates other proteases such as matrix metalloproteinases and urokinase plasminogen activator (uPA) and indirectly disrupts epithelial cell-cell contact and monolayer integrity [505-507]. On the other hand, both cathepsins and MMPs were seen to be associated with degradation of basement membrane. An immunohistochemical study has revealed a close relationship between cathepsin B and degradation of basement membrane [608]. MMP-13 was also seen to cleave lamininin-5 which is the main component of basement membrane, and this stimulates tumour cell invasion [483]. The increased cancer cell secretion of cathepsin-B and MMP-13 by galectin-3 can therefore decrease colon cancer cell epithelial integrity and aid tumour cell break up at primary tumour sites.

In this study, it was found that galectin-3 induced cathepsin B secretion through activation of PYK2/GSK3 signalling in SW620 and HCT116 human colon cancer cells. PYK2 expression has been reported to be positively associated with colon adenocarcinoma [545]. Inhibition on the SPON2/integrin β1/PYK2 cascade disrupts the trans-endothelial migration of monocytes and the cancer-promoting functions of TAMs *in vivo* in CRC [609]. Moreover, PYK2 was reported to regulate the proliferation of SW480 cells [545]. It has been shown that a high level of GSK3α is associated with poor prognosis of colon cancer patients [610]. GSK3β is required for maintaining mitosis through interacting with translocated promoter region (TPR)-dynein in colorectal cancer cells [611]. Galectin-3 can activate several signal transduction

pathways in cancer including EGFR, Hedgehog, Notch, TGF-β and Wnt pathways [612]. EGFR activation has been reported to activate PYK2 in breast cancer [613], while GSK3 is an important mediator of the Wnt/β-catenin pathway in colon tumorigenesis [581]. These findings are in line with the discovery in this study of galectin-3 induction of PYK2/GSK signalling in colon cancer cells.

It is known that the catalytic activity of cathepsin B is pH-dependent [529, 530], and the secretion of active cathepsin B can be induced by acidic conditions [531]. Interestingly, PYK2 has been reported to be also closely associated with pH alteration. A previous study has shown that PYK2 was a pH sensor and activator in the human kidney [614]. The activation of PYK2 is seen to occur faster in an acidic medium than pH neutral medium [614]. PYK2 activation induced NHE3 expression followed by releasing H+ to acidify the luminal side of the renal epithelial cells [614]. These findings suggested that PYK2 induces the acidification of extracellular pH. Thus, galectin-3-induced activation of PYK2/GSK3 can favourably create an acidic environment to induce cathepsin B secretion.

A proposed model of galectin-3 actions in promoting protease secretion are shown in Fig 6.1.

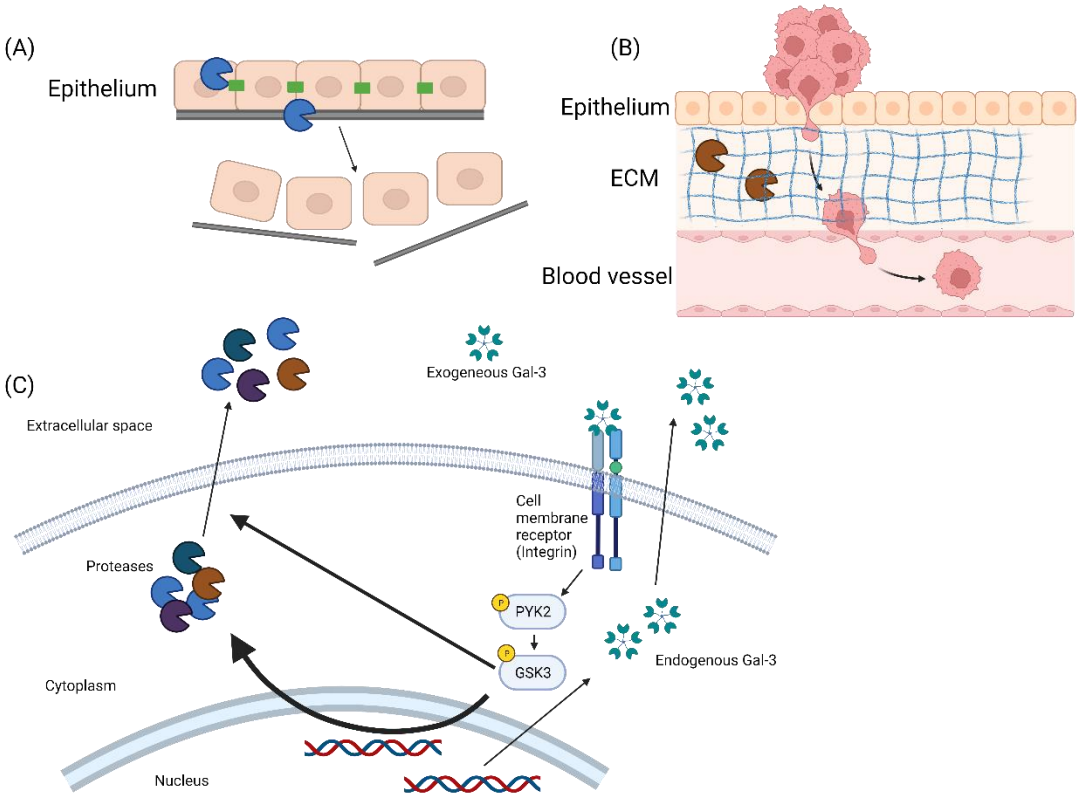

Fig 6.1. Proposed galectin-3 actions in protease secretion and subsequent effect on cancer cell invasion. (A) The galectin-3-induced secretion of the proteases to the extracellular space disrupts epithelium integrity, (B) that leads to increased tumour cell invasion through the basement matrix. (C) Galectin-3 induce protease secretion in a paracrine/autocrine manner. The secreted galectin-3 binds to its cell surface receptor and induces PYK2/GSK3 signalling to increase the secretion of proteases.

Implications for future research

Although this study has revealed that galectin-3-induced protease secretion promoted colon cancer invasion and disrupted epithelium integrity, there are still few aspects that need to be further explored. First, it was found that galectin-3 induced the secretion of multiple proteases, thus there is more to add in terms of the effect of increased protease secretion on cancer progression. The consequences of galectin-3-induced protease secretion could be more than promoting cell invasion due to various roles that proteases play in regulating cancer progression such as enhancing angiogenesis. Second, galectin-3 was seen to act extracellularly to induce protease secretion, and it leads to downstream activation of PYK2/GSK3. The cell surface activities involving galectin-3 binding partners and possible receptors have not been identified in this study. These could represent cell-type specific binding partners in colon cancer. Third, the cell lines we used in this study are only from colon cancer patients, therefore, the effect of galectin-3 on protease secretion can be tested in other cancer types such as breast cancer or melanoma. In addition, future research can be also addressed at using animal models to examine the effect of galectin-3-induced protease secretion on cancer metastasis *in vivo*.

Targeting galectin-3 and downstream PYK2 and GSK3 also represent potential therapeutic targets against metastatic tumour. Galectin-3 has already attracted a great number of interests in the field of cancer research and potential drug development. The use of galectin-3 inhibitors in this study was shown to decrease protease secretion and its associated cell invasion. In addition, the inhibitions of PYK2 and GSK3 also reduced galectin-3-mediated protease secretion. Therefore, combining targeting PYK2 and GSK3 with anti-galectin-3 therapy may provide a promising way to

therapeutically combat tumour metastasis in the future. These inhibitors including modified heparins are currently under development as a potent method to target elevated galectin-3 concentrations in the circulation of humans. Earlier this year, a new PYK2-derived peptide was found to sufficiently inhibit breast cancer metastasis [615]. The synthesized peptide successfully competed with the natural PYK2 protein to interact with cortactin and subsequently inhibited the formation of the invadopodia and metastasis *in vivo*. Our research explored the importance of targeting PYK2 in colon cancer in terms of forming invadopodia due to the crucial role of proteases plays in promoting cancer invasion.

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Appendix

Research output

Data presented in this thesis has been published in the following publication

Li S, Pritchard DM, Yu LG. Galectin-3 promotes secretion of proteases that decrease epithelium integrity in human colon cancer cells. Cell Death Dis. 2023 Apr 13;14(4):268. doi: 10.1038/s41419-023-05789-x. PMID: 37055381; PMCID: PMC10102123.

Li S, Pritchard DM, Yu LG. Regulation and Function of Matrix Metalloproteinase-13 in Cancer Progression and Metastasis. Cancers (Basel). 2022 Jul 3;14(13):3263. doi: 10.3390/cancers14133263. PMID: 35805035; PMCID: PMC9265061.