PANCREATIC STELLATE CELL SECRETION AND INTERLEUKIN-6 REGULATE PANCREATIC CANCER CELL PROLIFERATION AND EPITHELIAL-MESENCHYMAL TRANSITION THROUGH NUCLEAR FACTOR ERYTHROID-2

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a desmoplastic reaction mediated primarily by pancreatic stellate cells (PSC). However, the mechanisms by which PSC promote PDAC cell proliferation and motility are still unclear. Nuclear factor erythroid 2 (Nrf2), highly expressed in PDAC cells, is a transcription factor responsible for maintaining redox homeostasis. It was reported to regulate metabolic reprogramming recently and induces epithelial-mesenchymal transition (EMT) to promote tumor metastasis. The present study examined whether PSC secretory factors activate metabolic reprogramming to promote cell proliferation, and EMT via intracellular Nrf2 signaling. PSC-conditioned medium (PSC-CM) increased PDAC cell proliferation and elevated Nrf2 expression, enhancing Nrf2-regulated antioxidant genes expression through greater DNA binding. *NRF2* downregulation reduced PSC-mediated PDAC cell proliferation whereas overexpression of *NRF2* activity significantly increased, with PSC-CM treatment further enhanced this effect. These data strongly suggest that Nrf2 activity is required for PSC-mediated PDAC cell proliferation. PSC treatment also enhanced PDAC metabolic genes expression related to pentose phosphate pathway (PPP), glutaminolysis, and glutathione biosynthesis. This led to increased levels of ribose 5-phosphate (R5P), inosine 5'-monophosphate (IMP), glutamate, and malate metabolites in PSC-CM treated cells. Abrogation by G6PD inhibition indicated that PSC activates PPP to promote PDAC cell proliferation. Identification of PSC secretory factors that mediate these phenotypes showed that GRO-α was the most abundant cytokine, followed by IL-6 and SDF-1α. Only recombinant protein IL-6 and SDF-1α significantly induced PDAC cell proliferation (~150%), and upregulated *NRF2* and its target genes (*AKR1C1* and *NQO1*). IL-6 neutralization most strongly reduced cell proliferation (~50%) compared to SDF-1α. These indicated that IL-6 and SDF-1α secreted from PSC mediate PDAC cell proliferation via Nrf2 signaling activation. It was reported that IL-6 is important for

PDAC progression. Hence, the expression of *IL6* and its receptor (*IL6R*) were determined in PSC and PDAC cells (AsPC-1, BxPC-3, and Panc-1). Panc-1 cells were used to study IL-6 signaling in PSC-PDAC interaction because Panc-1 expressed the lowest *IL6* and highest *IL6R* levels. IL-6 neutralization reduced Panc-1 cell proliferation and Nrf2-induced metabolic genes. IL-6 neutralization caused PSC-induced mesenchymal to epithelial morphologic transition, and reduced the migration and invasion capacity; these were restored by tBHQ. Concurrently, upregulation of the mRNA levels was observed for *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *SNAIL*, *SLAUG*, and *TWIST2* genes, but not for epithelial marker *CDH1* encoding E-cadherin. *NRF2* mRNA was upregulated in IL-6-treated PDAC cells, indicating that Nrf2 mediates PSC-induced EMT and metabolic genes via Nrf2. Furthermore, inhibition of Stat3 signaling upregulated *E-Cadherin* while downregulated *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *SNAIL*, *SLUG*, and *TWIST2*, *NRF2* and Nrf2 target genes (*AKR1C1* and *NQO1*). Stat3 inhibition further suppressed Nrf2-mediated EMT-related gene expression. Therefore, PSC-secreted IL-6 promotes PDAC cell proliferation via Nrf2-mediated metabolic reprogramming, and induces EMT via Stat3/Nrf2 signaling. Targeting activated Stat3/Nrf2 pathways downstream of IL-6 might provide a novel therapeutic option to improve the prognosis of patients with PDAC.

ABSTRAK

Adenokarsinoma salur pancreas (PDAC) dicirikan dengan reaksi desmoplastic yang disumbang terutamanya oleh sel-sel bintang pankreas (PSC). Tetapi, mekanisme PSC mempengaruhi perkembangan percambahan sel dan pergerakkan PDAC masih tidak difahami. Faktor nuklear erythroid 2 (Nrf2) adalah pengawalatur utama bagi mengekalkan homeostasis redoks and didapati dengan kuantiti yang tinggi dalam sel-sel PDAC. Ia mengawalatur gen yang terlibat dalam reprogram metabolik dan berupaya mencetus peralihan mesenkimia epithelial (EMT) untuk menggalakkan pergerakan tumor. Penyelidikan ini mengkaji kebolehan PSC mengaktifkan reprogram metabolik untuk percambahan sel PDAC, dan EMT melalui isyarat intrasel Nrf2. Media terawat PSC (PSC-CM) menyebabkan peningkatan percambahan sel-sel PDAC dan ekspresi Nrf2. Ini menyebabkan peningkatan ekspresi gen antioksidan Nrf2 melalui peningkatan aktiviti pengikatan DNA. Kurangan ekspresi gen *NRF2* mengurangkan percambahan sel PDAC yang dicetuskan oleh PSC. Manakala, lebihan ekspresi gen *NRF2* meningkatkan percambahan sel PDAC. Ini menunjukkan kepentingan aktiviti Nrf2 dalam percambahan sel PDAC yang didorong oleh PSC. Rawatan PSC-CM juga meningkatkan ekpresi gen-gen metabolik yang terlibat dalam laluan pentosa fosfat (PPP), glutaminolisis, dan biosintesis glutation. Ini menyebabkan peningkatan metabolit ribose 5-phosphate (R5P), inosine 5'-monophosphate (IMP), glutamate, dan malate selepas rawatan PSC-CM. Perencatan G6PD didapati menghalang percambahan sel PDAC yang didorong oleh PSC-CM. Ini menunjukkan kepentingan PPP dalam percambahan sel PDAC. Pengenalpastian factor-faktor penting rembesan PSC yang berpotensi meningkatkan percambahan sel PDAC melalui isyarat Nrf2 menunjukkan bahawa GRO-α dijumpai dengan kuantiti yang tertinggi, diikuti oleh IL-6 dan SDF-1α. Cuma IL-6 dan SDF-1α menggalakkan percambahan sel PDAC (~150%) selepas dirawat dengan rekombinan protein masing-masing. Tambahan pula, tahap ekspresi *NRF2* and gen sasarannya

(*AKR1C1* and *NQO1*) juga dikurangkan. Peneutralan IL-6 menyebabkan pengurangan besar dalam percambahan sel (~50%) berbanding dengan SDF-1α. Ini menunjukkan bahawa IL-6 dan SDF-1α dari PSC menggalakkan percambahan sel PDAC melalui pengaktifan isyarat Nrf2. IL-6 dilaporkan memainkan peranan penting dalam perkembangan PDAC. Oleh itu, ekspresi *IL6* dan reseptornya (*IL6R*) ditentukan dalam sel-sel PSC dan PDAC (AsPC-1, BxPC-3, dan Panc-1). Sel-sel Panc-1 digunakan bagi mengkaji IL-6 isyarat dalam interaksi PSC-PDAC kerana Panc-1 mempunyai ekspresi IL6 yang terrendah dan *IL6R* yang tertinggi. Peneutralan IL-6 mengurangkan percambahan sel PDAC dan gen metabolik yang dikawal oleh Nrf2. Peneutralan IL-6 menyebabkan peralihan PSC daripada morfologi mesenchymal ke epitelium, dan mengurangkan penghijrahan dan pencerobohan kapasiti. Ini semua boleh dipulihkan apabila tBHQ ditambahkan. Serentak dengan itu, peningkatan gen diperhatikan bagi *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *SNAIL*, *SLUG*, dan *TWIST2* kecuali epitelium *CDH1* yang encod E-cadherin. Ekspresi gen *NRF2* ditingkatkan dalam sel-sel Panc-1 yang dirawat dengan IL-6. Ini menunjukan bahawa IL-6 mengaktifkan Nrf2 dalam pencetusan EMT dan reprogram metabolik. Selain itu, penghalangan Stat3 membawa kepada peningkatan gen E-cadherin dan pengurangan bagi gen *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *SNAIL*, *SLUG*, dan *TWIST2*, *NRF2*, dan gen sasaran Nrf2 (*AKR1C1* dan *NQO1*). Penghalangan Stat3 juga menpertingkatkan kesan kurangan gen *NRF2* dalam pengurangan gen EMT. Dengan ini, IL-6 dari PSC menggalakkan percambahan sel PDAC melalui reprogram metabolik yang diaktifkan oleh Nrf2, dan mendorong EMT melalui laluan Stat3/Nrf2. Penyasarkan laluan Stat3/Nrf2 dari IL-6 yang dirembeskan oleh PSC mungkin memberi pilihan terapeutik baru bagi meningkatkan prognosis pesakit PDAC.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABCG2	:	ATP Binding Cassette Subfamily G Member 2
AKR1	:	Aldo-keto reductase family 1
Akt	:	Protein kinase B
ARE	:	Antioxidant response element
ATRA	:	All-trans retinoic acid
α-SMA	:	Alpha-smooth muscle actin
bHLH	:	Basic helix-loop-helix
BMPs	:	Bone morphogenetic proteins
BrdU	:	Bromodeoxyuridine
BTB	:	Broad complex/tram track/bric-a-brac
bZIP	:	Basic leucine zipper domain
CA19-9	:	Carbohydrate 19-9
CAFs	:	Cancer-associated fibroblasts
CAT	:	Catalase
CBR1	:	Carbonyl reductase 1
CCL2	:	C-C motif chemokine ligand 2
CCN2	:	Connective tissue growth factor
cDNA	:	Complementary deoxyribonucleic acid
CDH1	:	Cadherin-1
CDH2	:	Cadherin-2
CDKN2A	:	Cyclin-dependent kinase 2A
CEA	:	Carcinoembryonic antigen
CLC	:	Cardiotrophinike cytokine
COL1A1	:	Collagen type 1 alpha 1 chain
COX	:	Cyclooxygenase

CNTF	:	Ciliar neurotrophic factor
CRP	:	C-reactive protein
CT-1	:	Cardiotrophin-1
Cul3-E3	:	Cullin E3 ubiquitin-based ubiquitin E3
CXCR4	:	Chemokine receptor type 4
DAPI	:	4’,6-diamidino-2-phenylindole
DCF	:	2’,7’-dichlorofluorescein
DCF-DA	:	2’,7’-dichlorofluorescein diacetate
DGR	:	Double glycine repeat
DHEA	:	Dehydroisoandrosterone
DMBA	:	7,12-dimethylbenz(a)anthracene
DMEM	:	Dulbecco’s Modified Eagle Medium
DMEM/F-12	:	Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12
DPBS	:	Dulbecco’s Phosphate Buffered Saline
EC6/Fib-CM	:	EC6/Fib-conditioned medium
ECM	:	Extracellular matrix
ELISA	:	Enzyme-linked immuno assay
EMMPRIN	:	Metalloproteinase inducer
EMT	:	Epithelial-mesenchymal transition
ERK1/2	:	Extracellular signal-regulated kinase 1/2
FAK	:	Focal adhesion kinase
FBS	:	Fetal bovine serum
FDA	:	Food and Drug Administration
FGF	:	Fibroblast growth factor
F1,6BP	:	Fructose-1,6-biphosphate
FN1	:	Fibronectin 1

F6P	:	Fructose-6-phosphate
FTH1	:	Ferritin heavy polypeptide 1
FTL	:	Ferritin light polypeptide
GADD45	:	Growth arrest and DNA damage inducible 45
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GCLC	:	Glutamate-cysteine ligase catalytic subunit
GCLM	:	Glutamate-cysteine ligase modifier
GEM	:	Genetically engineered model
Glu	:	Glutamate
GM-CSF	:	Granulocyte macrophage colony-stimulating factor
GOT1	:	Glutamic-oxaloacetic transaminase 1
G3P	:	Glyceraldehyde-3-phosphate
G6P	:	Glucose 6-phosphate
GP130	:	Glycoprotein 130
G6PD	:	Glucose-6-phosphate dehydrogenase
GPX2	:	Glutathione peroxidase 2
GRO-α	:	Growth regulated oncogene-alpha
GSR	:	Glutathione-disulfide reductase
GSTM3	:	Glutathione S-transferase M3
HGF	:	Hepatocyte growth factor
HIF	:	Hypoxia-inducible factor
HMOX1	:	Heme oxygenase 1
H_2O_2	:	Hydrogen peroxide
HPDE	:	Human pancreatic duct epithelial
HRP	:	Horseradish peroxidase
HSP	:	Heat shock protein

ICAM-1	:	Intercellular adhesion molecule-1
IDH1	:	Isocitrate dehydrogenase 1
IER3	:	Immediate early response-E3
IGF	:	Insulin growth factor
IgG	:	Immunoglobulin G
IL	:	Interleukin
ILK	:	Integrin-linked kinase
IκB	:	Inhibitor kappa B
IKKB	:	Inhibitor kappa B kinase beta
IMP	:	Inosine 5'-monophosphate
IVR	:	Intervening region
JAK	:	Janus tyrosine kinase
Keap1	:	Kelch-like ECH-associated protein 1
KRAS	:	Kirsten rat sarcoma viral oncogene homolog
LCMS	:	Liquid chromatography-mass spectrometry
LIF	:	Leukemia inhibitory factor
Maf	:	Musculoaponeurotic fibrosarcoma oncogene homolog
MAPK	:	Mitogen-activated protein kinase
MCP	:	Monocyte chemoattractant protein
ME1	:	Malic enzyme 1
MEK	:	Mitogen-activated protein/extracellular signal-regulated kinase kinase
miR	:	MicroRNA
MMPs	:	Matrix metalloproteinases
mRNA	:	Messenger ribonucleic acid
MRP2	:	Multidrug resistance-associated protein 2

MS	:	Mass spectrometry
MT	:	Metallothionein
MTHFD2	:	Musculoaponeurotic fibrosarcoma oncogene homolog 2
mTORC1	:	Mammalian target of rapamycin complex 1
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide
NaCl	:	Sodium chloride
$NADP^+$	:	Nicotinamide adenine dinucleotide phosphate
NADPH	:	Nicotinamide adenine dinucleotide phosphate
Neu	:	Neutralizing
NF-κB	:	Nuclear factor-kappa B
NOX	:	NADPH oxidase
NQ1	:	Naphthoquinone 1
NQO1	:	NAD(P)H quinone dehydrogenase 1
Nrf2	:	Nuclear factor erythroid 2
8-OH-G	:	8-hydroxyguanosine
OSM	:	Oncostatin M
PanINs	:	Pancreatic intraepithelial neoplasias
PanNETs	:	Pancreatic neuroendocrine tumors
PCR	:	Polymerase chain reaction
PDAC	:	Pancreatic ductal adenocarcinoma
PDGF	:	Platelet-derived growth factor
3-PG	:	3-phosphoglyceric acid
6-PG	:	6-phosphogluconate
PGD	:	Phosphogluconate dehydrogenase
Phospho	:	Phosphorylated
PI3K	:	Phosphoinositide 3-kinase

PKM2	:	Pyruvate kinase isozyme M2
PPAT	:	Phosphoribosyl pyrophosphate amidotransferase
PPP	:	Pentose phosphate pathway
5-PRA	:	Phosphoribosylamine
PSC	:	Pancreatic stellate cells
PSC-CM	:	Pancreatic stellate cells-conditioned medium
PTGR1	:	Prostaglandin reductase 1
PTPases	:	Protein tyrosine phosphatases
PVDF	:	Polyvinylidene difluoride
qRT-PCR	:	Real-time quantitative reverse transcriptase-polymerase chain reaction
RAGE	:	Advanced glycation end products
Rh	:	Recombinant human
RNA	:	Ribonucleic acid
RNAi	:	Ribonucleic acid interference
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
R5P	:	Ribose 5-phosphate
RpiA	:	Ribose 5-phosphate isomerase A
RPMI-1640	:	Roswell Park Memorial Institute Medium-1640
RSLC	:	Rapid separation liquid chromatography
SCID	:	Severe combined immunodeficiency
SDF-1α	:	Stromal cell-derived factor-1 alpha
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERPINE2	:	Serine protease inhibitor

SFM	:	Serum-free medium
shRNA	:	Short-hairpin RNA
sIL-6R	:	Soluble IL-6 receptor
SIP1	:	Smad interacting protein 1
siRNA	:	Small interfering RNA
SLC7A11	:	Solute carrier family 7 member
SLUG	:	Snail family zinc finger 2
SNAIL	:	Zinc finger protein SNAI1
SNP	:	Single-nucleotide polymorphism
SOD1	:	Manganese superoxide dismutase
SOD2	:	Copper-zinc superoxide dismutase
SOD3	:	Extracellular superoxide dismutase
SREBP	:	Sterol regulatory element binding proteins
SRXN1	:	Sulfiredoxin 1
Stat	:	Signal transducer and activator of transcription
TALDO1	:	Transaldolase 1
tBHQ	:	tert-Butylhydroquinone
TCA	:	Tricarboxylic acid
TGF	:	Transforming growth factor
T-HESC-CM	:	T-HESC-conditioned medium
TIMPs	:	Tissue inhibitors of metalloproteinases
TKT	:	Transketolase
TKTL1	:	Transketolase like 1
TLRs	:	Toll-like receptors
TMB	:	3,3’,5,5’-Tetramethylbenzidine
TNF	:	Tumor necrosis factor

TPA	:	12-O-tetradecanoylphorbol-13-acetate
TP53INP1	:	Tumor protein 53-induced protein 1
TWIST2	:	Twist family BHLH transcription factor 2
TXND1	:	Thioredoxin 1
UHPLC	:	Ultimate high performance liquid chromatography
VEGF	:	Vascular epithelial growth factor
VIM	:	Vimentin

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Pancreatic cancer represents the fourth leading cause of cancer-related deaths worldwide among both men and women, with more than 80% of the cases being caused by pancreatic ductal adenocarcinoma (PDAC) (Siegel, Miller, & Jemal, 2015). The prognosis of PDAC remains poor despite substantial recent improvements in diagnostic, surgical, and therapeutic approaches. PDAC is locally invasive and generally surrounded by a dense desmoplastic reaction that can involve the adjacent vital structures, thus limiting the number of patients who are suited to receive surgical resection at the time of diagnosis (B. Farrow, Albo, & Berger, 2008; Korc, 2007; Welsch, Kleeff, Esposito, Buchler, & Friess, 2007). Notably, the extremely dense desmoplastic infiltration is mainly contributed by pancreatic stellate cells (PSC) (Hwang et al., 2008). The lack of understanding of the contribution of stromal cells to the desmoplastic reaction may lead to the failure of conventional treatments.

Many studies have revealed the roles of PSC in tumor progression including cell proliferation, migration, invasion, and chemoresistance (Ali et al., 2015; Apte & Wilson, 2012; Hwang et al., 2008; Ozdemir et al., 2014). Activated PSC can secrete abundant cytokines and growth factors, such as interleukin(IL)-6, IL-8, transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), and insulin-growth factor (IGF)-1, as well as induce extracellular matrix (ECM) remodelling, all of which are important for the modulation of PDAC progression (Bachem et al., 2005; Hwang et al., 2008; J. Lu et al., 2014; Mantoni, Lunardi, Al-Assar, Masamune, & Brunner, 2011; Masamune, Watanabe, Kikuta, & Shimosegawa, 2009; Patel, Collins, Benyon, & Fine, 2010; Vonlaufen, Joshi, et al., 2008). However, the key mechanisms involved in promoting PDAC cell proliferation and motility through PSC factor secretion remain unknown.

PDAC requires high ROS levels for survival (Teoh, Sun, Smith, Oberley, & Cullen, 2007). However, this phenomenon is debatable as a recent study has found that

coupled with the *KRAS* mutation, Nrf2 confers a reducing intracellular environment that favors PDAC carcinogenesis, as demonstrated using a K-driven genetically engineered mouse model (DeNicola et al., 2011). The Nrf2 transcription factor, a master regulator of antioxidant-response element (ARE)-driven genes that mainly encode antioxidant and detoxifying enzymes (Bryan, Olayanju, Goldring, & Park, 2013), is known to combat oxidative stress. Under sustained activation of the PI3K/Akt pathway, Nrf2 can also induce the proliferation of several cancer cell lines by activating the metabolic pathways to enhance purine nucleotides synthesis and ROS detoxification (Mitsuishi, Taguchi, et al., 2012). Activated Nrf2 signaling can also induce epithelial-mesenchymal transition (EMT) in colorectal cancer (Liu et al., 2015), a key mechanistic cascade in tumor metastasis. However, there exists only limited scientific evidence demonstrating the role of Nrf2-mediated signaling pathways in either PSC-activated PDAC cell proliferation or EMT.

The cytokine IL-6, which is secreted by PSC and mainly regulates inflammation and immune response, has been reported to serve as a target of Nrf2 as it contains an ARE sequence within its promoter (Wruck et al., 2011). Conversely, IL-6 was shown to protect against trimethyltin-induced neurotoxicity *in vivo* by significantly reducing Nrf2 activity (Tran et al., 2012), indirectly suggesting that IL-6 could regulate Nrf2 activity in turn although whether Nrf2 represent a direct target of IL-6 remains unclear. Notably, IL-6 has been reported to play an important role in stepwise PDAC progression (Block, Hanke, Maine, & Baker, 2012; Goumas et al., 2015; Huang et al., 2010; Lesina et al., 2011; Y. Zhang et al., 2013), and the IL-6 secreted by PSC and other cancer-associated fibroblasts has been shown to promote tumor proliferation and invasion (Cirri & Chiarugi, 2011; Erez, Truitt, Olson, Arron, & Hanahan, 2010; Q. Z. Guo, 2014; Nagasaki et al., 2014). However, to date, no studies examine whether PSC may activate Nrf2 activity to promote PDAC cell proliferation and metastasis. Therefore, further investigation is needed to

determine whether IL-6 activates Nrf2 signaling to contribute to the pro-tumorigenic action of PSC in PDAC.

1.1 Study hypothesis

PSC has a key role in determining the pace of PDAC progression, particularly in modulating the processes of cell proliferation and motility toward aggressive phenotypes. Understanding the molecular mechanisms activated by the secretory factors from PSC may provide further insight to improve the poor prognosis of patients with PDAC. Accordingly, this study hypothesized that IL-6 secreted by PSC promotes PDAC cell proliferation and motility and invasion capacity via the activation of intracellular Nrf2 signaling pathways.

1.2 Specific objectives

1.2.1 To determine the effect of PSC secretion on PDAC cell proliferation.

1.2.2 To investigate the role of Nrf2 signaling in PSC-mediated PDAC cell proliferation

1.2.3 To determine the role of IL-6 secreted by PSC in activating Nrf2-mediated metabolic reprogramming in PDAC cells.

1.2.4 To investigate the role of IL-6 secreted by PSC in inducing EMT phenotypes in PDAC cells.

1.2.5 To determine the mechanism by which IL-6 secreted by PSC affects Nrf2 signaling to induce EMT in PDAC cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Pancreatic cancer

2.1.1 Statistic, incidence, and mortality

Pancreatic cancer is the seventh most common cancers in the world (*Cancer Facts & Figures 2017*, 2017). It is the fourth leading cause of all cancer-related deaths among men and women, with 5-year average incidence, survival, and death rates per 100,000 population are 1%, 9%, and 80%, respectively (*Cancer Facts & Figures 2017*, 2017). In the United States, it is estimated that 53,670 people will be diagnosed, and 43,090 people will die from this disease in 2017. By 2020, pancreatic cancer could be the second most prevalent cancer worldwide (*Cancer Facts & Figures 2017*, 2017). It was reported that African American are more susceptible to pancreatic cancer compared to Asian, Hispanic, or Caucasian (Khawja et al., 2015). For example, they have higher pancreatic cancer incidence and mortality rates in the United States, with greater rates in men than in women (Institute, 2011). Comparatively, there were less cases of pancreatic cancer reported in Malaysia. Only 1,829 out of 103,507 cases (1.7% incidence rate) reported in 2007 to 2011 were diagnosed as pancreatic cancer, with men and women contributed 57% (1,041 cases) and 43% (788 cases), respectively. The 5-year average survival and death rates are 3% and 80%, respectively (Azizah Ab, Nor Zaleha, Noor Hashimah, Asmah, & Mastulu, 2011).

2.1.2 Types of pancreatic cancer

Pancreatic cancer is classified into two types based on the location of the pancreas affected. Majority of the cases (99%) were found in the exocrine of the pancreas while a small number of cases found in the endocrine part (Harris, 2013; Oberg, Knigge, Kwekkeboom, & Perren, 2012). The exocrine pancreas consists of about 90% of acinar and 10% of ductal epithelial cells (Feldman, Friedman, & Brandt, 2010). Despite

abundant acinar cells in the exocrine pancreas, acinar cell carcinoma is rare and only contributes about 5% of total exocrine pancreatic cancers. In contrast, pancreatic ductal adenocarcinoma (PDAC), which arises from ductal epithelial cells is the most common type of pancreatic cancer, representing about 85% of all pancreatic cancer cases (Govindan, 2011; Ryan, Hong, & Bardeesy, 2014).

Pancreatic neuroendocrine tumors (PanNETs) is the most common endocrine pancreatic cancer. It arises from neuroendocrine cells, and can be grouped into functioning and non-functioning types (Klimstra, Modlin, Coppola, Lloyd, & Suster, 2010). The functioning type of PanNETs secrete a large quantity of hormones such as gastrin, insulin, and glucagon to control the levels of blood sugar and give rise to early detection. In contrast, the non-functioning type of PanNETs do not secrete hormones for early detection and overt clinical symptoms.

2.1.3 Symptoms and risk factors

PDAC diagnosis is difficult although there are some detectable common symptoms, such as pain in the upper abdomen or back (Tobias & Hochhauser, 2010). Abdominal pain was reported as the main symptom in about two-third people in being diagnosed, followed by 46% of jaundice, 13% have jaundice without pain, and some people may have unexplained weight loss (Bond-Smith, Banga, Hammond, & Imber, 2012). About 50% of PDAC new cases are diagnosed with pain or jaundice (De La Cruz, Young, & Ruffin, 2014). PDAC patients with an unexplained weight loss, mostly caused by loss of appetite or exocrine malfunction resulting in poor digestion (Bond-Smith et al., 2012).

There are several risk factors for PDAC, in which age, gender, and ethnicity are viewed as the most common. PDAC is rarely diagnosed before the age of 40, with most cases occurring to those over the age of 60. PDAC is more commonly diagnosed in men

compared to women. In comparison to Caucasian, African American have 1.5 times higher risk being diagnosed in the United States (*Cancer Facts & Figures 2017*, 2017). Additionally, the risk for PDAC increases with the number of years of smoking and quantity of cigarettes smoked (Bosetti et al., 2012). Obese people with body mass index greater than 35 have 1.5-fold increased risk of developing PDAC (Bond-Smith et al., 2012). Genetic inheritance also contributes to about 5-10% of PDAC cases (Reznik, Hendifar, & Tuli, 2014; Ryan et al., 2014). Furthermore, patients with hereditary pancreatitis contributes to about 30-40% increased lifetime risk to have PDAC while chronic pancreatitis contributes to about 3-fold increase in risk.

2.1.4 Treatments

The most effective therapy for PDAC thus far is surgical removal of the tissue; however, this option is only applicable in 20% of new cases (Bond-Smith et al., 2012). Despite successful surgery, relapse may occur due to remnants of malignant cells (Ryan et al., 2014). When cancer spreads, it compresses other organs such as duodenum or colon; therefore, a bypass surgery can be used for palliation (Bond-Smith et al., 2012). Palliative surgery is also used to treat other complications such as bile ducts or intestines obstruction caused by the tumor (De La Cruz et al., 2014).

To those who are unsuited to undergo surgery, chemotherapy is often the approach to extend and to improve their quality of life. Gemcitabine has been demonstrated to prolong the median survival duration in patients with PDAC (Thota, Pauff, & Berlin, 2014). However, the use of gemcitabine alone is insufficient to extend the life and its quality further. In 2005, erlotinib was approved by Food and Drug Administration (FDA) for pancreatic cancer as it helps to increase the overall survival (6.4 months) when administered in combination with gemcitabine ("Cancer Drug Information: FDA Approval for Erlotinib Hydrochloride," 2013). Besides, FOLFIRINOX chemotherapy

regime using four drugs (folinic acid, fluorouracil, irinotecan, and oxaliplatin) or nab-paclitaxel can be applied to patients who response well with gemcitabine treatment because it was found to have a higher efficacy than gemcitabine (Borazanci & Von Hoff, 2014). The combination use of FOLFIRINOX or nab-paclitaxel with gemcitabine were considered as a suitable adjuvant chemotherapy choice for PDAC patients with good performance status. In contrast, gemcitabine will remain as the primary option for PDAC patients with bad performance status (Thota et al., 2014).

2.1.5 Progression of pancreatic cancer

In recent years, several studies have claimed that PDAC may arise from pre-cancerous lesions, known as pancreatic intraepithelial neoplasias (PanINs) (Hezel, Kimmelman, Stanger, Bardeesy, & Depinho, 2006; Hruban, Wilentz, & Kern, 2000). PanINs can be categorized into low-, intermediate-, and high-grade lesions. PanIN-1A and PanIN-1B are low-grade lesions, which usually harbor activated *KRAS* mutation. PanIN-2A and PanIN-2B are intermediate-grade lesions, which featured with a low expression of cyclin-dependent kinase 2A (*CDKN2A*) and *KRAS* mutations. PanIN-3, on the other hand, is developed from carcinoma in situ. It has the marked features of nuclear atypia, budding of cells into the lumen of duct-like structures, and mitotic figures, which are a reflection of increased cellular proliferation and the occasional presence of *TP53* mutations (Hezel et al., 2006; Hruban et al., 2006; Hruban et al., 2000). *In vivo* mouse studies demonstrated that the phenotypic impact of mice carrying $KRAS^{G12D}$ was limited to development of PanIN. However, $KRAS^{G12D}$ expression together with a partially inactivated *TP53* allele or *INK4a* locus deletion resulted in an earlier appearance of PanIN and progressed rapidly to highly invasive and metastatic cancer (Aguirre et al., 2003; Hingorani et al., 2005). These evidences indicate that PDAC progression from PanINs requires the incorporation of multiple mutations.

The amount of stroma is associated with the stages of PanINs. The pancreatic ducts of PanIN-1 and -2 have a small amount of normal stroma. Whereas, enhanced stroma formation can be seen in PanIN-3 lesions and invasive carcinoma, together with inflammatory infiltrate (Korc, 2007). Many studies have demonstrated that PDAC progression is accompanied by increasing amount of stroma (Clark et al., 2007; Korc, 2007; Mahadevan & Von Hoff, 2007). For example, the formation of pancreatic cancer from PanINs in a KPC mouse model (with both *KRAS* and *TP53* mutation) was accompanied by the accumulation of fibrotic stroma (Clark et al., 2007). Similarly, accumulation of fibro-inflammatory stroma was found during PanIN formation in an iKras mouse model. Stromal cell proliferation was suppressed following Kras inactivation at the PanIN stage in the same model, leading to fibroblast inactivation and extracellular matrix (ECM) remodeling (Collins, Bednar, et al., 2012; Waghray, Yalamanchili, di Magliano, & Simeone, 2013).

2.2 Pancreatic tumor microenvironment

2.2.1 Tumor microenvironment as a hallmark of cancer

Cancer hallmarks represent a biological tool to understand better the complexities of cancer. There are eight cancer hallmarks acquired in the process of tumor development and progression. Their acquisition depends on two enabling characteristics, which are genomic stability and the control of inflammation by immune cells in pre-malignant or malignant lesions. The acquisition of these hallmark traits can be contributed by tumor microenvironment, which is another dimension of complexity in a tumor (Hanahan & Weinberg, 2011; Negrini, Gorgoulis, & Halazonetis, 2010).

Tumor microenvironment consists of individual specialized cell types, in addition to cancer cells produced in the process of tumorigenesis (Diaz-Cano, 2012; Hanahan & Weinberg, 2011). The individual specialized cell types collectively termed stromal

elements, which include cancer stem cells, endothelial cells, pericytes, inflammatory immune cells, and cancer-associated fibroblasts as shown in Figure 2.1. Each of these cell types has their individual or synergistic role with another cell types to promote tumorigenesis. The reciprocal tumor-stroma interactions play a major role in the stepwise tumor progression (Egeblad, Nakasone, & Werb, 2010; Kessenbrock, Plaks, & Werb, 2010). For example, the cancer cells recruit and activate the adjacent stromal cells to form a pre-neoplastic stroma, which in turn promote the cancer cell phenotypes. Cancer cells may also further evolve genetically and send the signal to the stroma, thereby reprogram the normal stromal cells to increase cancer cell motility. Finally, cancer cells invade and metastasize to adjacent normal tissues with the fuel of signals sent from the stroma. (Alphonso & Alahari, 2009; Hanahan & Weinberg, 2011; Quail & Joyce, 2013).

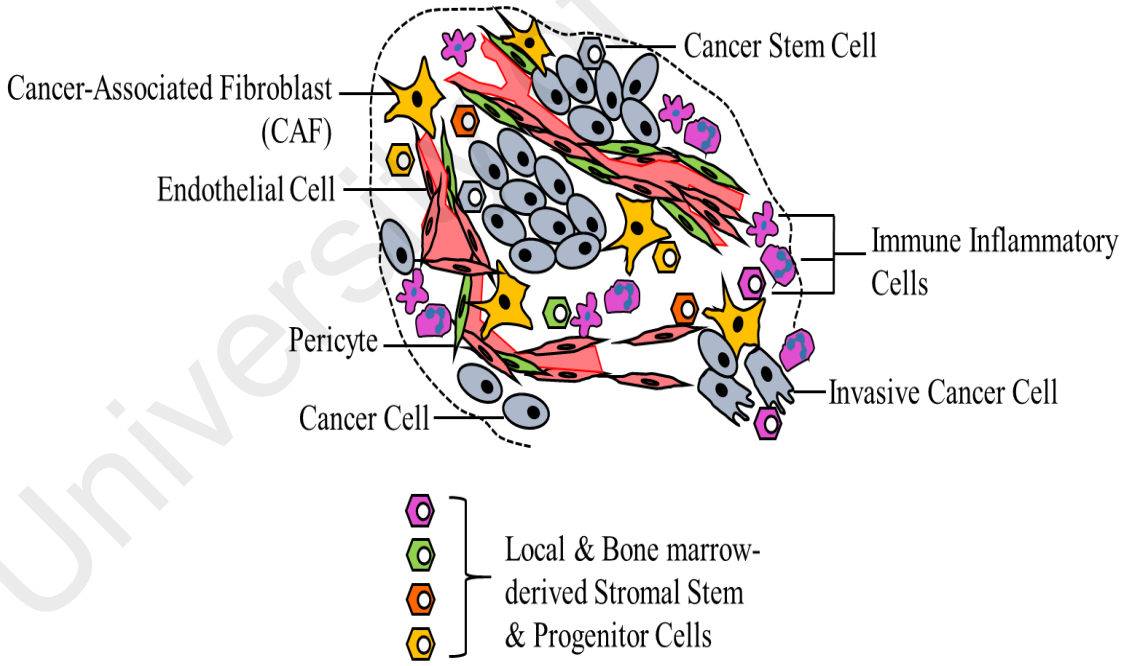

Figure 2.1: The cells of the tumor microenvironment. The tumor microenvironment consists of individual specialized cell types, such as noninvasive and invasive cancer cells, cancer stem cells, cancer-associated fibroblasts, endothelial cells, immune inflammatory cells, and pericytes.

2.2.2 Components of pancreatic cancer stroma

Tumor stroma consists of both cellular and extracellular components as shown in Figure 2.2. Each of these has a distinct role in creating an active stroma to support PDAC progression (B. Farrow et al., 2008). The cellular components of PDAC mainly consist of activated fibroblast and pancreatic stellate cells (PSC), which produce extracellular matrix (ECM), such as collagens and fibronectin (Bachem et al., 2005). The secretion from fibroblasts such as hepatocyte growth factor has been shown to enhance pancreatic cancer cell growth and invasion (Muerkoster et al., 2004; Ohuchida et al., 2004; Qian et al., 2003). Moreover, the cellular of stroma also contains chemokines and cytokines-producing cells, such as aberrant endothelial cells, foci of inflammatory cells, pericytes, and macrophages, which may promote fibroblasts and PSC activation (B. Farrow et al., 2004). Besides, nerve growth factors (NGFs) and bone marrow-derived stem cells are also the cellular component of the stroma. NGFs and bone marrow-derived stem cells have been reported to have the ability to differentiate into PSC and fibroblasts (Sangai et al., 2005; Zhu et al., 2002). The adjacent endocrine islets, which secrete high levels of insulin can be also found in the stroma (B. Farrow et al., 2008). Extracellular matrix (ECM) mainly constitutes the extracellular component and it is comprised of collagen, glycoproteins, and proteases that regulate the tissue structuring to facilitate pancreatic cancer invasion (B. Farrow, O'Connor, Hashimoto, Iwamura, & Evers, 2003). All these create a unique tumor microenvironment, which is a hallmark of pancreatic cancer.

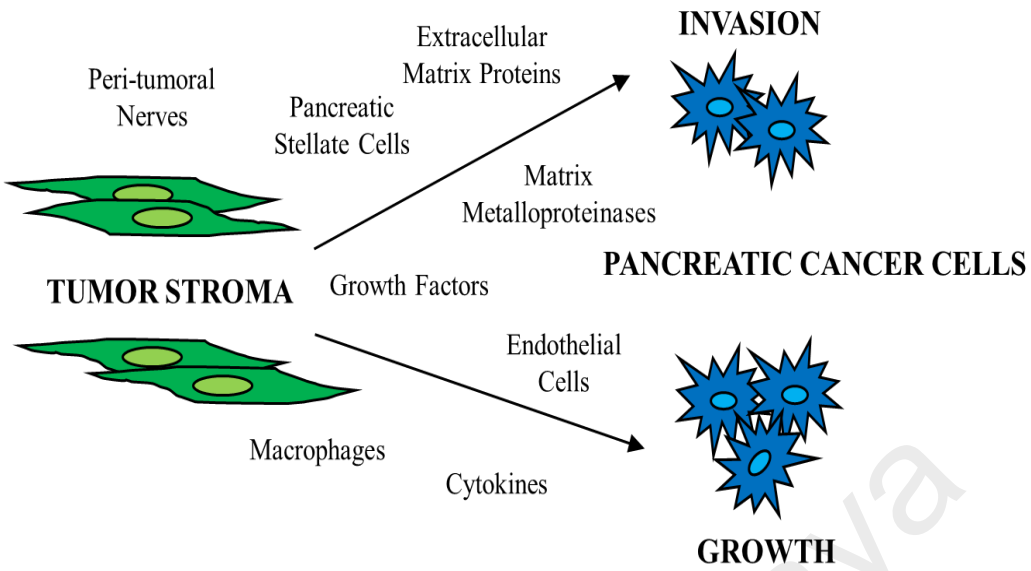

Figure 2.2: The pancreatic tumor microenvironment. The tumor microenvironment of PDAC consists of both cellular and extracellular components.

2.2.3 Cancer-associated fibroblasts (CAFs)

Cancer-associated fibroblasts (CAFs) are the main cell type that can be found in tumor stroma. These can be divided into two cell types: (1) cells that create the structure foundation to support normal epithelial tissues and share similarities with fibroblasts, and (2) myofibroblasts, whose properties and biological functions can be differentiated by markers expressed by tissue-derived fibroblasts. The expression of alpha-smooth muscle actin (α-SMA) can be used to identify myofibroblasts. They are rarely found in the normal healthy epithelial tissues, however, in certain tissues from liver and pancreas, its expression can be high (Hanahan & Weinberg, 2011).

CAFs are the activated form of myofibroblasts or normal fibroblasts, with the most important source is PSC. They have been demonstrated in many studies to promote tumor cell proliferation, angiogenesis, invasion, and metastasis. The importance role of CAFs in mediating tumor phenotypes are found by transplanting both cancer epithelial cells and CAFs together into mice (Bhowmick, Neilson, & Moses, 2004; Dirat, Bochet, Escourrou, Valet, & Muller, 2010; Kalluri & Zeisberg, 2006; Pietras & Ostman, 2010;

Rasanen & Vaheri, 2010; Shimoda, Mellody, & Orimo, 2010). They are capable of secreting ECM and soluble factors, which include chemokines, cytokines, and growth factors. Therefore, they are implicated in the formation of desmoplasia stroma, which is the hallmark of PDAC (Hanahan & Weinberg, 2011).

2.2.4 Pancreatic stellate cells (PSC)

Pancreatic stellate cells were isolated and cultured by Bachem and his group in 1998 (Apte et al., 1998; Bachem et al., 1998). PSC resemble the characteristics of hepatic stellate cells as confirmed in their morphology, functional, and gene expression (Erkan et al., 2010). In the pancreatic tumor stroma, PSC are the predominant mesenchymal cells (Apte et al., 2004). They may originate from mesenchymal, endodermal or neurodermal. They are seldom found in the normal pancreas but exist in high amount in the benign pancreatic inflammatory or malignant disease (E. G. Farrow, Davis, Ward, & White, 2007).

The name of stellate cells was determined from their shape (*stella* in Latin means "a star") (Keane, Strieter, & Belperio, 2005). PSC are found in the periacinar spaces in the normal pancreas. They share some similarities with myofibroblast cells. In the quiescent state, they contribute to about 4-7% of the pancreatic cells. The pathobiology of chronic pancreatitis and pancreatic cancer can be affected by the secretion of PSC (Apte et al., 1998; Bachem et al., 1998; Erkan et al., 2007; Erkan et al., 2009). During their activation or transformation to myofibroblast-state, retinoid-containing fat droplets in the cytoplasm are lost, with a concomitant expression of α-SMA (Apte et al., 1998; Bachem et al., 1998).

Activated PSC were reported to produce ECM component, and secrete proinflammatory cytokines, chemokines, and growth factors (Apte et al., 1998; Bachem et al., 1998; Bachem et al., 2005; Hwang et al., 2008; Masamune & Shimosegawa, 2009;

Omary, Lugea, Lowe, & Pandol, 2007; Shimizu, 2008; Vonlaufen, Joshi, et al., 2008; Watanabe et al., 2004). The secretion further activate PSC and induce cell responses with cancer cells in both autocrine and paracrine fashion (Apte et al., 1998; Bachem et al., 1998; Bachem et al., 2005; Hwang et al., 2008; Masamune & Shimosegawa, 2009; Omary et al., 2007; Shimizu, 2008; Vonlaufen, Joshi, et al., 2008). A few studies have suggested that PSC have many cellular functions. They produce matrix-degrading enzymes of the matrix metalloproteinases (MMPs) family and tissue inhibitors of metalloproteinases (TIMPs) (Masamune et al., 2009). The incorporation of MMPs and TIMPs with ECM turnover can affect the regulation of normal tissue structure (Phillips et al., 2003). Additionally, MMP-2 may contribute to the pancreatic cancer progression (Schneiderhan et al., 2007). PSC are located nearby the ductal and vascular structures; thus, they can regulate the pancreatic ductal and vascular tone by increasing the expression of cytoskeletal protein α-SMA and endothelial-1 and confer contractile potential (Masamune, Satoh, Kikuta, Suzuki, & Shimosegawa, 2005).

PSC secrete different cytokines, chemokines, and growth factors, including IL-6, IL-1β, PDGF-BB, TGF-β1, and tumor necrosis factor(TNF)-α, etc (Masamune et al., 2009). Cell adhesion molecules (intercellular adhesion molecule (ICAM)-1) and chemokines (IL-8, RANTES, and monocyte chemoattractant protein (MCP)-1) from PSC contribute to the recruitment of inflammatory cells in the inflamed pancreas (Andoh et al., 2000; Masamune, Kikuta, et al., 2002; Masamune, Sakai, et al., 2002). Furthermore, PSC are involved in the innate immunity activation against microorganism infection by expressing Toll-like receptors (TLRs) proteins (Masamune, Kikuta, Watanabe, Satoh, Satoh, et al., 2008; Vonlaufen et al., 2007). For example, TLR2 and TLR4 have been demonstrated to combat the gram-positive and gram-negative bacteria, respectively. TLR3 recognizes virus infection via their double-stranded RNA while TLR5 recognizes flagellin in the bacteria. Also, PSC have the functions to fight against foreign bodies via

endocytose and phagocytose actions, suggesting that PSC are involved in the local immune functions (Masamune, Kikuta, Watanabe, Satoh, Satoh, et al., 2008; Shimizu, Kobayashi, Tahara, & Shiratori, 2005). PSC also render pancreatic acinar cells to undergo apoptosis and necrosis to maintain organ homeostasis (Tahara, Shimizu, & Shiratori, 2008).

Under hypoxic condition, PSC also contribute to angiogenesis by producing vascular endothelial growth factor (VEGF) in a high amount (Masamune, Kikuta, Watanabe, Satoh, Hirota, et al., 2008). In addition, PSC express angiogenesis-related molecules, such as vasohibin-1, angiopoietin-1 and its receptor Tie-2, and VEGF receptors (Flt-1 and Flk-1) (Masamune, Kikuta, Watanabe, Satoh, Hirota, et al., 2008). The secretion may facilitate pancreatic cancer and chronic pancreatitis as these diseases were associated with fibrosis and higher VEGF expression (Kuehn, Lelkes, Bloechle, Niendorf, & Izbicki, 1999).

2.2.5 PSC in pancreatic cancer progression

Many lines of evidence showing that a bidirectional relationship exists between PSC and PDAC cells, which favors PDAC progression. In PDAC, when Panc-1 cells were co-cultured with normal skin fibroblast, desmoplasia was induced with increasing amount of ECM (collagen I, III, and fibronectin), TGF-β1, and fibroblast growth factor (FGF)-2. The production of collagen I and PDGF-AA was stimulated in Panc-1 cells transfected with TGF-β1. In addition, tyrosine phosphorylation was increased several-fold higher in fibroblasts when co-cultured with Panc-1/TGF-β1 (Lohr et al., 2001). Besides, the invasiveness of Suit-2 or Capan-1 PDAC was stimulated when co-cultured with irradiated normal human lung fibroblast (MRC5). This effect was further enhanced when PDAC cells were also irradiated and co-cultured with irradiated MRC5 cells (Mahadevan & Von Hoff, 2007). The increased invasiveness might be due to the

activation of mitogen-activated protein kinase pathway (MAPK) with c-Met expression as observed in Suit-2 cells exposed to conditioned medium from irradiated MRC5 (Mahadevan & Von Hoff, 2007).

The growth factors and chemokine families can act as autocrine or paracrine mediators for tumor-stroma interactions as reported by many studies using mouse models. A rich stroma was induced after orthotopically transplanting TGF-β1-transfected Panc-1 cells into nude mouse pancreas (Bierie & Moses, 2006). In addition, PanINs were induced by Notch signaling pathway, cyclooxygenase(COX)-2, and MMP-7 using a Kras mouse model of PDAC (Hingorani et al., 2003). Furthermore, PanIN lesions formation and rapid progression to invasive and metastatic PDAC were found in mouse with pancreas-specific Cre-mediated activation of mutant $KRAS^{G12D}$ and deletion of a conditional *CDKN2/INK-4a*/Arf tumor suppressor allele. The tumors dissected were found to resemble human PDAC with proliferative stromal component and PanIN lesions with the ability to transform into poorly differentiate state (Aguirre et al., 2003).

In the case of PSC, their crosstalk with PDAC cells has been shown to promote PDAC progression. The mitogenic and fibrogenic mediators secreted by PDAC cells were reported to activate PSC. For example, the conditioned medium derived from PDAC cells (AsPC-1, Panc-1, MiaPaCa-2) increased the proliferation (more than 5-fold), and enhanced the matrix synthesis in PSC, in which these effects were reduced after neutralizing PDGF, FGF-2, and TGF-β1 (Apte et al., 2004; Bachem et al., 2005; Vonlaufen, Joshi, et al., 2008; Vonlaufen, Phillips, et al., 2008; Z. Xu et al., 2010). Furthermore, metalloproteinase inducer (EMMPRIN), a type of ECM secreted by PDAC cells was found to increase MMP-2 secretion by PSC (Phillips et al., 2010; Schneiderhan et al., 2007). MMP-2 disrupts normal basement membrane formation during cancer progression. Therefore, it is associated with invasive phenotype in pancreatic cancer (Phillips et al., 2010).

Bachem et al. (2005) demonstrated that PSC and PDAC cells co-injected subcutaneously into the flanks of nude mice led to a larger volume of the tumor with a significant amount of stromal compartment, larger than those produced by cancer cells alone. Likewise, nude mice co-injected with PSC and PDAC cells harboring overexpression of serine protease inhibitor *SERPINE2* resulted in more extensive tumor growth, increased fibrillar collagen of ECM component deposition, and protected PDAC cells from apoptosis (Neesse et al., 2007). Nonetheless, the subcutaneous mouse model is not an ideal choice for PDAC as they do not allow the study of tumor behavior in an appropriate microenvironment. Thus, an orthotopic model was developed to better understanding the tumor-stroma interaction in pancreatic cancer (Hwang et al., 2008; Vonlaufen, Joshi, et al., 2008). This model revealed that co-injection of PSC and pancreatic cancer cells showed dense bands of fibrosis, and the presence of PSC with higher expression of α-SMA (Hwang et al., 2008; Vonlaufen, Joshi, et al., 2008).

In addition, PSC co-opts PDAC cells to form a growth permissive and tumor facilitatory environment. Through the secretory factors, PSC actively participate in mediating PDAC progression, including survival, proliferation, migration, invasion, and metastasis (Hwang et al., 2008; Vonlaufen, Joshi, et al., 2008; Z. Xu et al., 2010). Using an *in vivo* orthotopic mouse model, intra-pancreatic co-injection of PSC and PDAC cells led to large tumor volume and also greater distant metastases compared to those caused by PDAC cells alone (Vonlaufen, Joshi, et al., 2008). In addition, Hwang et al. (2008) further showed that higher human PSC proportion to PDAC cells increased the incidence of tumor formation and no tumor developed when mice received PSC alone. In the same study, the conditioned medium of human PSC promoted PDAC cell proliferation, colony formation, and resistance to radiation therapy (Hwang et al., 2008). It has also been reported that PSC play a major role in seeding because they act as a metastatic fuel to facilitate the migration and invasion of pancreatic cancer to new tissues when co-

migrating (Z. Xu et al., 2010). Furthermore, direct and indirect co-culture models have demonstrated that PSC are essential to promote PDAC progression, in particular, proliferation, migration, and invasion (Fujita et al., 2009; Vonlaufen, Joshi, et al., 2008). These findings support that PSC are critical for PDAC progression.

2.3 Oxidative stress in pancreatic cancer

2.3.1 Reactive oxygen species (ROS) in cancer cells

Oxygen-free radicals contain one or more unpaired electrons, which decide their degree of reactivity (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). These include reaction oxygen species (ROS) and reactive nitrogen species (RNS), which are generated through exogenous and endogenous sources. The endogenous sources of ROS generation include macrophages and neutrophils that fight against pathogen infection during inflammation, and the byproducts of electron transport reactions catalyzed by mitochondria and cellular metabolism. In contrast, the exogenous sources include metal-catalyzed reactions and irradiation by UV lights, X-rays, and gamma rays (Cadenas, 1989; Evans & Halliwell, 1999).

ROS have a dual role in biological systems because they can exert both deleterious and beneficial effects based on their concentration (Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). At lower concentration or suitable physiological concentrations, ROS are involved in cellular response to anoxia, including activating cellular signaling systems and providing biological defense against infectious agents. ROS at high concentrations cause oxidative damage to cellular molecules, such as nucleic acids, protein, lipids and membranes (Poli, Leonarduzzi, Biasi, & Chiarpotto, 2004). This is often termed as oxidative stress. Consequently, these damages result in the development of aging diseases, such as cancer. Moreover, ROS are involved in intracellular signaling cascades by acting as secondary messengers, which are required to induce and maintain the cancer

phenotypes (Valko et al., 2006). However, if the ROS levels are too high, it eventually causes cancer cell death through various mechanisms. Therefore, the physiological concentration of ROS is crucial to decide their deleterious or beneficial function to cancer cells (Halliwell, 1996; Poli et al., 2004).

2.3.2 The involvement of ROS at three stages model of carcinogenesis

Carcinogenesis is a multi-stage process, which transforms normal cells to pre-cancerous lesion and ultimately to cancer (Trueba, Sanchez, & Giuliani, 2004). This process is characterized by different underlying mechanisms. The earliest discovered mechanism is the "initiation-promotion-progression" model involving both viruses and chemical carcinogens (Berenblum & Shubik, 1947; Reddy & Fialkow, 1983) followed by the "oncogene/tumor suppressor gene" and "integrative theory" (Valko et al., 2006). The multi-stage can be divided into initiation, promotion, and progression. ROS can act in each of these three stages as shown in Figure 2.3.

Initiation involves a non-lethal mutation in DNA to produce an altered cell. In this stage, the DNA damage as indicated by the levels of 8-hydroxyguanosine (8-OH-G) is considered mild and fixable. However, if the DNA damage is severe and irreversible, the dividing cells halt the cell cycle temporarily at G1, S or G2 phase for repair before re-execution (Trueba et al., 2004). ROS, for example, hydroxyl radicals generated via Fenton-type mechanism are required to cause DNA damage. Investigations on benign tumors suggested that the amount of 8-OH-G adduct formation was positively correlated with the tumor size (Trueba et al., 2004). The initiation process can be further proceeded by the release of free intracellular calcium from its stores and also extracellular influx induced by oxidative stress (Dreher & Junod, 1996).

The promotion stage is induced when more initiated cells formed by an imbalance of cell proliferation and (or) programmed cell death (apoptosis). An identifiable focal

lesion is formed in this stage, which continuously requires the presence of tumor promoters; thus, it is considered a reversible process. Many of these tumor promoters have a function to inhibit the activity of antioxidants. Therefore, the levels of ROS in this stage is a key to determine the execution of progression stage as low to moderate levels of ROS are essential for cell division and tumor growth, but high levels of ROS can cause cell death (Dreher & Junod, 1996).

Progression is the last stage of carcinogenesis. The levels of ROS act in this stage is relatively less compared to initiation and promotion stages. Many cellular biological changes occur in this stage. For example, the cells acquired mesenchymal-like morphology and have greater motility during the conversion. This stage is irreversible. It's features include additional chromosome integrity disruption and genetic instability, which are required during the transformation of normal cells to cancer cells (Klaunig & Kamendulis, 2004).

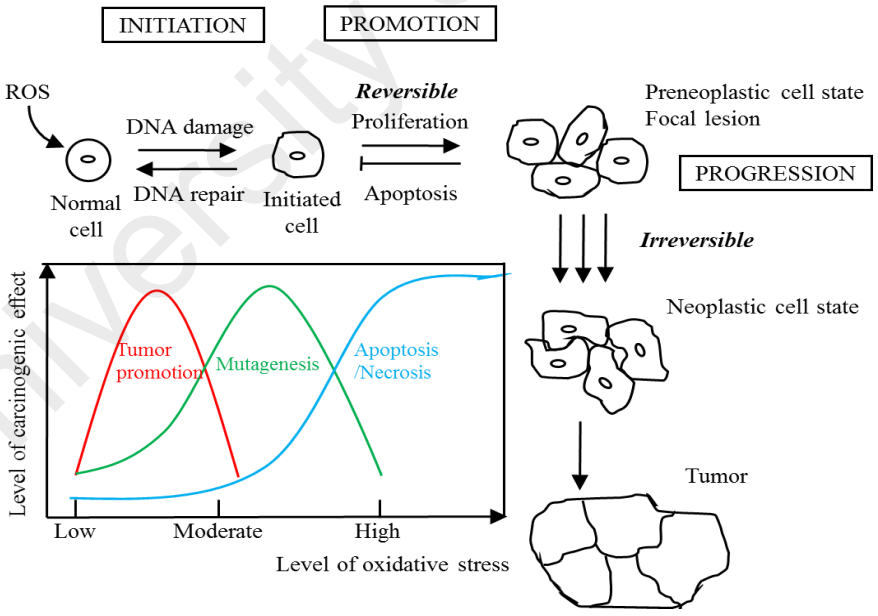

Figure 2.3: The level of ROS at three stages of the carcinogenic process. Intracellular ROS are required to cause DNA damage to macromolecules during the initiate stage. In the promotion stage, the amount of ROS is increased and the continuously presence of the tumor stimuli is needed, thus it is a reversible process. The amount of ROS needed in the progression stage is relatively less compared to initiation and promote stages. This stage is irreversible and characterized with chromosomal integrity disruption and genomic instability. Adapted from Valko et al (2006).

2.3.3 Roles of ROS in pancreatic cancer progression

Cancer cells have elevated levels of ROS compared to normal cells, which are essential for tumor development and progression. Meanwhile, cancer cells also have an active antioxidant system to combat against oxidative stress. This phenomenon suggests that cancer cells require balance, specifically low to moderate levels of intracellular ROS for proper functions (Liou & Storz, 2010).

PDAC has been previously described to be dependent on high intracellular ROS levels for survival. For example, the antioxidant compounds and suppression of superoxide anion levels elicited apoptosis (Cullen et al., 2003; Mouria et al., 2002; Teoh et al., 2007). However, a recent study from DeNicola et al. (2011) demonstrated that PDAC acquired lower intracellular ROS levels to drive its progression. This finding was supported by the studies that exogenous hydrogen peroxide (H_2O_2) treatment triggered PDAC cell death due to the high intracellular basal levels of ROS (A. Lewis et al., 2005; Osada et al., 2008). ROS from NADPH oxidase (NOX) source have been implicated in PDAC development (Wu et al., 2011). For example, downregulation of *NOX2* and *RAC* gene expression decreased PDAC cell growth because the levels of ROS generated was reduced in PDAC cells (Du, Liu, Smith, Tsao, & Cullen, 2011). NOX4-derived ROS induced by IGF-1 and IGF-2 protected MiaPaCa-2 and Panc-1 cell growth from apoptosis, probably through activation of Janus Tyrosine Kinase(JAK)2/Signal transducer and activator of transcription(Stat)1/3 pathway (Friess, Guo, Nan, Kleeff, & Buchler, 1999; J. K. Lee et al., 2007; Vaquero, Edderkaoui, Pandol, Gukovsky, & Gukovskaya, 2004). Furthermore, TGF-β-induced EMT can be mediated by NOX-derived ROS by activating p38 MAPK and inhibiting protein tyrosine phosphatase 1B (Hiraga, Kato, Miyagawa, & Kamata, 2013). Besides, ROS can activate cell survival pathways, including JAK2/Stat1/3, p38 MAPK, and nuclear factor kappa (NF-κ)B (Yu & Kim, 2014), as well as cytokines (IL-1β, IL-6, IL-8, TNF-α, and TGF-β) expression for pancreatic

carcinogenesis (Friess et al., 1999; Green, 2003; Ju, Lim, Kim, & Kim, 2011; J. K. Lee et al., 2007; Wigmore et al., 2002). For example, the expression of these pro-inflammatory cytokines is higher in pancreatic cancer patients compared to healthy patients (Miron, Miron, Milea, & Cristea, 2010). Taken together, these evidences show that intracellular ROS at physiological levels are required for PDAC progression.

2.3.4 Interaction between ROS and CAFs

2.3.4.1 ROS contribution to myofibroblast differentiation

The cellular origin of myofibroblasts can be from many sources. In the view of adenocarcinomas, they can derive from epithelial cells via EMT (Neilson, 2006; Radisky et al., 2005; Zavadil, Haley, Kalluri, Muthuswamy, & Thompson, 2008; Zeisberg, Potenta, Xie, Zeisberg, & Kalluri, 2007). However, genetic alterations are rare in fibroblasts dissected from human breast tumors, suggesting that EMT is not the main origin for myofibroblasts (Qiu et al., 2008). Myofibroblasts can be derived from cells produced in bone marrow, including fibrocytes or mesenchymal stem cells, and other mesenchymal cell types, including endothelial cells, pericytes or pre-adipocytes (Dirat et al., 2011; Direkze et al., 2004; Jeon et al., 2008; Kidd et al., 2012; Paunescu et al., 2011; Quante et al., 2011; Spaeth et al., 2009; Zeisberg et al., 2007). Many studies have also reported that tumor-associated fibroblasts were mainly local resident fibroblasts (Erez et al., 2010; Hinz et al., 2007; Kojima et al., 2010; Ronnov-Jessen & Petersen, 1993; Toullec et al., 2010).

Many signaling pathways activated by ROS have been reported to induce the transition of normal fibroblasts into myofibroblasts. For example, pharmacological inhibition of ROS generated by mitochondria reduced the expression of NOX4 subsequently inhibited TGF-β-driven myofibroblasts differentiation (Jain et al., 2013). Besides, it has been shown that SDF-1 chemokine promotes myofibroblasts

differentiation in a ROS-dependent manner (Kojima et al., 2010; Toullec et al., 2010). Remarkably, mice depleted of $JunD^{-/-}$ or $Nrf2^{-/-}$ causing chronic oxidative stress due to the inactivation of antioxidant transcription factors, resulting in fibroblasts became activated and converted to myofibroblasts (Laurent et al., 2008).

Oxidative stress that contributes to myofibroblast differentiation can be derived from fibroblasts and cancer cells within the tumor bed as given in Figure 2.4. For example, highly reactive and diffusible H_2O_2 generated by NOX4 induce fibroblast differentiation to myofibroblasts (Niethammer, Grabher, Look, & Mitchison, 2009). In addition, myeloid cells infiltrating the tumor bed can be an additive ROS source while MMPs were reported to regulate the mitochondrial respiratory chain to increase intracellular ROS levels (Niethammer et al., 2009; Radisky et al., 2005).

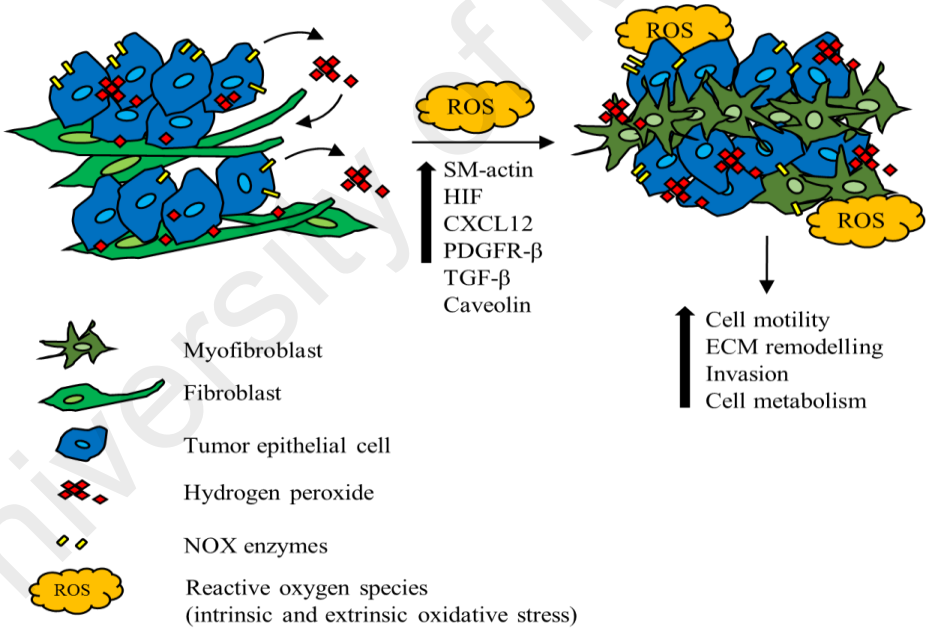

Figure 2.4: The roles of ROS in myofibroblast differentiation and crosstalk with tumor epithelial cells. Highly reactive and diffusible H_2O_2 induce chronic oxidative stress in the tumor microenvironment, with the higher amount generated by NOX from tumor epithelial cells and to a small extent from myeloid cells and fibroblasts. ROS regulate a few secretory factors from fibroblasts, such as hypoxia-inducible factor (HIF), SDF-1, PDGF, TGF-β, caveolin, etc, which are crucial for conversion of myofibroblasts from fibroblasts. Besides, ROS from activated fibroblasts stimulates paracrine signals to promote tumor cell motility, ECM remodeling, cellular metabolism, all of which are importance for tumor progression and metastasis.

2.3.4.2 Modulation of CAFs invasive properties by ROS

Many studies have revealed that CAFs possess tumor-prone potential as compared to their normal counterparts. Also, they are the sources of paracrine signals, which promote multiple cancer phenotypes, including cell survival, proliferation, neo-angiogenesis, invasion, inflammation, and ECM remodeling (Hanahan & Coussens, 2012; Hanahan & Weinberg, 2011; Hu et al., 2009; Hwang et al., 2008; Kojima et al., 2010; Olumi et al., 1999; Orimo et al., 2005; Polyak, Haviv, & Campbell, 2009; Shinohara et al., 2007; Vicent et al., 2012; G. Yang et al., 2006). Signaling pathways triggered by the pro-invasive signals (IL-6, VEGF, CXCL12, CXCL14, etc) can increase ROS levels in the stromal fibroblasts to promote cancer cell proliferation, neo-angiogenesis, and metastatic dissemination (Allinen et al., 2004; Bhowmick, Chytil, et al., 2004; Gerald et al., 2004; X. Guo, Oshima, Kitmura, Taketo, & Oshima, 2008; Orimo et al., 2005; Pietras, Sjoblom, Rubin, Heldin, & Ostman, 2003; Spaeth et al., 2009). In addition, CAFs promote squamous cancer cell migration most probably via Rho-dependent pathway (Gaggioli et al., 2007). This pathway is important for collective migration of cancer cells, as remodeling of ECM is occurring in a ROS-dependent manner (Sanz-Moreno et al., 2011).

Furthermore, CAFs also induce cancer invasion and metastasis by promoting EMT program. For example, overexpression of *MMP3* led to Rac1b expression and mitochondrial ROS generation, which subsequently induced EMT program in cancer cells (Radisky et al., 2005). Moreover, MMPs secreted by CAFs can enhance ROS generation in cancer cells via COX-2, which is required for EMT, stemness properties, and tumor dissemination (Giannoni, Bianchini, Calorini, & Chiarugi, 2011; Giannoni et al., 2010; Giannoni, Parri, & Chiarugi, 2012). The ROS-induced paracrine signals exerted by secretion of CAFs are regulated by protein tyrosine phosphatases (PTPases), such as tyrosine kinase receptors and integrins (Frijhoff, Dagnell, Godfrey, & Ostman, 2014;

Giannoni et al., 2011). Therefore, the oxidative tumor microenvironment is crucial to induce fibroblast differentiation to myofibroblasts and the paracrine signals generated can influence the invasion and metastasis of epithelial tumor cells.

2.4 Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2 (Nrf2) system in stress response and anabolic metabolism

2.4.1 Nrf2 and its regulation by Keap1

Keap1/Nrf2 is a major signaling pathway to combat oxidative stress and xenobiotic damage. Nrf2 is a basic region-leucine zipper (bZip)-type transcription factor that maintains redox homeostasis by regulating the antioxidant response element (ARE)-driven cytoprotective genes, encoding mainly antioxidants and detoxification enzymes (No, Kim, & Song, 2014). The intracellular activity of Nrf2 depends on its seven functional domains (Neh1 to Neh7). Neh2 domain is the major regulatory domain, with two bindings sites (termed ETGF and DLG motifs) for Keap1. Neh1 domain has DNA binding motifs while Neh3, Neh4, and Neh5 are involved in regulating the transactivation of Nrf2 target genes. Neh2, Neh6, and Neh7 mainly regulate the Nrf2 stability as shown in Figure 2.5A (Jaramillo & Zhang, 2013; McMahon, Thomas, Itoh, Yamamoto, & Hayes, 2006; Tong, Kobayashi, Katsuoka, & Yamamoto, 2006).

Keap1 is a dominant negative regulator for Nrf2. It has three functional domains, such as broad complex/tram track/bric-a-brac (BTB), intervening region (IVR), and Kelch domain (double glycine repeat (DGR)) as shown in Figure 2.5B. The Kelch domain with six Kelch repeats is used for Nrf2 binding while the cysteine residues of IVR have a role in oxidative stress sensing. The BTB domain is mainly used for Nrf2 ubiquitination by binding to Cullin E3 ubiquitin-based ubiquitin E3 (Cul3-E3) ligase as shown in Figure 2.5B (Lo, Li, Henzl, Beamer, & Hannink, 2006; Zipper & Mulcahy, 2002). Keap1 regulates Nrf2 activity via two mechanisms: the hinge and latch, and the Keap1-Cul3

dissociation models. In the hinge and latch model, Keap1 binds to the ETGF motif in the first place followed by the binding of DLG motif on the adjacent dock Kelch-repeat domain of Keap1. After Keap1 dimerizing, Keap1 interacts with Cul3-E3 ubiquitin ligase complex for Nrf2 polyubiquitination and proteasomal degradation (McMahon et al., 2006; Tong et al., 2007; D. D. Zhang, Lo, Cross, Templeton, & Hannink, 2004). In the Keap1/Cul3 dissociation model, oxidative stress disrupts the interaction between Keap1 and Cul3 E3 ligase (Taguchi, Motohashi, & Yamamoto, 2011).

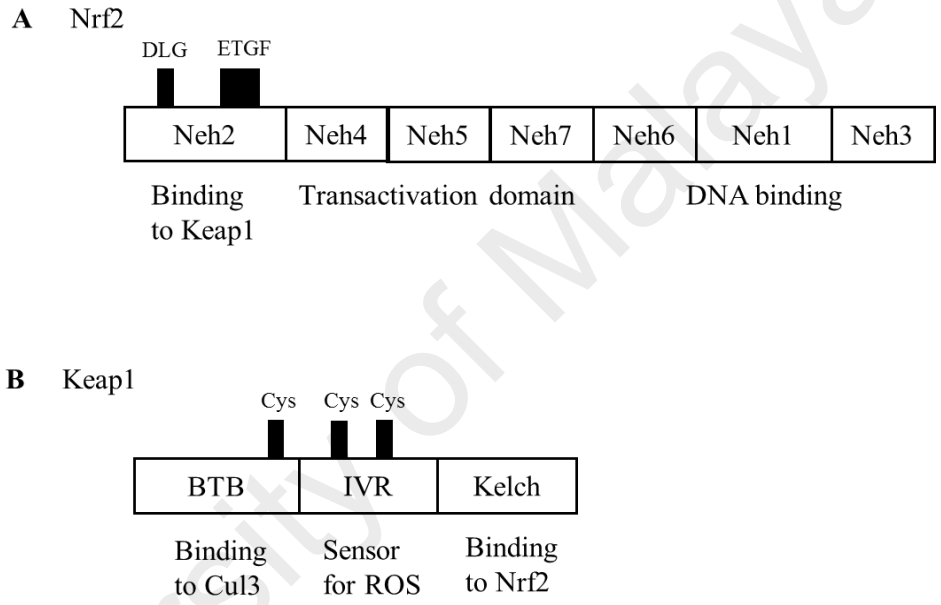

Figure 2.5: The different domains in the structures of Keap1 and Nrf2. Nrf2 has three functional domains, with Neh2 is the major regulatory domain containing two binding motifs (DLG and ETGF) to Keap1. Transactivation domain is represented by Neh4, Neh5, and Neh7 while Neh1, Neh3, and Neh6 are DNA binding domain. Similarly, Keap1 also harbors three functional domains. IVR acts as a sensor for ROS while Kelch and BTB are used for Nrf2 and Cul3 binding, respectively.

Under basal conditions, Keap1 forms a complex with Nrf2 and subjected to polyubiquitination before degradation by the 26S proteasomal protein (Xiang, Namani, Wu, & Wang, 2014). When response to oxidative stress or electrophiles, Nrf2 dissociates from Keap1 binding due to modification of cysteine residues of Keap1 (Taguchi et al., 2011). Nrf2 becomes stabilized and translocates into the nucleus, where it binds to ARE together with musculoaponeurotic fibrosarcoma (Maf) as a heterodimer in the promoter

of target genes for encoding cytoprotective genes to maintain redox homeostasis. When the intracellular ROS return to basal levels, Nrf2 translocates from nucleus to cytoplasm and binds to Keap1 for degradation as shown in Figure 2.6 (Sun, Zhang, Chan, & Zhang, 2007).

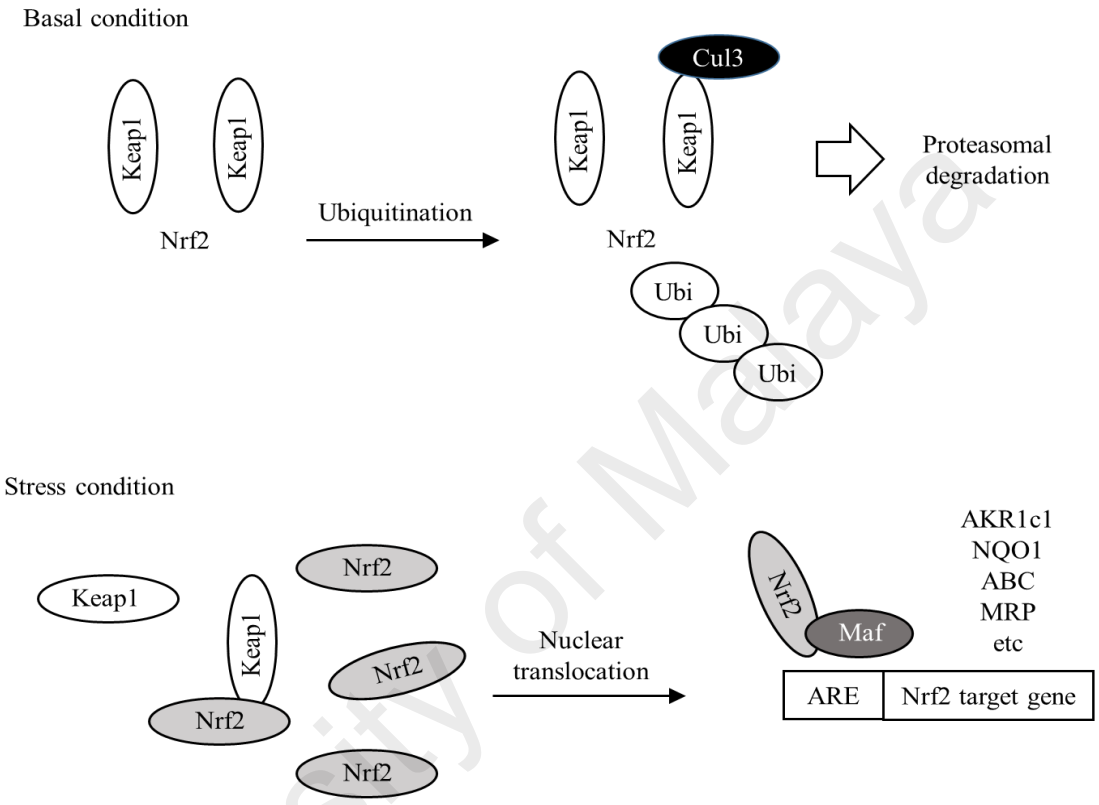

Figure 2.6: The Keap1/Nrf2/ARE signaling pathway. Under basal condition, Keap1 forms a complex with Nrf2 and subjected to polyubiquitination before degradation by the 26S proteasomal protein. In response to oxidative stress and electrophiles attack, Nrf2 dissociates from Keap1 binding and translocates into the nucleus to drive the cytoprotective genes to maintain redox homeostasis.

2.4.2 Nrf2/ARE target genes

Nrf2 binds to the ARE in the promoter of its target genes to regulate cellular responses against oxidative stress and electrophiles attack. Nrf2 target genes encode for enzymes that are involved in many cytoprotection functions, including antioxidant, phase II detoxifying enzymes, NAPDH production, drug transporters, intermediary metabolism, and proteasomal regulation (A. J. Hayes, Skouras, Haugk, & Charnley, 2015). The genes

that are regulated by Nrf2 in human have been studied using microarray analysis and *KEAP1* knockout in human cancer cells (J. D. Hayes, McMahon, Chowdhry, & Dinkova-Kostova, 2010). Nrf2 target genes can be classified into 5 groups: (1) antioxidants (glutamate-cysteine ligase (*GCL*) catalytic (*GCLC*), GCL modifier (*GCLM*), glutathione-disulfide reductase (*GSR*), glutathione peroxidase 2 (*GPX2*), sulfiredoxin 1 (*SRXN1*), solute carrier family 7 member (*SLC7A11*), and thioredoxin 1 (*TXND1*)); (2) drug detoxification enzymes and drug transporters (aldo-keto reductase 1b1 (*AKR*)*1B1*, *AKR1C1*, *AKR1C2*, *AKR1C3*, *AKR1C4*, carbonyl reductase 1 (*CBR1*), glutathione S-transferase M3 (*GSTM3*), multidrug resistance-associated protein 2 (*MRP2*), naphthoquinone 1 (*NQ1*), and prostaglandin reductase 1 (*PTGR1*)); (3) NADPH production enzymes (glucose-6-phosphate dehydrogenase (*G6PD*), phosphogluconate dehydrogenase (*PGD*), isocitrate dehydrogenase 1 (*IDH1*), and malic enzyme 1 (*ME1*)); (4) metal-binding proteins (ferritin heavy polypeptide 1 (*FTH1*), ferritin light polypeptide (*FTL*), metallothionein(MT)1 (*MT1*), and MT2); and (5) stress response proteins (growth arrest and DNA damage inducible 45 (*GADD45*), heme oxygenase 1 (*HMOX1*), heat shock protein(*HSP*)*40*, and *HSP70*) (J. D. Hayes et al., 2010).

KEAP1 downregulation in human keratinocytes led to upregulation of 23 genes, with *AKR* genes were induced to the highest (MacLeod et al., 2009). Thus, aldo-keto reductases (AKR) can be a useful biomarker for Nrf2 activation. As Keap1 can regulate the ubiquitination of NF-κB inhibitor (inhibitor kappa B (IκB) kinase beta (IKK-β)), thus upregulation of some Nrf2 target genes could be caused by the increase of NF-κB activity (D. F. Lee et al., 2009). Additionally, more than 100 Nrf2 target genes have been profiled in mice, with 70% of the genes are not limited to ROS detoxification. Most of the target genes are involved in inflammation and immunity (J. D. Hayes et al., 2010).

2.4.3 Tumor suppressor and oncogenic functions of Nrf2

The tumor suppressor function of Nrf2 has been reported in many studies. Using the *NRF2* knockout mice (*NRF2*$^{-/-}$), Nrf2 has been shown to mediate the tumor formation induced by the chemical carcinogens. For example, gastric cancer is more likely to occur in *NRF2* knockout mice in response to benzo(a)pyrene exposure compared to wild-type mice (Ramos-Gomez, Dolan, Itoh, Yamamoto, & Kensler, 2003). Furthermore, higher incidence of gastrointestinal and bladder tumors were reported in *NRF2*-deficient mice after exposure to azoxymethane followed dextran sodium sulfate and N-nitrosobutyl(4-hydroxybutyl)amine, respectively compared to wild-type mice (Fahey et al., 2002; Khor et al., 2008; Osburn et al., 2007). In addition, mice that were exposed to 7,12-dimethylbenz(a)anthracene (DMBA) or 12-O-tetradecanoylphorbol-13-acetate (TPA) could induce skin tumor (C. Xu et al., 2006). The protective role of Nrf2 against the carcinogenesis induced by the chemical carcinogens is mostly through the intracellular ROS detoxification and cellular DNA damage prevention (Hirayama et al., 2003; Morito et al., 2003).

In the mice that harbor single-nucleotide polymorphism (SNP) in the promoter region of *NRF2* gene, they are more susceptible to develop lung cancer during oxidative stress. For example, an SNP (rs6721961) in the promoter region of *NRF2* was found to increase the risk to develop non-small-cell lung cancer in human (Marzec et al., 2007; Suzuki et al., 2013; Yamamoto et al., 2004).

The oncogenic functions of Nrf2 are reported to favor the progression of several cancer types, although many evidences showed that its activation has a protective role against many diseases. Elevated levels of Nrf2 has been found in cancer of the head and neck, breast, lung, endometrial, ovarian, and pancreas. In addition, high levels of Nrf2 are associated with a poor prognosis, probably due to the impact of Nrf2 in promoting cancer cell proliferation, and conferring chemoresistance and radioresistance (Y. R. Kim et al.,

2010; Lister et al., 2011; Shibata, Kokubu, et al., 2008; Shibata, Ohta, et al., 2008; Singh, Bodas, Wakabayashi, Bunz, & Biswal, 2010; Singh et al., 2006; L. M. Solis et al., 2010; X. J. Wang et al., 2008; P. Zhang et al., 2010). *NRF2* is overexpressed in many PDAC. Human tissues with RNAi-mediated *NRF2* knockdown caused a decrease in proliferation of PDAC cells (Suit-2, MiaPaca-2, and FAMPAC) and enhanced their sensitivity to chemotherapeutic drugs, including gemcitabine, 5-fluorouracil and cisplatin, and also gamma radiation (Lister et al., 2011). Similarly, certain PDAC cells and tissues have elevated Nrf2 protein expression, which resulted in increased drug resistance via the upregulation of drug transporter gene expression, such as ATP Binding Cassette Subfamily G Member 2 (*ABCG2*) (Hong et al., 2010; Singh et al., 2006).

2.4.4 Molecular basis of Nrf2 activation in cancer cells

In many human tumors, *NRF2* are overexpressed due to several mechanisms. One of the prominent mechanisms is somatic mutations in *KEAP1*, *NRF2* or *CUL3*, although deletion or genomic amplification of *NRF2* is rarely found in cancer. Somatic *KEAP1* mutation is induced by amino acid (glycine-to-cysteine) substitution, which leads to the less binding affinity of mutant *KEAP1* and subsequently, E3 ubiquitin ligase lost its adaptor function to Nrf2. Almost all *KEAP1* mutations reported thus far are distributed in the Kelch/DGR domain. The first identification was reported in human lung cancer cell lines and tissues. *KEAP1* mutations are also present in other cancer types, including breast, gallbladder, liver, ovarian, endometrial, lung papillary, and pancreas (Konstantinopoulos et al., 2008; Q. K. Li, Singh, Biswal, Askin, & Gabrielson, 2011; Lister et al., 2011; Shibata, Kokubu, et al., 2008; Shibata, Ohta, et al., 2008; Singh et al., 2006; Sjoblom et al., 2006; Wong et al., 2011). Heterozygous mutation is the most common *KEAP1* mutation identified in cancer. This mutation generates three types of dimers, such as wild-type homodimer, wild-type-mutant heterodimer, and mutant homodimer at a ratio of 1:2:1

(Ohta et al., 2008; Padmanabhan et al., 2006; Shibata, Kokubu, et al., 2008; Singh et al., 2006; Suzuki, Maher, & Yamamoto, 2011). The wild-type-mutant heterodimer does not support Nrf2 ubiquitination, and heterozygous *KEAP1* mutations have reduced Keap1 activity to about 75%. It has been reported that 75% reduction in Keap1 activity is sufficient to cause Nrf2 accumulation in the nucleus (Taguchi et al., 2010).

NRF2 mutations appear less in human cancers compared to *KEAP1*. However, these mutations were found in several cancer types of head and neck, lung, and esophageal (Y. R. Kim et al., 2010; Shibata et al., 2011; Shibata, Ohta, et al., 2008). About 43% mutations in *NRF2* gene are clustered in DLG motif while 57% clustered in ETGF motif (Shibata, Ohta, et al., 2008). These two motifs are crucial for binding in the Kelch/DGR domain of Keap1. Mutations in these regions resulted in Nrf2 stabilization, nuclear translocation, and increased expression of its target genes.

Nrf2 stabilization can also be induced by epigenetic modification of *KEAP1* gene. The DNA hypermethylation of *KEAP1* gene was reported in prostate, lung, colorectal, and gliomas cancers (Muscarella, Barbano, et al., 2011; Muscarella, Parrella, et al., 2011; X. J. Wang et al., 2008; P. Zhang et al., 2010). The DNA hypermethylation occurred at the specific CpG sites of the promoter region, induced local chromatin remodeling, and inhibited transcriptional machinery from binding to DNA sequences of *KEAP1* (Copple, 2012). Furthermore, poor clinical outcomes were reported in lung and gliomas cancers due to the epigenetic alterations in *KEAP1* gene (Muscarella, Barbano, et al., 2011; Muscarella, Parrella, et al., 2011).

2.4.5 Dysregulation of Keap1/Nrf2 signaling in pancreatic cancer

The Keap1/Nrf2/ARE signaling pathway is a critical cellular defense mechanism to protect normal cells from oxidative stress. However, in PDAC cells harboring activated oncogenes, upregulation of ARE-driven genes is importance to provide survival and

proliferative advantages within the metabolic cancer microenvironment, and help to withstand the attack from immune regulation and cytotoxic drugs (J. D. Hayes & McMahon, 2009; Lau, Villeneuve, Sun, Wong, & Zhang, 2008). Many studies have reported that oncogenic activation can control transcription of Nrf2 gene. An insightful study carried out by DeNicola et al. (2011) demonstrated that oncogenic activation of $KRAS^{G12D}$ determined Nrf2 activity by activating Nrf2 transcription and reducing the ROS levels in primary fibroblasts via MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK)/Jun signaling pathway. Oncogenic activation of $BRAF^{V619E}$ and MYC^{ERT12} also increased *NRF2* gene expression and 60% of its target genes, thereby creating a more reduced intracellular environment which is favorable for tumorigenic activity. Jun and Myc small interfering(si)RNA results further support this observation, in which $KRAS^{G12D}$ and $BRAF^{V619E}$ used Jun and Myc to upregulate *NRF2* transcription (DeNicola et al., 2011).

The mutational prevalence of *KEAP1* and *NRF2* is PDAC is approximately one percent, which is considerably low when compared to other types of cancer. In the study carried out by DeNicola et al. (2011), more than 100 pancreatic cancer resection specimens were sequenced to detect the somatic mutations in *KRAS*, *NRF2*, and *KEAP1* genes. *KRAS* mutation was identified in most of the specimens in accordance to reported in the literature while no mutation was found for *NRF2* in all specimens examined. Only three nonsynonymous *KEAP1* mutations were observed (DeNicola et al., 2011). This observation is also supported by mutational analysis in PDAC cells. For example, Lister et al. (2011) reported that there was no *NRF2* mutation in all PDAC cells tested and only synonymous mutations in *KEAP1*. Additionally, there were no mutations of *KEAP1* and *NRF2* detected in 24 PDAC tissues examined (Jones et al., 2008; Lister et al., 2011). Therefore, overexpression of *NRF2* in pancreatic cancer most likely is not caused by the

somatic mutations in *NRF2* or *KEAP1* as reported in many biliary tract cancers (Shibata, Kokubu, et al., 2008).

A few studies have demonstrated that a direct increase of Nrf2 protein levels in PDAC cell lines and tissues. Elevated Nrf2 levels were mostly found in the cytoplasm of the malignant epithelial cells (DeNicola et al., 2011; Hong et al., 2010; Lister et al., 2011). For example, strong immunohistochemistry staining was detected in the cytoplasmic part in 48 out of 57 pancreatic cancer specimens compared to benign tissue. However, there is no nuclear staining difference found in both cancer and benign tissues (Lister et al., 2011). Similarly, Hong et al. (2010) also described that strong cytoplasmic Nrf2 staining was showed in both PDAC and normal tissues, the only difference with that strong nuclear staining in PDAC tissue compared to normal tissue. Furthermore, it has been reported that the high nuclear Nrf2 expression was associated with poor survival rate following pancreatic cancer resection (Soini et al., 2014). In contrast, Keap1 protein levels were detected higher in cellular nuclear in PDAC tissue compared to benign tissue. However, its detection was only in about 30% of pancreatic cancer specimen (Lister et al., 2011). These studies suggest that more Nrf2 protein levels were detected in PDAC compared to Keap1.

The role of Nrf2/ARE system in PDAC has first demonstrated in cell lines and clinical samples, in which moderate to strong immunohistochemical staining of Nrf2/ARE proteins was found in malignant epithelium. One example is NAD(P)H:quinone oxidoreductase 1 (NQO1), a prototypical Nrf2 downstream protein, highly overexpressed (about 10-fold) in pancreatic cancer tissue compared to normal tissue (Logsdon et al., 2003). Besides, high expression of NQO1 was also found in PanINs, suggesting that it may activate during early stage of pancreatic cancer (Awadallah et al., 2008; A. M. Lewis et al., 2005). Nonetheless, the use of NQO1 as pancreatic cancer biomarker may be poor because it also found in pancreatitis tissue (A. M. Lewis et al.,

2005; Lyn-Cook et al., 2006). The gene polymorphisms of *NQO1* was rarely detected in pancreatic cancer specimens compared to normal pancreatic tissue (Lyn-Cook et al., 2006). The important role of Nrf2 in upregulating this protein was further supported by DeNicola et al. (2011) that PanINs mice with *NRF2*-deficient were found negative for NQO1.

Aldo-keto reductase family 1 is another Nrf2/ARE downstream protein that is overexpressed in PDAC and many PanIN lesions. For example, *AKR1B10* has been found to be overexpressed in more than 70% in well- and moderately differentiated pancreatic cancer specimens compared to specimens with poorly and normal pancreas (Chung et al., 2012). While *AKR1C1* was expressed in a panel of PDAC cells (MiaPaCa-2, Panc-1, FAMPAC, PaCa-2, and Suit-2), which associated with Nrf2 expression. *NRF2* downregulation in these cell lines decreased the protein levels of AKR1C1 (Lister et al., 2011). These Nrf2 target proteins play a major role in pancreatic cancer as their inhibition could impede tumorigenesis and reduce activated Kras protein (H. Li et al., 2013).

2.4.6 Increased Nrf2 activity in pancreatic tumorigenesis

Increased Nrf2 activity plays a major role in oncogenic transformation in chronic pancreatitis and pancreatic tumorigenesis. In a PDAC mouse model with conditional transgenic Kras knock-in, PanINs formation, tumor volume, and cellular proliferation were reduced while elevated ROS levels were maintained (DeNicola et al., 2011). Paraxidocally, DeNicola et al. (2011) revealed a novel finding that the mutagenic oxidation of DNA caused by oxidative stress was reduced by activating the ROS detoxification program mediated by Kras, Braf, and Myc oncogenes. These oncogenes increased Nrf2 activity by binding to its promoter via MAPK signaling and independent of Keap1 expression (DeNicola et al., 2011). This observation is specific, as Nrf2/ARE

system cannot be activated by other proteins, such as Notch 1 and β-catenin (DeNicola et al., 2011).

Besides, the loss of tumor protein 53-induced protein 1 (*TP53INP1*) is associated with increased levels of Nrf2 (Al Saati et al., 2013). *TP53INP1* is a p53 target gene, which involves in cell cycle arrest, apoptosis, and p53-driven oxidative stress response. *TP53INP1* highly expressed in normal pancreas tissue and PanIN-1A but its expression only found in fewer PanIN-2s. The expression of *TP53INP1* is absent in high grade PanIN and PDAC (Gironella et al., 2007), suggesting that its expression is typically lost in the early stages of pancreatic carcinogenesis. The loss of *TP53INP1* expression has been shown to accelerate murine PanINs formation, and this was associated with an elevated Nrf2 expression (Al Saati et al., 2013). The absence of *TP53INP1* has led to lesser antioxidant activity, which may be induced by both p53-dependent or -independent mechanisms (Cano et al., 2009). This finding suggests that diminished TP53INP1 activity may increase secondary intracellular ROS levels and inactivate Keap1 activity to promote nuclear Nrf2 localization. The crosstalk between p53 and Nrf2 signaling may activate the alternative mechanism for increased Nrf2 levels under *TP53INP1* deficiency in pancreatic cancer (Rotblat, Melino, & Knight, 2012).

Chronic pancreatitis has long been suggested as a prominent risk factor for developing pancreatic cancer (Rivera et al., 1997; Whitcomb, 2004). One of the features in chronic pancreatitis is oxidative stress as contributed by alcohol metabolism, activated immune cells, and endoplasmic reticulum stress (Altomare, Grattagliano, Vendemiale, Palmieri, & Palasciano, 1996; O'Byrne & Dalgleish, 2001; Sah et al., 2014). Increased expression of *NRF2* and its target genes (*NQO1* and *HMOX1*) were found during proinflammatory state of chronic pancreatitis to confer protection from oxidative stress (Yang et al., 2012). When oncogenic *KRAS* was also present, pancreatitis-driven PanIN lesions from acinar-ductal metaplasia was accelerated. This progression was augmented

with increased Nrf2 levels (DeNicola et al., 2011; Y. Zhang et al., 2013). Furthermore, the contribution of PanIN progression to PDAC induced by oncogenic Nrf2 activation also depends on the IL-6/Stat3 pathway (Lesina et al., 2011; Y. Zhang et al., 2013).

2.5 Role of Nrf2 in metabolic reprogramming to promote cancer cell proliferation

2.5.1 Nrf2 promotes anabolic pathways in cancers

Nrf2/ARE system has been shown to activate the genes encoding detoxifying enzymes and antioxidants to combat oxidative stress and electrophiles. More recently, Nrf2 has the ability to regulate the metabolic pathways that are required to induce lung cancer cell proliferation. The key genes that are regulated by Nrf2 have been identified, whose products are involved in pentose phosphate pathway (PPP), producing both ribose-5-phosphate (R5P) and reducing equivalents nicotinamide adenine dinucleotide phosphate (NADPH), including glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD), transketolase (TKT), transaldolase 1 (TALDO1), and malic enzyme 1 (ME1). In addition, Nrf2 also regulates glutamine consumption by mainly regulating isocitrate dehydrogenase 1 (IDH1) to produce NADPH and glutathione biosynthesis (Mitsuishi, Taguchi, et al., 2012). Moreover, PI3K/Akt signaling pathway activation is needed in Nrf2-mediated metabolic gene transcription for lung cancer cell proliferation as shown in Figure 2.7.

Nrf2 alone is not sufficient to initiate cancer development. For example, *KEAP1* knockdown in mice to approximately 5% of its mRNA levels led to constitutive Nrf2 activation in many tissues, but no spontaneous cancer was found (Taguchi et al., 2010). Indirectly, this suggests that Nrf2 activity does not play a crucial role to initiate cancer development. Instead, it accelerates cancer cells proliferation and stress resistance once cancer cells are initiated.

PPP consists of the oxidative and non-oxidative arms. It is required to fulfill two cellular requirements. The first function is to produce R5P for purine nucleotide synthesis while the second function is to produce NADPH as reducing equivalents. R5P is a precursor for nucleotide synthesis, which is produced by the oxidative and non-oxidative arms of PPP. The oxidative arm is an irreversible mechanism, and it is used to produce NADPH. The oxidative arm also promotes cell growth by increasing thymidine incorporation via G6PD activity, which is a critical enzyme of the oxidative arm involved in NADPH production (Tian et al., 1998). The direction of the non-oxidative arm is determined by the cellular requirements between NADPH and R5P (Wamelink, Struys, & Jakobs, 2008). When the need for NADPH production is higher, the oxidative arm-derived pentose phosphates are subjected to glycolysis in a recycle mode (Boros et al., 2000). However, cancer cells always need a higher amount of nucleotide synthesis. Hence, both the oxidative and non-oxidative arms shift forward to produce R5P (Boros et al., 2000). The importance of the non-oxidative arm in cancer cell growth can be seen with an increased expression of transketolase like(TKTL)1, an active mutant form of TKT. It has been associated with a poor prognosis in urothelial and colon cancer patients (Langbein et al., 2006) while inhibition of TKTL1 suppresses hepatocellular carcinoma cell proliferation (S. Zhang, Yang, Guo, & Cai, 2007). Moreover, Nrf2 was able to render R5P in a low concentration and efficiently shifted glucose flux to both the oxidative and non-oxidative arms for purine nucleotides production by increasing the enzymes levels in both arms of PPP (Mitsuishi, Taguchi, et al., 2012). With PI3K/Akt signaling pathway mediation, Nrf2 increased the glucose uptake and glycolytic activity to supply the glycolytic intermediates (Elstrom et al., 2004).

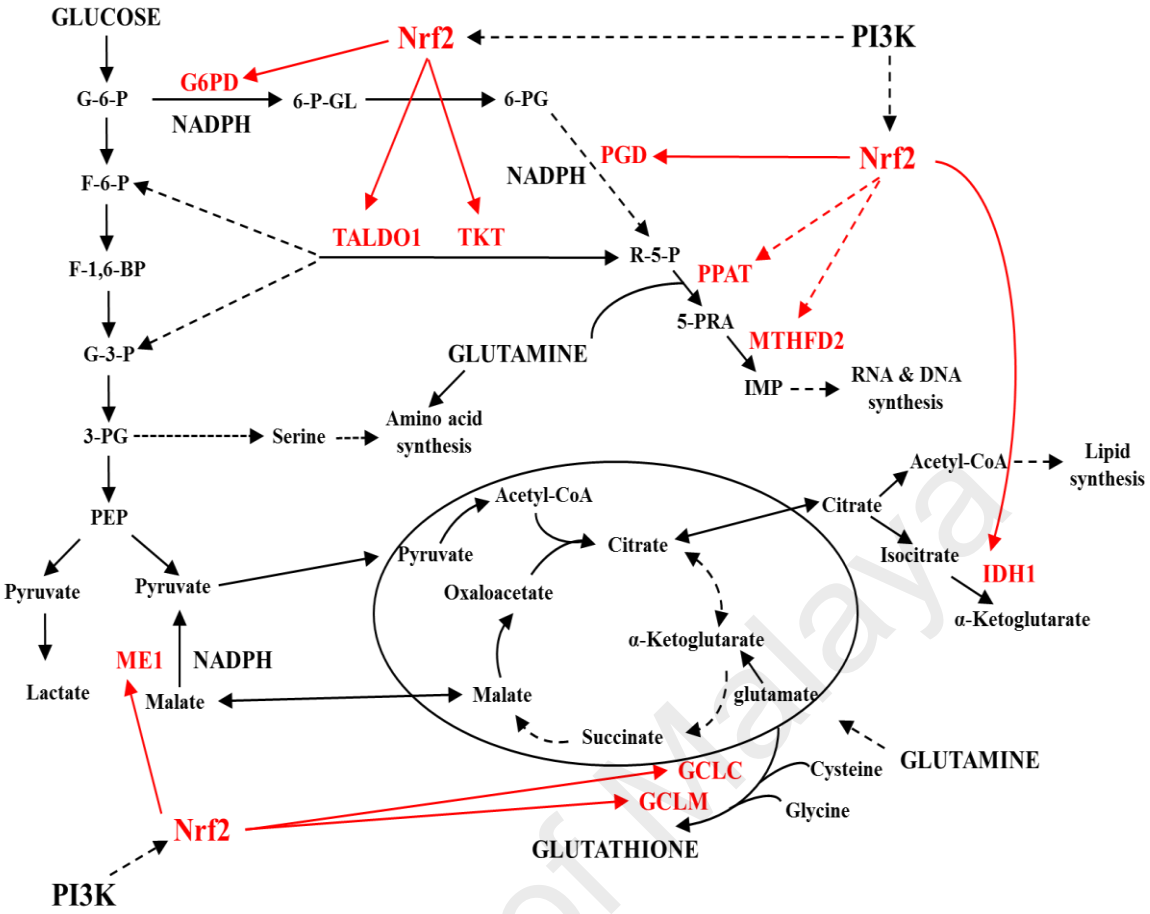

Figure 2.7: Metabolic pathways and their regulation in proliferating cells by Nrf2 transcription factor. The diagram illustrates the involvement of Nrf2 in the metabolic pathways of glycolysis, pentose phosphate pathway (PPP), TCA cycle, glutamine metabolism, and glutathione biosynthesis. Those red solid arrows or dash arrows indicate that genes that are directly or indirectly regulated by Nrf2, respectively.

2.5.2 Other regulators of PPP

In addition to Nrf2 and c-Myc, other regulators of PPP include oncogenic *KRAS* (*KRAS*^{G12D}), mammalian target of rapamycin complex 1 (mTORC1), and tumor suppressor p53. Oncogenic *KRAS*^{G12D} enhances the non-oxidative arm of PPP in pancreatic tumors with the mediation of myc (Ying et al., 2012). DeNicola et al. (2011) reported that oncogenic *KRAS*^{G12D}*-MYC* axis induced *NRF2* gene expression and also its activity in pancreatic cancer (DeNicola et al., 2011). Indirectly, this indicates that there is a positive association between Nrf2 and non-oxidative arm of PPP.

It has been reported that mTORC1 regulates metabolic flux by increasing glycolysis and the oxidative arm of PPP (Duvel et al., 2010). One of the downstream effectors of mTORC, sterol regulatory element binding proteins (SREBP1 and SREBP2) have been shown to regulate the enzymes involved in PPP under PI3K/Akt signaling pathway activation (Duvel et al., 2010). Since the activation of Nrf2 also required PI3K/Akt signaling to induce PPP, this may indicate that Nrf2 and SREBP have a synergistical role in the regulation of the oxidative arm of PPP.

Lastly, tumor suppressor p53 has been reported to prevent the formation of G6PD dimer and inhibit PPP (Jiang et al., 2011). In this regard, NADPH production was inhibited by wild-type p53 and led to cancer cell death due to pro-apoptotic effect of high intracellular ROS levels. However, tumor-associated p53 mutant did not inhibit G6PD but allowed for a high NADPH production to create a relatively more reduced environment to promote cancer cell proliferation (Jiang et al., 2011). Therefore, tumor-associated p53 mutant may play a beneficiary role to mediate Nrf2-induced PPP in cancers.

2.5.3 Detoxification of ROS for cell survival and proliferation

Under the control of Nrf2, the redox homeostasis in cancer is regulated by Nrf2-mediated metabolic reprogramming mainly involved PPP, glutaminolysis, and glutathione biosynthesis. Many studies have demonstrated that frequently mutated tumor suppressor and oncogenic pathways render high intracellular ROS levels (A. C. Lee et al., 1999; Nogueira et al., 2008; Szatrowski & Nathan, 1991; Vafa et al., 2002). Therefore, an efficient ROS detoxification program is a requisite for cell proliferation. Glutathione is widely known as a key molecule to maintain redox homeostasis by detoxifying ROS and reducing the oxidized proteins (MacLeod et al., 2009).

Many evidence have shown that Nrf2 directly activates the genes that are involved in glutathione biosynthesis, especially *GCLM* and *GCLC* subunits as shown in Figure 2.7 (Bea, Hudson, Chait, Kavanagh, & Rosenfeld, 2003; Moinova & Mulcahy, 1999; Sekhar et al., 2003; W. A. Solis et al., 2002). Furthermore, Nrf2 also increases the expression of cystine transporter *SLC7A11* (xCT), whose product is able to increase the cysteine availability for glutathione synthesis (Sasaki et al., 2002). In addition, Nrf2 has the potential to mediate directly the gene expression of four major enzymes producing NADPH, including *G6PD* and *PGD* of PPP, *ME1*, and *IDH1* of glutaminolysis, which are involved in glutaminolysis (Mitsuishi, Taguchi, et al., 2012). Therefore, Nrf2 produces glutathione and NADPH to create a reduced environment for cancer growth.

The activation of metabolic genes involved in NADPH production can be synergistically regulated by Nrf2 and glycolytic pyruvate kinase isozyme type M2 (PKM2). Inhibition of PKM2 by high ROS concentration redirects the glucose into PPP to generate NADPH for ROS detoxification (Anastasiou et al., 2011). Silencing of *NRF2* gene expression was found to increase the metabolites concentration of PPP for NADPH production while decreased the metabolites concentration required for nucleotide synthesis (Mitsuishi, Taguchi, et al., 2012). Therefore, high levels of intracellular ROS may activate Nrf2 transcription, which enhances the PPP utilization to produce NADPH in order to maintain redox homeostasis.

2.6 Roles of interleukin(IL)-6 in pancreatic cancer

2.6.1 IL-6 signaling

IL-6 is a multifunctional cytokine that mainly regulates inflammation and immune responses. It is grouped into a cytokine family consisting of oncostatin M (OSM), IL-11, cardiotrophin-1 (CT-1), ciliar neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and cardiotrophinike cytokine (CLC) (Heinrich et al., 2003; Heinrich, Behrmann,

Muller-Newen, Schaper, & Graeve, 1998). There are two types of IL-6 receptors: (1) cell membrane IL-6 receptor (IL-6R), which has a low binding affinity for glycoprotein 130 receptor (gp130) component to JAK; (2) soluble IL-6 receptor (sIL-6R) binds to the membrane receptor β chain - gp130, leading to the intracellular signal activation (Imada & Leonard, 2000; Scheller, Ohnesorge, & Rose-John, 2006). The signaling derived from IL-6/IL-6R is called classic signaling while sIL-6R-activated IL-6 signaling is called trans-signaling (Holmer, Goumas, Waetzig, Rose-John, & Kalthoff, 2014). The activated JAK signaling induced by IL-6/IL-6 receptor binding leads to the activation of Stat3 as shown in Figure 2.8 (Heinrich et al., 2003; Imada & Leonard, 2000). gp130 was found to have no intrinsic kinase, but it can be associated with JAK1, JAK2, and tyrosine kinase 2 (TYK2) of the JAK family (Calo et al., 2003; Heinrich et al., 2003; Heinrich et al., 1998). The activated Stat3 signaling regulates the expression of apoptotic regulatory genes which includes *MYC*, *XIAP*, *MCL-1*, and *FAS* (Darnell, 1997). In addition to Stat3 signaling pathway, IL-6 is also capable of activating Ras, MAPK, P13K/Akt, Cox-2, and Wnt signaling pathways (Arad, Bar-Oz, Ergaz, Nir, & Barak, 2010; Jee et al., 2004).

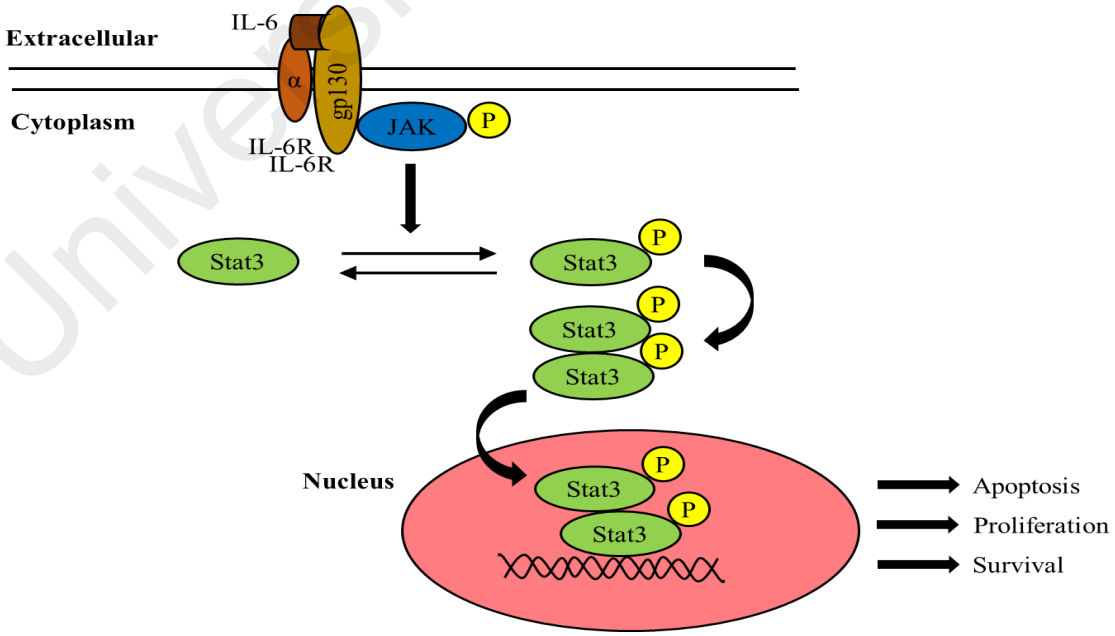

Figure 2.8: IL-6/JAK/Stat3 signaling pathway. After IL-6 binds to IL-6R, a cascade of phosphorylation JAK kinase is induced that leads to the activation of Stat3. Activated Stat3 translocates into the nucleus, where it drives the transcription of its target genes that are involved in apoptosis, proliferation, and survival.

2.6.2 A key role of IL-6 in pancreatic cancer development and progression

Many studies have reported that patients with PDAC or chronic pancreatitis have higher levels of IL-6 in their serum compared to healthy controls (Barber, Fearon, & Ross, 1999; Bellone, Carbone, et al., 2006; Ebrahimi, Tucker, Li, Abbruzzese, & Kurzrock, 2004; Lesina et al., 2011; Mroczko, Groblewska, Gryko, Kedra, & Szmitkowski, 2010; Okada et al., 1998; Talar-Wojnarowska et al., 2009; Wenger et al., 1999; Wigmore et al., 2002). Furthermore, IL-6 levels were positively correlated with the tumor stage, size, and distant metastases in PDAC patients (Mroczko et al., 2010; Okada et al., 1998; Talar-Wojnarowska et al., 2009; Wenger et al., 1999). PDAC patients with resectable tumors had higher IL-6 serum levels compared to those with non-resectable tumors (Mroczko et al., 2010). Moreover, high IL-6 serum levels were associated with poor survival and performance status (Bellone, Smirne, et al., 2006; Ebrahimi et al., 2004; Mroczko et al., 2010). IL-6 serum levels can be an independent predictor of PDAC poor prognosis (Mitsunaga et al., 2013). Therefore, IL-6 can be a useful clinical tumor biomarker for pancreatic cancer diagnosis rather than using the carbohydrate 19-9 (CA19-9) and carcinoembryonic antigen (CEA) or the inflammatory molecule (C-reactive protein, CRP) (Mroczko et al., 2010). However, its ubiquitous expression in many diseases, indicating that IL-6 is not very specific to distinguish pancreatic cancer from other cancer types (Yao et al., 2014).

Several intracellular proteins govern the expression of IL-6 in PDAC cells. One of which is mesothelin, which is claimed to be a useful PDAC biomarker (Johnston et al., 2009). Its expression has been shown to increase IL-6 expression in PDAC cells and patient samples (Bharadwaj, Marin-Muller, Li, Chen, & Yao, 2011). Furthermore, the proliferation of normal mesothelin-expressing cells was increased with exogenous IL-6 treatment. In the contrary, *IL6* knockdown led to the reduction of mesothelin-overexpressing cell proliferation (Bharadwaj et al., 2011). In addition, the receptor for

advanced glycation end products (RAGE) was reported to control IL-6 expression (Bharadwaj et al., 2011; Kang et al., 2012). Using short-hairpin(sh)RNA, downregulation of *RAGE* expression reduced the IL-6 secretion in human Panc2.03 and murine Panc02 cells, and also diminished the ATP production induced by exogenous IL-6 (Kang et al., 2012). Moreover, oncogenic *KRAS*, zinc transporter, and microRNA(miR)-21 were also reported to be a contributor to increasing IL-6 expression (Ancrile, Lim, & Counter, 2007; Bao et al., 2012; Y. Zhang et al., 2010).

Exogenous IL-6 treatment can increase the expression of VEGF and MMP-2. It plays a crucial role in angiogenesis and invasion (Huang et al., 2010; Masui et al., 2002; R. F. Tang et al., 2005). It has been shown to create a pro-tumorigenic environment for PDAC by inducing the expression of granulocyte macrophage colony-stimulating factor (GM-CSF) and Th2 cytokines of IL-5, IL-7, IL-6-10, and IL-13 (Feurino et al., 2007). However, the cell proliferation-promoting role of IL-6 is only observed in certain PDAC cells, especially those cells with mesothelin overexpression (Huang et al., 2010; Kang et al., 2012; Saito et al., 1998).

Most of the studies used $KRAS^{G12D}$ mouse model to analyze the role of IL-6 in PDAC development and progression *in vivo* because its Cre expression in the pancreas can induce PanIN lesions that recapitulate human PanINs. The PanINs formation could progress to invasive and metastatic PDAC; thereby this model is an appropriate system to study PDAC development. Compared to control mice, the pancreas of $KRAS^{G12D}$ mice showed elevated *IL6* mRNA mostly contributed by infiltrating immune cells, including F4/80-positive macrophages (Lesina et al., 2011). Oncogenic $KRAS^{G12D}$-induced elevated IL-6 expression increased the frequency of PanIN lesions, especially macrophage-derived IL-6 seems to be necessary for PanIN progression (Lesina et al., 2011). Furthermore, inhibition of IL-6 trans-signaling with sgp130 ($Kras^{G12D}$, sgp130Fc) helps to reduce PanIN development (M. Fischer et al., 1997; Lesina et al., 2011). Interestingly, primary

cells derived from *KRAS*^{G12D} mice had Stat3 phosphorylation after IL-6 treatment. This result suggests a switch from trans-signaling to classic IL-6 signaling in the later stages of tumor development (Lesina et al., 2011).

Zhang et al. (2013) used iKras* mouse model and showed that *IL6* mRNA was elevated in iKras* pancreata with embryonic Kras activation compared to control. They also found that pancreatitis-induced neoplastic lesions in iKras* mice generated more high-grade PanINs compared with iKras* *IL6*$^{-/-}$ mice (Y. Zhang et al., 2013). Intriguingly, the stromal of PanINs was transformed into normal pancreatic parenchyma in the absence of IL-6 (Y. Zhang et al., 2013). IL-6 also protected PDAC cells from oxidative stress caused by oncogenic *KRAS*^{G12D} by enhancing the activity of Nrf2 (Y. Zhang et al., 2013). A subcutaneous mouse model was also used to study the PDAC cell progression after *IL6* knockdown. However, an inconsistent effect in different PDAC cells was seen (Ancrile et al., 2007). One possible explanation is that this model does not mimic the complex microenvironment of pancreatic cancer (Bibby, 2004). These evidences indicate that IL-6 is important for precursor lesions development and progression of PDAC as summarized in Figure 2.9. Therefore, targeting IL-6 either secreted by infiltrating stromal cells or cancer cells themselves may provide new insights for PDAC therapies.

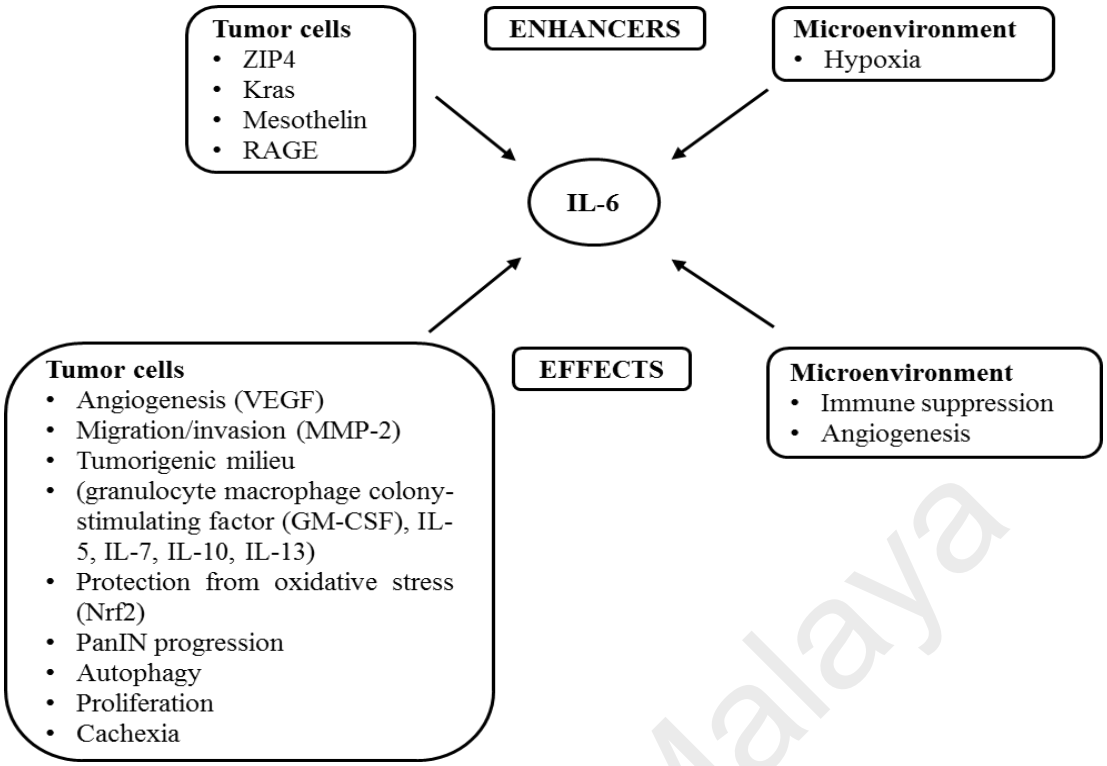

Figure 2.9: The pro-tumorigenic roles of IL-6 in pancreatic cancer. IL-6 plays a major role in PDAC development and progression. It produced by secretory factors from tumor cells and under hypoxic tumor microenvironment. In turn, IL-6 acts on tumor cells and microenvironment by activating the genes as listed in the bracket, that are involved in different cellular signaling pathways to promote PDAC tumorigenesis.

2.7 Epithelial-mesenchymal transition (EMT)

2.7.1 Characteristics of EMT

Tumor cells are mostly carcinoma and derived from epithelial tissue, and most of the invading and metastasizing tumor cells are reported to share the characteristics of mesenchymal cells. This finding suggests a transition of tumor cells from epithelial to mesenchymal characteristics (Maier, Wirth, & Beug, 2010; Thiery, Acloque, Huang, & Nieto, 2009) via a process called epithelial-mesenchymal transition (EMT) as shown in Figure 2.10.

Epithelial cells have cobble-stoned or polygonal shape, strong cell-cell adhesion organized in cell layers, apico-basolateral polarization, which limits their mobility. These characteristics distinguish them from other cell types. In contrast, mesenchymal cells

show reverse characteristics of epithelial cells, whereby they appear to have spindle-shaped, focal cell-cell adhesion organized in three-dimensional matrix, anterior-posterior polarity, which confers them to have greater migratory potential. Cells losses their epithelial properties and acquired mesenchymal characteristics during EMT. Studies using molecular analysis revealed that E-cadherin, occluding, claudin, and certain cytokines are markers of epithelial cells while mesenchymal cells acquired the markers of N-cadherin, fibronectin, vimentin, etc. The EMT process is reversible, in which cells can employ mesenchymal-epithelial transition (MET) process to change back to their epithelial state (Kalluri & Weinberg, 2009).

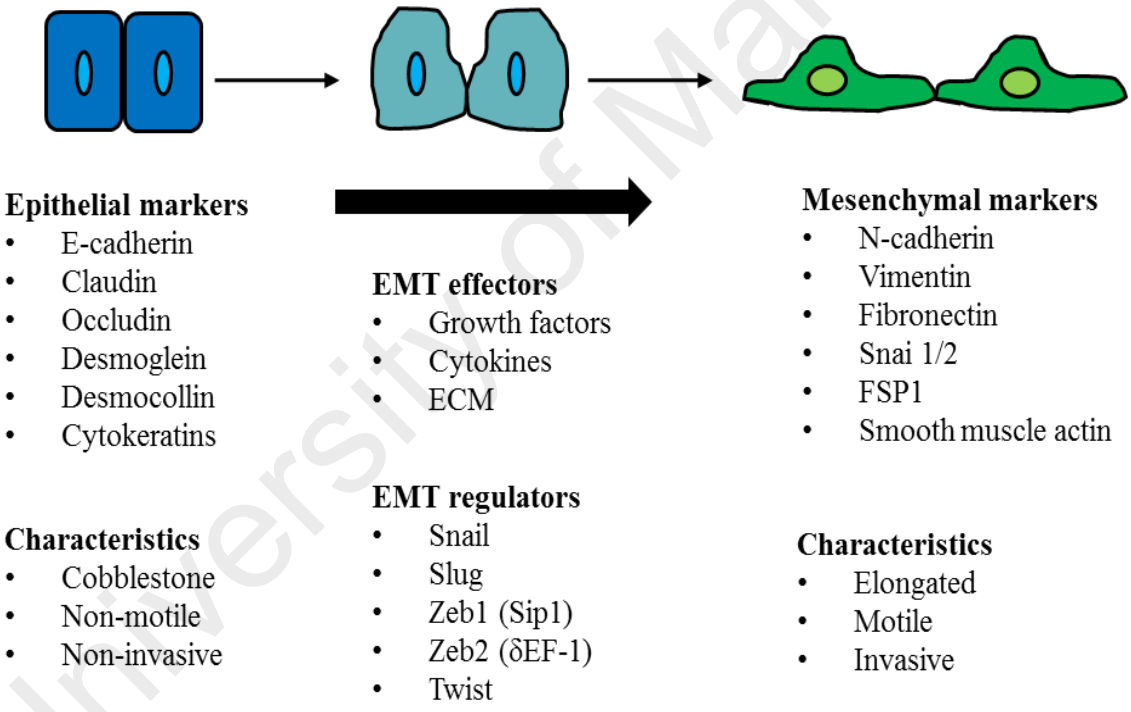

Figure 2.10: Epithelial-mesenchymal transition. During the progression of cancer, the tumor epithelial cells lose their characteristics and gain the characteristics of mesenchymal cells. EMT can be induced and regulated by growth factors (PDGF, TGF-β, etc), cytokines (IL-6, IL-8, etc) and ECM components. The epithelial markers (E-cadherin, cytokeratins, etc) and mesenchymal markers (N-cadherin, vimentin, etc) are regulated by EMT transcription factors (Snail, Slug, Sip1, Twist, etc).

2.7.2 EMT induction in pancreatic cancer

Extracellular signals emitted by soluble factors or non-soluble factors can induce EMT, in particular, the members of TGF-β and others growth factors, including EGF, FGF, or HGF family members (Thiery & Sleeman, 2006). The receptors that are important to induce EMT signaling include receptor tyrosine kinases, TGF-β receptors, and receptors from ECM. The signal relayed by several signaling pathways has been shown to upregulate the expression of EMT transcription regulators (Snail, ZEB, and basic helix-loop-helix (bHLH)), which resulted in the repression of E-cadherin (Peinado, Olmeda, & Cano, 2007).

Soluble factors that activate EMT in PDAC can be grouped into three (Maier, Wirth, et al., 2010). The three groups are (1) bone morphogenetic proteins (BMPs), which are members of TGF-β superfamily; (2) growth factors that function via receptor tyrosine kinases (for examples, hepatocyte growth factor (HGF) and VEGF); (3) third group is growth factors that emit their signals via other pathways, for example, TNFα or periostin. These growth factors can be produced by stromal cells (for example PSC) or cancer cells themselves, which are necessary for EMT induction in PDAC (Maier, Wirth, et al., 2010). BMPS, specifically BMP-2, BMP-4, and BMP-7 were reported to promote EMT in Panc-1 cells, in which their function depends on SMAD1 activation, inactivation of TβRIII, and increased MMP-2 activity (Gordon, Kirkbride, How, & Blobe, 2009; Hamada et al., 2007). Additionally, increased motility and invasiveness were associated with the inactivation of TβRIII (Gordon, Dong, Chislock, Fields, & Blobe, 2008). The mediation of tyrosine kinase receptors to EMT program in PDAC is often reported with the binding of their respective ligands. For example, decreased expression of Axl led to reduced binding to its receptor, c-Met and these associated with the downregulation of EMT effectors, including *MMP-9*, *TWIST*, *SNAIL*, and *SLUG* (Koorstra et al., 2009). Moreover, PDAC L3.6pl cells were showed to increase migration and invasion, and EMT after

activating vascular endothelial growth factor receptor-1 (VEGFR-1, Flt-1) by VEGF-A or VEGF-B (A. D. Yang et al., 2006). The stimulation of EMT by TNFα in PDAC can occur without the mediation of SMAD4 or enhanced the expression of Snail transcription factor (Baran et al., 2009; Maier, Schmidt-Strassburger, et al., 2010). Periostin is mainly secreted by PSC and to a lesser extent by cancer cells themselves. It promotes invasion of PDAC cells by producing fibrous environment. However, periostin may be able to promote MET as well (Baril et al., 2007; Erkan et al., 2007; Kanno et al., 2008; Ruan, Bao, & Ouyang, 2009).

EMT program is regulated by transcription repressors, including ZEB, Snail, and bHLH (basic helix-loop-helix) families (Peinado et al., 2007). *ZFHXIA* and *ZFHXIB* genes encode for zinc finger transcription factors, ZEB1 (δEF1) and ZEB2 (SIP1, for SMAD-interacting protein). The expression of ZEB1 can be induced by the activation of IKK/NF-κB in PDAC and it acts as a transcriptional repressor for E-cadherin (Eger et al., 2005; Maier, Schmidt-Strassburger, et al., 2010). Furthermore, ZEB1 is capable of inactivating the miR-200 family members (miR-141 and miR-200c), which are the negative regulators of EMT promoters TGF-β2 and ZEB1 (Burk et al., 2008). Furthermore, ZEB1 also plays a role in other biological processes in PDAC, including stemness via the inhibition of miRNAs (Wellner, Brabletz, & Keck, 2010; Wellner et al., 2009). The role of ZEB2 in mediating EMT in PDAC can be observed in a way that *ZEB2* mRNA levels is negatively associated with E-cadherin expression, but positively associated with advanced tumor stages. In summary, ZEB1 and ZEB2 are regulated by co-factors with two roles of activating or repressive (Peinado et al., 2007).

Twist and E47 (encoding by *E2A* gene) are examples of basic helix-loop-helix transcription factors which are essential for EMT. E47 and Twist transcription factors have been shown to inactivate EMT-related genes, especially E-cadherin that encoded by *CDH1* (Peinado et al., 2007). This effect is also observed in Snail superfamily of zinc-

finger transcriptional repressors, which consists of Snail (SNAI1) and Slug (SNAI2) (Peinado et al., 2007). Snail expression was reported to be associated with an EMT inducer, integrin-linked kinase (ILK) (Schaeffer et al., 2010). Furthermore, ectopic Snail expression in PDAC BxPC-3 cells is correlated with EMT, invasion, and metastasis induction (Nishioka et al., 2010).

2.7.3 PSC induce invasiveness and EMT in pancreatic cancer

There are accumulating evidence showing that PSC promote pancreatic cancer invasiveness by inducing EMT. When PDAC cells were co-cultured with PSC, higher expression levels of vimentin and Snail were observed while E-cadherin and membrane-associated β-catenin levels were decreased compared to mono-cultured cells (Fujita et al., 2009). In addition, Kikuta et al. (2010) showed that PDAC cells (Panc-1 and Suit-2) were morphologically changed to fibroblast-like with scattered and loose cell contacts when co-cultured with PSC. They further demonstrated that the expression of E-cadherin, cytokeratin 19, and membrane-associated β-catenin was decreased, whereas higher expression was observed for vimentin and Snail in co-cultured cells (Kikuta et al., 2010). The decreased E-cadherin expression induced by PSC was not altered by TGF-β neutralizing antibody, suggesting that TGF-β is not involved in PSC-induced EMT (Kikuta et al., 2010). Furthermore, the release of thrombospondin-2 from PSC stimulated the migration and invasion in Panc-1 cells (B. Farrow, Berger, & Rowley, 2009). When stromal cell-derived factor 1 (SDF-1) was neutralized in the conditioned medium from PSC, the migration and invasion of AsPC-1, BxPC-3, and SW1990 were reduced (Gao, Wang, Wu, Zhao, & Hu, 2010). Besides, IL-6 secreted by PSC was showed to promote invasive ability of Panc-1 and MiaPaCa-2 cell by inducing Stat3 signaling (Q. Z. Guo, 2014). Hwang et al. (2008) demonstrated that the proportion of developed metastases in mice co-injected with PSC and PDAC cells (BxPC-3) was relatively higher compared to

mice injected with PDAC cells alone. Similar observation was reported by Vonlaufen et al. (2008) that co-injection of PSC with MiaPaCa-2 cells exhibited enlarged tumor size, regional and distant metastasis, and fibrotic bands containing activated PSC in the tumors.

2.7.4 Potential role of IL-6 to promote EMT in pancreatic cancer

Many lines of evidence have showed that IL-6 can induce EMT event to drive the invasiveness in several cancers types of breast, lung, and prostate (Blunt et al., 2015; Sullivan et al., 2009; Xie et al., 2012; Yadav, Kumar, Datta, Teknos, & Kumar, 2011; Zhao et al., 2014). The role of IL-6 in inducing EMT in breast, ovarian, and lung cancers is mainly via JAK/Stat3 signaling pathway (Colomiere et al., 2009; Sullivan et al., 2009). The potential roles of IL-6 in inducing EMT in PDAC cells were also observed. For example, downregulation of *IL6* expression from CAFs with retinoic acid inhibited the migration and EMT in AsPC-1 and Panc-1 cells (Ali et al., 2015; Guan et al., 2014; Ohlund, Elyada, & Tuveson, 2014). Hamada et al. (2016a) also demonstrated that IL-6 secreted by PSC promoted migration and the expression of EMT-related markers (Snail and Cadherin-2) by activating Stat3 signaling in Panc-1 and Suit-2 cells. These evidences indicate the important role played by IL-6 in regulating EMT.

2.7.5 Involvement of Nrf2 in promoting EMT

There are less evidence showing the involvement of Nrf2 in EMT in cancer. However, its role has been demonstrated in other diseases. For example, Nrf2 was associated with TGF-β1 to induce EMT event in chronic renal diseases. Nrf2 activation was repressed after TGF-β1 binded to E-cadherin, a potent epithelial marker (Choi et al., 2014; W. D. Kim, Kim, Cho, Lee, & Kim, 2012). In the context of cancer, Nrf2 was reported to have a double-edge sword role in regulating EMT program and may be cancer type-specific. The positive regulation of Nrf2 in EMT phenotypes was showed after

silencing *NRF2* gene. Blockage of Nrf2 signaling suppressed the migration and invasion of esophageal squamous cancer cells under hypoxic condition (Shen et al., 2014). Moreover, the activation of Nrf2 signaling with its activator, *tert*-butylhydroquinone (tBHQ) was able to increase the expression of vimentin and Slug while decreased E-cadherin levels in pre-malignant human pancreatic ductal epithelial and PDAC cells (Colo357) (Arfmann-Knubel et al., 2015). However, increased tumor cell motility in SMAD-dependent manner and growth in soft agar were observed in colon and hepatocellular carcinoma cells after Nrf2 suppression mediated by either Keap1-directed ubiquitination or RNAi (Rachakonda et al., 2010). These findings have suggested the importance role of Nrf2 in EMT event. However, its role in PSC-mediated EMT in PDAC is still unclear.

CHAPTER 3: METHODOLOGY

3.1 Chemicals

Dehydroisoandrosterone (DHEA) was purchased from Acros Organics (Geel, Belgium) and 2',7'-dichlorofluorescein diacetate (DCF-DA) was obtained from Life Technologies (CA, USA). Glucose 6-phosphate, 6-phosphogluconate, ribose 5-phosphate, inosine 5'-monophosphate, glutamate, malate, 4-nitrobenzoic acid, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (MO, USA). All-trans retinoic acid (ATRA) and CYT-387 were purchased from Cayman Chemical (Michigan, USA). *tert*-butylhydroquinone (tBHQ) was obtained from International Laboratory (San Francisco, CA, USA). Crystal violet powder was obtained from Rocky Mountain Reagents (CO, USA), while Stattic was purchased from Santa Cruz Biotechnology (Texas, USA).

3.2 Cell culture

Human pancreatic adenocarcinoma cell lines AsPC-1 (CRL-1682), BxPC-3 (CRL-1687), and Panc-1 (CRL-1469), and immortalized human normal endometrial fibroblast T-HESC (CRL-2923) were purchased from American Type Culture Collection (VA, USA). Human primary pancreatic stellate cells (PSC) were provided by a collaborator, Associate Professor Dr. Masamune Atsushi from Tohoku University. PSC were isolated from the resected pancreas tissues of one patient undergoing operation for pancreatic cancer (Masamune, Kikuta, Watanabe, Satoh, Hirota, et al., 2008) under the approval of the Ethics Committee of Tohoku University School of Medicine (article#: 2015-1-433). Human endometrial adenocarcinoma primary fibroblast cell (EC6/Fib) were isolated from resected endometrium tissues of patients undergoing operation for endometrial cancer (Subramaniam et al., 2013) under the approval of the Ethics Committee of University Malaya Medical Centre (Reference no.: 865.19). Stringent cares

were needed to maintain the viability of primary PSC and EC6/Fib and to avoid contamination. AsPC-1, BxPC-3, and EC6/Fib cells were maintained in RPMI-1640 media (ThermoFisher Scientific, MA, USA) while Panc-1 cells were maintained in DMEM media (ThermoFisher Scientific). PSC and T-HESC cells were maintained in DMEM/F-12 media (ThermoFisher Scientific). All media were supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and 1% penicillin sodium/streptomycin sulfate (Life Technologies) at 37 ^{o}C in 95% humidified atmosphere of 5% CO_2. All the cells were routinely tested for *Mycoplasma* infection by using Venor^{TM} GeM Mycoplasma PCR detection kit (Sigma-Aldrich, Missouri, USA).

3.3 Preparation of PSC-conditioned media (PSC-CM)

PSC were grown in 100 mm cell culture dish to 70-80% confluency for 48 hours using DMEM/F-12 complete media. At 48 hours, the cells were washed once with 10 ml of Dulbecco phosphate buffered saline (DPBS) (Life Technologies) before changing to 10 ml of DMEM/F-12 media containing 0 or 1% FBS for additional 72 hours. The supernatant was then collected and concentrated with Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore, MA, USA) by centrifugation at 5000 x g at 4^{o}C for 1 hour. The protein concentration was quantified using Bradford assay (Bio-Rad, CA, USA). The conditioned media was aliquoted into microcentrifuge tubes and stored at -80^{o}C freezer before use.

3.4 Cell viability and proliferation assays

3.4.1 MTT assay

Briefly, 1 x 10^4 of PDAC cells (AsPC-1, BxPC-3, and Panc-1) were plated into 96-well plate for 24 hours before serum-starving for additional 24 hours. The cells were then treated with complete media, control media (media containing 1% FBS) or PSC-CM

(1.0 µg/µl) with or without recombinant human (rh) proteins (GRO-α, SDF-1α, and IL-6) in the presence of control media (BioLegend, CA, USA) or IL-6 neutralizing (Neu) antibody (BioLegend) for subsequent 24-72 hours. At the end of treatment, MTT solution (5 mg/ml) was added into each well, followed by 4 hours incubation at 37 ^{o}C. The formazan crystals were dissolved with sodium dodecyl sulfate (SDS) before reading the absorbance using CHAMELEON™V multi-technology plate reader (Hidex, Turku, Finland) at 570 nm with reference wavelength of 630 nm.

3.4.2 BrdU assay

To measure cell proliferation, we determined BrdU incorporation into the cellular DNA using an ELISA-based approach (Cell Signaling Technology, MA, USA). BxPC-3 and AsPC-1 cells (1 x 10^4) were plated for 24 hours before treating with complete media, control media or PSC-CM. At the end of treatment, 10 µl of 10X BrdU solution was added into each well for additional 24 hours. The cells were then fixed with 100 µl of fixing/denaturing solution for 30 minutes at room temperature. The fixed cells were labeled with 100 µl of 1X detection antibody (1 hour) and horseradish peroxidase (HRP)-conjugated secondary antibody (30 minutes) at room temperature before washing three times with 1X wash buffer. TMB substrate (100 µl) was then added to the labeled cells for 30 minutes at room temperature. Color development was terminated with 100 µl of STOP solution, and the absorbance was measured at 450 nm using CHAMELEON™V multi-technology plate reader.

3.4.3 Cell counting using trypan blue

Briefly, 1 x 10^4 cells of AsPC-1 and BxPC-3 were plated into 96-well plate for 24 hours and serum-starved for an additional 24 hours. The cells were then treated with complete media, control media, T-HESC-CM or EC6/Fib-CM for subsequent 72 hours.

At the end of treatment, cells were rinsed with DPBS before trypsinizing with 0.25% trypsin-EDTA (Life Technologies). The cell suspension was transferred into microcentrifuge tubes and mixed with 0.04% trypan blue (Life Technologies) in 1:1 ratio before direct cell counting using hemocytometer. Results were expressed as the percentage of control that was calculated based on the number of viable cells with treatment divided by number of cells treated without treatment.

3.5 Intracellular ROS measurement

Briefly, 1 x 10^4 cells of PDAC cells were plated into 96-well plate for 24 hours before serum-starving for additional 24 hours. The spent media were discarded and the cells were washed once with DPBS. AsPC-1 and BxPC-3 cells were then treated with PSC-CM for 72 hours after Nrf2 gene silencing and overexpression. In another experiment, oxidative stress was induced in Panc-1 cells with H_2O_2 (600 µM) before treatment of rhIL-6 (100 ng/ml) and tBHQ (50 µM) for 2 hours. Redox-sensitive dye, DCF-DA (10 µM) were added to the treated cells for 20 minutes at 37^{o}C in darkness. The cells were then washed once with DPBS before lysing with 1% Triton-X 100 for 5 minutes at room temperature. The green DCF fluorescence was observed with inverted fluorescence microscope (Nikon Eclipse T*i*, Tokyo, Japan), and measured at 485/535 nm using CHAMELEON™V plate reader.

3.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

3.6.1 Total RNA extraction

TRIzol reagent (Invitrogen, CA, USA) was used to extract total RNA from PSC and PDAC cells after treatment. The spent media were discarded and the cells were washed once with DPBS. TRIzol (500 µl) were added and the cell lysates were transferred into 1.5 ml microcentrifuge tube and placed on ice 10 min or stored at -80^{o}C freezer. To

separate the organic and inorganic phases chloroform (250 µl) was added and the tube was then shaken for 15 seconds before incubating at room temperature for 5 minutes. The mixture solution was then centrifuged at 16000 x g for 20 minutes. After centrifugation, three layers were formed. The first layer is the aqueous phase containing the RNA and DNA while second (interphase) and bottom layers (organic phase) mainly contain proteins. The first layer was transferred to a fresh 1.5 ml microcentrifuge tube followed by addition of 550 µl isopropanol to precipitate RNA. Glycogen (1 µl) was added to carry RNA in order to increase RNA precipitation. The mixture was mixed gently by several times of inversion before incubating at room temperature for 10 minutes. The mixture solution was then centrifuged at maximum speed (~24000 x g) for 20 minutes at 4^{o}C. The isopropanol was discarded carefully followed by addition of 75% ethanol in DEPC-treated water (1 ml). This step was used to remove any impurities in order to increase the purity of the RNA extracted. This step was repeated twice, before dissolving the extracted RNA in DEPC-treated water (20 µl) by heating at 60^{o}C for 5 minutes. The RNA concentration and purity were verified by optical density (A260/A280 ≥ 1.65) using NanoDrop-2000 spectrophotometer (ThermoFisher Scientific).

3.6.2 Reverse transcription (RT)

One µg of total RNA was used to be reverse transcribed using RevertAid^{TM} first strand cDNA RT kit (ThermoFisher Scientific). For every 20 µl reaction, the total RNA sample (1 µg) was mixed with random hexamer primer (1 µl), 5x reaction buffer (4 µl), ribolock RNAse inhibitor (20 U), 10 mM dNTP mix (2 µl), RevertAid RT (200 U) (1 µl), and DEPC-treated water (up to 12 µl). The RT reaction was aliquoted in the tube and briefly vortexed and centrifuged to eliminate any air bubbles. The reaction was then incubated in the thermal cycler (MJ Research PTC-100, USA). The samples were

incubated at 25°C for 5 minutes followed by 42°C for 1 hour. The reaction was terminated by heating at 70°C for 5 minutes, before holding at 4°C.

3.6.3 qRT-PCR

The reaction set up for qRT-PCR assays was performed according to the manufacturer's instructions for the 5X HOT FIREPol® evagreen® qPCR mix plus (ROX) (Solis BioDyne, Tartu, Estonia). For each reaction mix (which contains 20 µl), 4 µl of 5X HOT FIREPol® evagreen® qPCR mix plus, 1 µl of forward and reverse primers, 1 µl of cDNA template, and 13 µl of DEPC-treated water were mixed, vortexed, and then centrifuged briefly to avoid any bubbles formation. The qRT-PCR assay was then performed with one cycle at initial activation at 95°C for 15 minutes, followed by 40 cycles of step 1-denaturation at 95°C for 15 seconds, step 2-annealing at 55-62°C for 15 seconds, and step 3-elongation at 72°C for 15 seconds. The primer sequences used were listed in Table 3.1.

Table 3.1: List of primers used for qRT-PCR

Primer	Sequences	Annealing (°C)
GAPDH	F: 5'-CCCATCACCATCTTCCAGGA-3' R: 5'-GTTGTCATGGATGACCTTGGC-3'	55
KEAP1	F: 5'-CAGATTGGCTGTGTGGAGTT-3' R: 5'-GCTGTTCGCAGTCGTACTTG-3'	55
NRF2	F: 5'-GAGAGCCCAGTCTTCATTGC-3' R: 5'-TTGGCTTCTGGACTTGGAAC-3'	55
HMOX1	F: 5'-GGTAAGAACCAGGTCCGTCA-3' R: 5'-GGGCACTAACTCCCGTTACA-3'	62
CAT	F: 5'-CATGCTGAATGAGGAACAGA-3' R: 5'-TTGTCCAGAAGAGCCTGGAT-3'	62

AKR1C1	F: 5'-ATTCCCATCGACCAGAGTTG-3'	
	R: 5'-TTTGGGATCACTTCCTCACC-3'	62
NQO1	F: 5'-GAAGAGCACTGATCGTACTGGC-3'	
	R: 5'-GGATACTGAAAGTTCGCAGGG-3'	62
SOD1	F: 5'-GGTGGGCCAAAGGATGAAGAG-3'	
	R: 5'-CCACAAGCCAAACGACTTCC-3'	62
SOD2	F: 5'-GACAAACCTCAGCCCTAACG-3'	62
	R: 5'-CTGATTTGGACAAGCAGCAA-3'	
SOD3	F: 5'-ATGCTGGCGCTACTGTGTTC-3'	62
	R: 5'-CTCCGCCGAGTCAGAGTTG-3'	
G6PD	F: 5'-ACCGCATCGACCACTACCT-3'	62
	R: 5'-TGGGGCCGAAGATCCTGTT-3'	
PGD	F: 5'-ATGGCCCAAGCTGACATCG-3'	62
	R: 5'-AAAGCCGTGGTCATTCATGTT-3'	
TKT	F: 5'-CCTACACCGGCAAATACTTCG-3'	62
	R" 5'-GCCTCCCATACAGAGCCCT-3'	
TALDO1	F: 5'-CTCACCCGTGAAGCGTCAG-3'	62
	R: 5'-GTTGGTGGTAGCATCCTGGG-3'	
PPAT	F: 5'-AATTGTCAGCCCTTCGTTGTT-3'	62
	R: 5'-CCTTAATCGAGCAGCATTTACCA-3'	
MTHFD2	F: 5'-AGGACGAATGTGTTTGGATCAG-3'	62
	R: 5'-GGAATGCCAGTTCGCTTGATTA-3'	
ME1	F: 5'-GAGTGCTGACATCTGACATTGA-3'	62
	R: 5'-TTGGCTTCCGAAACACCAAAC-3'	
IDH1	F: 5'-AGAAGCATAATGTTGGCGTCA-3'	62
	R: 5'-CGTATGGTGCCATTTGGTGATT-3'	

GCLC	F: 5’-GGCGATGAGGTGGAATACAT-3’ R: 5’-GGGTAGGATGGTTTGGGTTT-3’	62
GCLM	F: 5’-CATTTACAGCCTTACTGGGAGG-3’ R: 5’-ATGCAGTCAAATCTGGTGGCA-3’	62
IL6	F: 5’-ACTCACCTCTTCAGAACGAATTG-3’ R: 5’-CCATCTTTGGAAGGTTCAGGTTG-3’	60
IL6R	F: 5’-CCCCTCAGCAATGTTGTTTGT-3’ R: 5’-CTCCGGGACTGCTAACTGG-3’	60
CDH1	F: 5’-CGAGAGCTACACGTTCACGG-3’ R: 5’-GGGTGTCGAGGGAAAAATAGG-3’	60
CDH2	F: 5’-TGCGGTACAGTGTAACTGGG-3’ R: 5’-GAAACCGGGCTATCTGCTCG-3’	60
VIM	F: 5’-GACGCCATCAACACCGAGTT-3’ R: 5’-CTTTGTCGTTGGTTAGCTGGT-3’	60
FN1	F: 5’-CGGTGGCTGTCAGTCAAAG-3’ R: 5’-AAACCTCGGCTTCCTCCATAA-3’	60
COL1A1	F: 5’-GAGGGCCAAGACGAAGACATC-3’ R: 5’-CAGATCACGTCATCGCACAAC-3’	60
SIP1	F: 5’-GTGGAAGAGTTGATGCCTCGG-3’ R: 5’-GCTACCACAACATCTGGACATT-3’	60
TWIST2	F: 5’-GTCCGCAGTCTTACGAGGAG-3’ R: 5’-GCTTGAGGGTCTGAATCTTGCT-3’	60
SNAIL	F: 5’-TCGGAAGCCTAACTACAGCGA-3’ R: 5’-AGATGAGCATTGGCAGCGAG-3’	60
SLUG	F: 5’-TGTGACAAGGAATATGTGAGCC-3’ R: 5’-TGAGCCCTCAGATTTGACCTG-3’	60

3.6.4 qRT-PCR analysis

Comparative $\Delta\Delta C_T$ method was used to calculate relative quantification, provided the amplification efficiency of both reference gene (glyceraldehyde 3-phosphate dehydrogenase, *GAPDH*) and target gene were similar. Both sample and calibrator data were first normalized in order to account for variation in sample quality and quantity. Normalized (ΔC_T) values were calculated based on following equations:

$\Delta CT(sample) = C_T(target) - C_T(reference)$

$\Delta C_T(calibrator) = C_T(target) - C_T(reference)$

The $\Delta\Delta C_T$ is then determined using the following formula:

$\Delta\Delta C_T = \Delta C_T(sample) - \Delta C_T(calibrator)$

The expression of the target gene normalized to the reference gene and relative to calibrator

$= 2^{-\Delta\Delta C_T}$

3.7 Western blotting

Total cell lysates were harvested using RIPA lysis buffer (ThermoFisher Scientific) containing 25 mM Tris HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS, with additional (10 µl) of protease and phosphatase inhibitors. Briefly, after washing twice with ice-cold DPBS, the cells were then resuspended in 100 µl of ice-cold RIPA lysis buffer for 5 minutes. The cell lysate was gathered by using ice-cold cell scraper before collecting and transferring into ice-cold microcentrifuge tube. The cell debris was pelleted by centrifuging at 14000 x g for 15 minutes at 4^{o}C. The supernatant was then transferred into fresh 1.5 ml microcentrifuge tube and stored at -80^{o}C freezer after determining the concentration of protein using Bradford assay.

The cytoplasmic and nuclear cell lysates were prepared using NE-PER nuclear and cytoplasmic extraction reagents (ThermoFisher Scientific). Briefly, cells were harvested using 0.25% trypsin-EDTA. The cell suspension was centrifuged at 500 x g for 5 minutes. The cell pellet was washed once with 500 µl of DPBS and centrifuged at 500 x g for 5 minutes. Ice-cold CER I (200 µl) was added to the cell pellet and resuspended thoroughly followed by vigorous vortexing for 15 seconds. Then, 11 µl of ice-cold CER II were added and vortexed vigorously for 5 seconds followed by incubation on ice for 1 minute. The cell lysate was then vortexed vigorously for 5 seconds followed by centrifugation at 16000 x g for 5 minutes at 4^{o}C. The supernatant (cytoplasmic extract) was then immediately transferred to a clean pre-chilled 1.5 ml microcentrifuge. The insoluble cell pellet containing nuclei was resuspended in 100 µl of ice-cold NER before vortexing vigorously for 15 seconds. The cell lysate was then placed on ice and continued vortexing for 15 seconds every 10 minutes, for a total of 40 minutes. The microcentrifuge tube was subsequently centrifuged at 16000 x g for 10 minutes. The supernatant (nuclear extract) was immediately transferred to a clean pre-chilled 1.5 ml microcentrifuge tube. Protein concentration of extracts was quantified using Bradford assay.

Proteins (20-30 µg) were resolved on 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transferring to polyvinylidene difluoride (PDVF) membrane. The membrane was first blocked in 5% non-fat dry milk before probing overnight at 4^{o}C with human Nrf2 (Novus Biologicals, CO, USA), IL-6, phospho-Stat3(Tyr 705), Stat3 (Cell Signaling Technology, MA, USA) antibodies at 1:1000 dilution. Beta-actin (Santa Cruz Biotechnology, Texas, USA) was used as loading control for total cell extract while cyclophilin A and lamin B1 (Cell Signaling Technology) were used as loading control for cytoplasmic and nuclear extract, respectively at 1:1000 dilution. The membrane was then washed five times with 1X PBST buffer with 5 minutes each before probing with secondary antibody conjugated with HRP (Santa Cruz

Biotechnology) at 1:10000 dilution. Detection was performed using WesternBright Quantum chemiluminescence detection reagent (Advansta, CA, USA) and visualized using gel documentation system (Biospectrum 410, UVP, CA, USA).

3.8 Immunofluorescence staining

Briefly, cover slips were placed into 24-well plate and coated with human fibronectin (1 µg/ml) (Corning, NY, USA) for 1 hour in 5% CO_2 incubator at 37°C. Media (500 µl) containing 4 x 10^3 cells were then plated for 24 hours. The spent media were discarded and the cells were washed once with 500 µl of DPBS before treating with complete media, control media or PSC-CM for additional 72 hours. The cells were fixed in ice-cold 4% formaldehyde for 30 minutes before permeabilizing in 0.1% Triton-X 100 for 1 hour at 4°C. Intracellular Nrf2 protein was probed with human Nrf2 primary antibody (BioLegend) at 1:2000 dilution followed by Alexa fluor® 488 donkey anti-rabbit IgG secondary antibody at 5 µg/ml (Molecular Probes, Oregon, USA). The cells were stained with mounting medium containing DAPI (Vector Laboratories, CA, USA), before viewing using Apotome2 fluorescence microscope (Carl Zeiss, Jena, Germany).

3.9 ARE-promoter transactivation activity

To measure DNA binding activity of Nrf2 on ARE promoter, dual-luciferase reporter assay system (Promega, Madison, USA) was used. Briefly, media (100 µl) containing 1 x 10^4 cells of AsPC-1 and BxPC-3 were plated into 96-well plate for 24 hours. The spent media was then discarded and the cells were washed once with 100 µl of DPBS. pGL4.37[*luc2P*/ARE/Hygro] (Promega) vector containing firefly luciferase construct, and pGL4.74[hRluc/TK] (Promega) vector containing renilla luciferase construct were co-transfected in the ratio of 10:1 using Lipofectamine LTX with PLUS reagent (Life Technologies). After 5 hours of transfection, the cells were treated with

complete media, control media, PSC-CM or tBHQ (50 µM) for additional 72 hours. After treatment, the spent media was discarded and the cells were washed once with 100 µl of DPBS. 1X passive lysis buffer (PLB) (20 µl) were added into respective wells and shaken for 15 minutes at room temperature in order to lyse the cells, before centrifuging for 30 seconds at 4^{o}C. The supernatant (cell lysate) was transferred to fresh 1.5 ml microcentrifuge tube and stored on ice if ready to be used or stored at -80^{o}C freezer until use. Luciferase assay reagent (LAR) II (100 µl) were pre-dispensed into 96-well white plate, followed by addition of cell lysate (20 µl) and gently mixed by pipetting for 2 to 3 times. Immediately, the activity of firefly luciferase was measured using Tecan Infinite M1000 Pro multiplate reader (Männedorf, Switzerland). Next, 100 µl of Stop and Glo reagent were added into respective wells to stop the luciferase reporter activity. The plate was then shaken gently before measuring the activity of renilla luciferase. The firefly and renilla luciferase activities were then normalized to the amount of protein. The ARE induction level was determined by dividing the normalized firefly luciferase activity to renilla luciferase activity.

3.10 Transient *NRF2* gene silencing

Cells (1 x 10^4) were plated in 100 µl of serum-free media containing into 24-well plate. After 24 hours, the spent media was discarded and the cells were washed once with 100 µl of DPBS. Before transfection, 100 nM of ON-TARGETplus siRNA targeting *NRF2* or *G6PD*, and ON-TARGETplus siControl (Thermo Scientific) were diluted in 1X OptiMEM (Life Technologies). The mixture was then mixed with DharmaFECT 2 (ThermoFisher Scientific) and incubated at room temperature for 20 minutes. The cells were then transfected for either 48 hours (for validation using qRT-PCR) or 72 hours (for validation using western blotting).

Nrf2 target genes (*HMOX1*, *CAT*, *AKR1C1*, *NQO1*, *SOD1*, *SOD2*, and *SOD3*) expression was measured using qRT-PCR. The *NRF2*-silenced cells were then treated with control media or PSC-CM for additional 72 hours before examining the effect on cell proliferation, intracellular ROS levels, and metabolic genes expression (*G6PD*, *PGD*, *TKT*, *TALDO1*, *PPAT*, *MTHFD2*, *ME1*, *IDH1*, *GCLC*, and *GCLM*).

3.11 Transient *NRF2* Gene overexpression

To induce *NRF2* gene expression, serum-free media (500 µl) containing 1 x 10^4 cells were plated into 24-well plate for 24 hours. The spent media was then discarded and the cells were washed once with 500 µl of DPBS. The pCMV6-AC-GFP vector (100 ng) which contains either *NRF2* ORFs (Cat no.: PS100010) or control ORFs (Cat no.: RG204140) (OriGene, MD, USA) was individually incubated with 1X OptiMEM for 30 minutes. Both vectors were mixed with Lipofectamine and PLUS reagent for 20 minutes at room temperature before transfection for 5 hours. *NRF2* and its target gene expression (*AKR1C1* and *NQO1*) were then measured using qRT-PCR. In another experiment, *NRF2* overexpressed cells were treated with control media or PSC-CM for additional 72 hours before determining the cell proliferation, intracellular ROS levels, and metabolic gene expression.

3.12 Measurement of extracted metabolite concentration

3.12.1 Metabolite extraction

The metabolite extraction was prepared according to Sheikh et al. (2011) (Sheikh, Khanna, Byers, Fornace, & Cheema, 2011). Briefly, 2 x 10^6 cells of AsPC-1 and BxPC-3 were plated into four 100 mm cell culture dishes. After 24 hours, the spent media was discarded and the cells were washed once with 2 ml of DPBS before incubating with serum-free media for additional 24 hours. The cells were then treated with control media

or PSC-CM for additional 72 hours. After treatment, the cells were washed once with 2 ml of DPBS and detached using 0.25% trypsin-EDTA. The cell suspension was transferred to pre-chilled 15 ml conical tubes. At the same time, cells (1×10^7) were transferred into 1.5 ml microcentrifuge tubes and centrifuged at 1500 rpm for 10 minutes. The cell pellet was washed once with 500 µl of DPBS by centrifugation at 1500 rpm for 10 minutes. The cell pellet was then resuspended in 150 µl of liquid chromatography-mass spectrometry (LCMS) grade water before lysing by two cycles of freeze thaw (30 seconds at -20°C, followed by 90 seconds in a 37°C water bath) and sonication (30 seconds). Cell suspension (5 µl) was aliquoted and its protein concentration was measured with Bradford assay.

Methanol (600 µl) containing internal standard (4-nitrobenzoic acid) was added to the cell suspension and vortexed. The cell suspension was then incubated on ice for 15 minutes, followed by addition of 600 µl of chloroform. The microcentrifuge tubes were vortexed and centrifuged at 13000 rpm for 10 minutes at 15°C. The two phases were carefully transferred to fresh microcentrifuge tube by avoiding the interface. Chilled acetonitrile (600 µl) was added to each tube, vortexed, and incubated at -80°C for 2 hours. The microcentrifuge tubes were centrifuged at 13000 rpm for 10 minutes at 4°C. The supernatant was transferred to fresh microcentrifuge tubes and dried under vacuum. The two microcentrifuge tubes for each sample were combined using 150 µl of 50% acetonitrile/water prior to UHPLC-MS/MS analysis.

3.12.2 UPLC-ESI-Q-TOF-MS analysis

For UHPLC-MS/MS analysis, samples (10 µl) were injected into ThermoFisher Scientific Dionex Ultimate 3000 Rapid Separation liquid chromatography (RSLC) (MA, USA) coupled to an Orbitrap Fusion™ Tribrid™ mass spectrometer (ThermoFisher Scientific). Separation was achieved on a Hypersil GOLD aqueous column, with a dimension of 100

x 2.1 mm (ThermoFisher Scientific) using a 20 minutes gradient with the mobile phases consist of 95% water and 5% acetonitrile. MS analysis was performed using ThermoFisher Scientific Xcalibur 3.0 software.

3.13 Cell morphological observation

After treating 24 h with ATRA (20 µM), the morphological change of PSC was observed under phase contrast using an inverted research microscope (Nikon Eclipse Ti, Tokyo, Japan). The cell morphology was also observed in Panc-1 cells after 24 h treatment with serum-free media (SFM), PSC-CM with or without IL-6 Neu antibody (4 µg/ml) or rhIL-6 or tBHQ (1 µM).

3.14 Scratch wound healing assay

Briefly, 1.5 x 10^5 of Panc-1 cells were plated in 24-well plate for 24 h to reach 80-90% confluency. The cells were serum-starved for subsequent 24 h before wounding the monolayers with a sterile 200 µl pipette tip. After washing the wounded monolayers with DPBS, the cells were treated with SFM or PSC-CM, with or without IL-6 Neu antibody, rhIL-6 or tBHQ (1 µM) for 24 h. The wound closure was examined and photographed using an inverted light microscope (Olympus, PA, USA) while the distances migrated by the cell monolayer to close the wounded area during these times were measured using an ocular micrometer at 0 and 24 h. Results were expressed as a relative migration index, i.e. the distance (µm) of migrated cells after 24 h was divided by the distance of non-migrated at 0 h for all untreated and treated cells to address the distance variation in different wells of 24-well plate after wounding at 0 h. The normalized distance migrated by the treated cells were compared relatively to the distance migrated by untreated cells.

3.15 *In vitro* transwell migration and invasion assays

A transwell system using 8 µm pore size cell culture inserts (BD Biosiences, NJ, USA) was used for cell migration assay. Similar transwell system precoated with 0.3 mg/ml of extracellular matrix substitute Matrigel (Corning, NY, USA) for 5 h in SFM was used for invasion assay. SFM or PSC-CM with or without IL-6 Neu antibody, rhIL-6, or tBHQ (1 µM) was added in the lower chamber. Panc-1 cells (1×10^5) were placed into the upper chamber in 200 µl of DMEM containing 1% FBS. After 20 h incubation, the migrating or invading cells were fixed and permeabilized using 3.7% formaldehyde and 100% methanol, respectively. The non-migrated or -invaded cells were scraped off using pre-wetted cotton swabs. The migrated or invaded cells were stained with 0.4% crystal violet, examined, and photographed using an Olympus inverted light microscope. Results were expressed as a migration index (cells/field) by counting the number of migrated and invaded cells were counted in four random microscopic fields.

3.16 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad, CA, USA). The difference between two groups was analyzed by two-tailed Student *t* test. The difference between three or more groups was analyzed by one-way analysis of variance multiple comparisons (ANOVA). Bars in the figures were considered statistically significant different at $*P < 0.05$. Data shown as the mean ± standard deviation (SD).

CHAPTER 4: RESULTS

4.1 PSC secretion promotes PDAC cell proliferation in a dose- and time-dependent manner

The effects of PSC secretion on PDAC cell proliferation were determined using an indirect co-culture system. Secretion of PSC was collected from conditioned medium (PSC-CM) and applied to AsPC-1 and BxPC-3 cells for 72 h before examining their cell viability using an MTT assay and proliferation using BrdU incorporation and trypan-blue cell counting. AsPC-1 and BxPC-3 cells treated with PSC-CM showed an increase in cell viability in a dose- and time-dependent manner (Figure 4.1). Notably, 1 µg/µl PSC-CM caused a significant increase in the cell viability of BxPC-3 (185 ± 12%) and AsPC-1 (153 ± 4%) after 72 h compared to that in control medium (1% FBS). The increased cell viability was partly due to enhanced cell proliferation, as evidenced by increased BrdU incorporation in these cells (BxPC-3: 55 ± 0.1%; AsPC-1: 32 ± 0.1%), which did not significant differ from the range induced by complete media (10% FBS) (BxPC-3: 68 ± 5%; AsPC-1: 37 ± 2%) (Figure 4.2A). Trypan-blue stained cell counting also showed increased cell viability in a dose-dependent manner, with 1 µg/µl PSC-CM significantly increasing the cell number of BxPC-3 (180 ± 3%) and AsPC-1 (157 ± 2%) (Figure 4.2B). These data indicated that PSC secretion could promote PDAC cell proliferation.

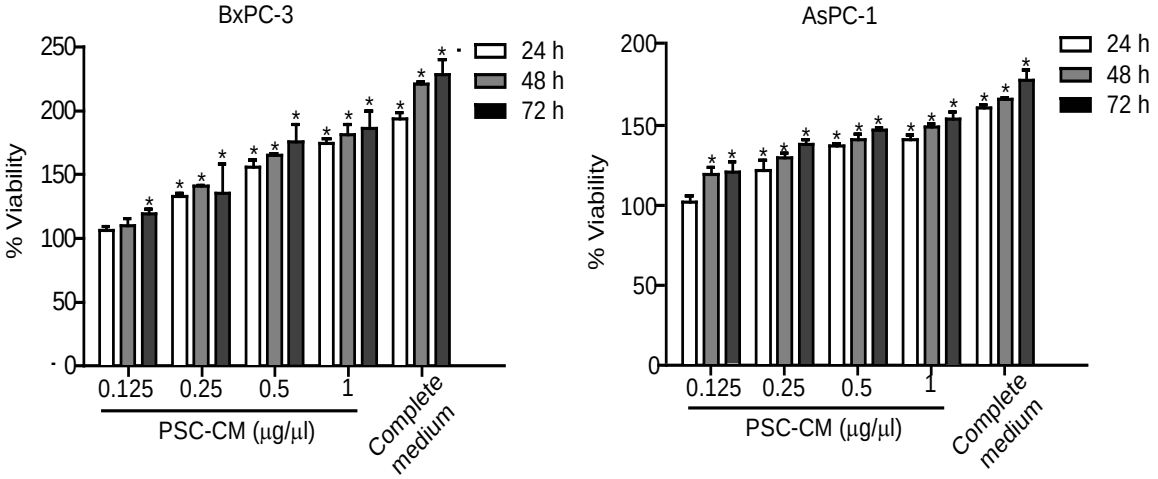

Figure 4.1: PSC-CM promotes AsPC-1 and BxPC-3 cell viability in a dose- and time-dependent manner as evaluated by the MTT assay. BxPC-3 (top panel) and AsPC-1 (bottom panel) cells were treated with PSC-CM (0.125-1 µg/µl) for 24-72 h before the determination of cell viability using an MTT assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed thrice.

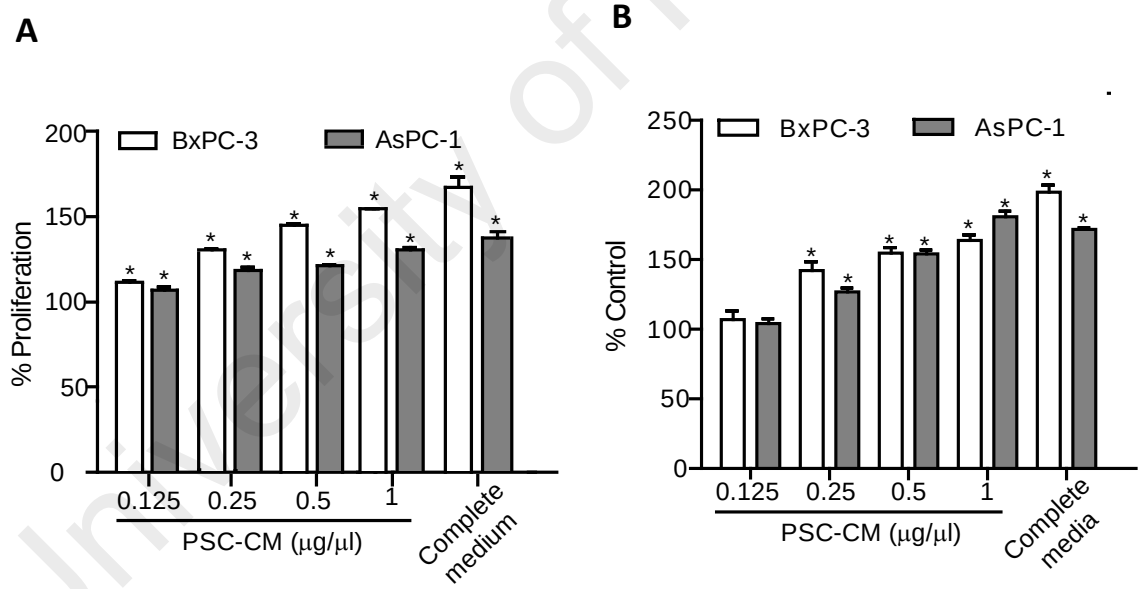

Figure 4.2: PSC-CM promotes AsPC-1 and BxPC-3 cell proliferation as evaluated by BrdU the assay and trypan-blue cell counting method. Cells were treated with PSC-CM (0.125-1 µg/µl) for 72 h before the determination of cell proliferation using a BrdU assay (A) or trypan-blue cell counting (B). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). Each experiment was performed twice.

To determine whether the proliferation-promoting effect of PSC secretion was specific, AsPC-1 and BxPC-3 cells were also treated with the secretion collected from PSC cultured in serum-free medium (SFM) or the conditioned medium from human normal endometrial fibroblast cells (T-HESC-CM) and human endometrial adenocarcinoma primary fibroblast cells (EC6/Fib-CM). The result showed that PSC-CM devoid of FBS also dose-dependently increased the cell viability, with 0.1 µg/µl PSC-CM significantly increasing the viability of BxPC-3 (151 ± 0.9%) and AsPC-1 (143 ± 2.6%) cells (Figure 4.3A), which is incomparable to the effect induced by 1 µg/µl PSC-CM on both cells. Notably, T-HESC-CM decreased BxPC-3 cell viability in a dose-dependent manner (35 ± 2.4% at 1 µg/µl) whereas no significant effect on AsPC-1 cells was observed (Figure 4.3B). Treatment of EC6/Fib-CM dose-dependently reduced the viability of both cell types, with greater effect on BxPC-3 (69 ± 1.8%) than on AsPC-1 (35 ± 2.4% at 1 µg/µl) (Figure 4.3C). Similar results were observed with the cell counting assay, with 1 µg/µl T-HESC-CM caused the greatest decrease in BxPC-3 cell viability among the tested concentrations (32 ± 2.1%) but had no significant effect on AsPC-1 cells (Figure 4.4A). Conversely, after treating with EC6/Fib-CM, the cell viability was decreased in a dose-dependent manner, with the highest reduction observed in BxPC-3 (45 ± 3.1%) and AsPC-1 (47 ± 2.1%) at 1 µg/µl (Figure 4.4B). These data indicated that the proliferation-promoting effect of PSC secretion on PDAC cells was specific.

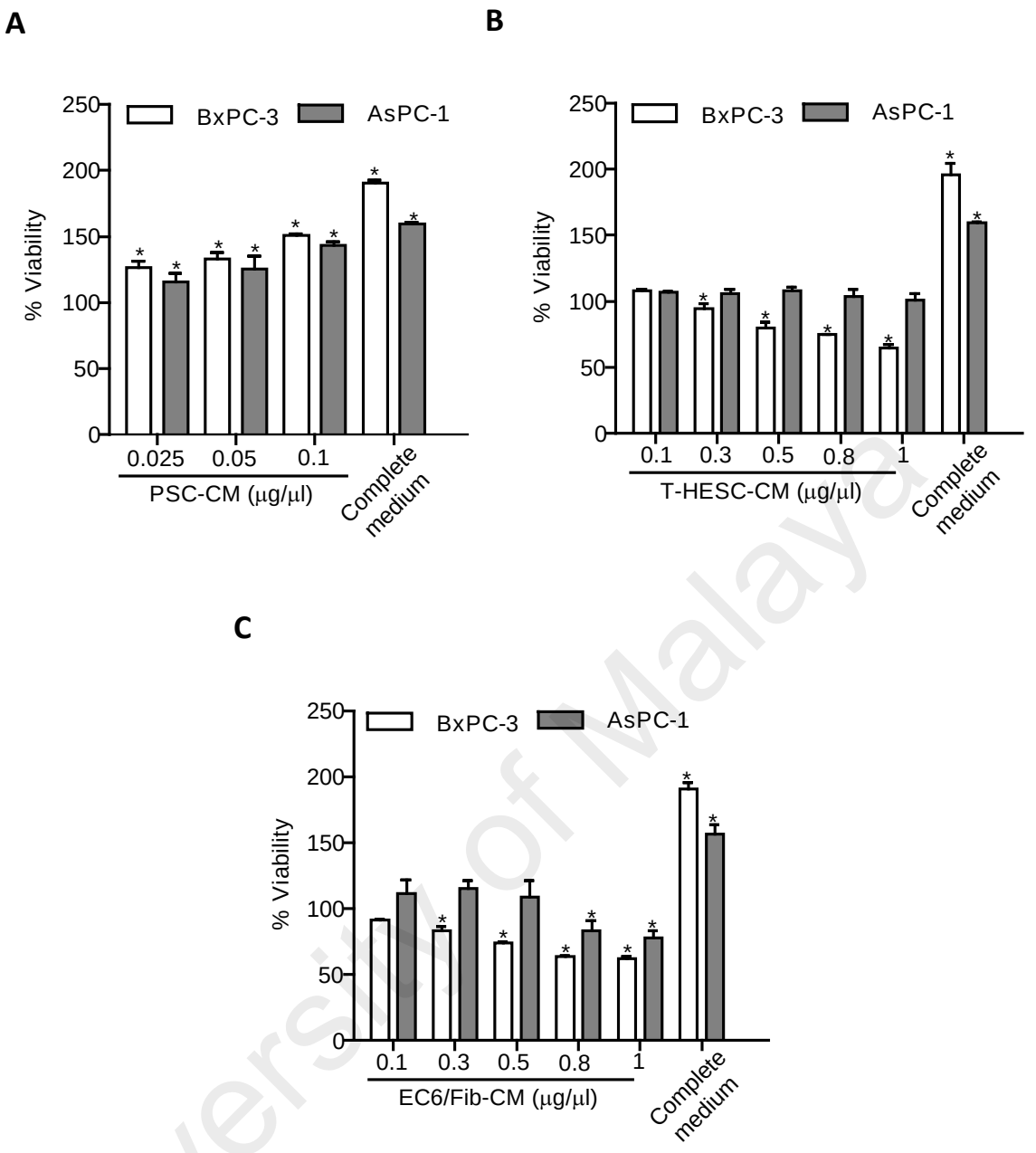

Figure 4.3: PSC-CM (FBS-free), T-HESC-CM, and EC6/Fib-CM treatment promote AsPC-1 and BxPC-3 cell viability as evaluated by the MTT assay. Cells were treated with PSC-CM (FBS-free) (A), T-HESC-CM (B), and EC6/Fib-CM (C) for 72 h before the determination of cell viability using an MTT assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed twice.

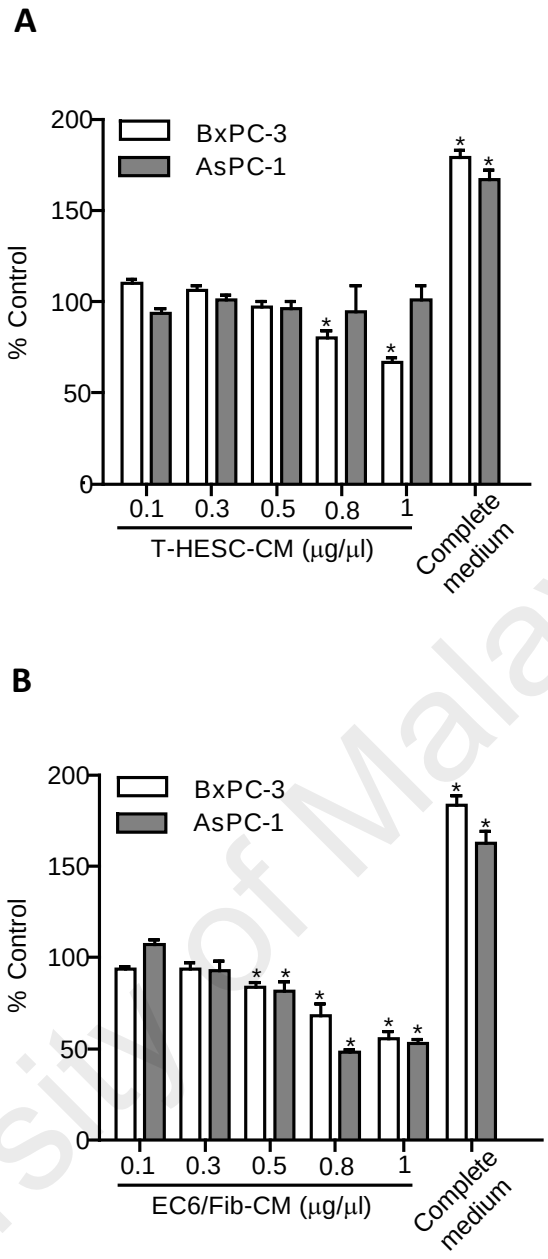

Figure 4.4: T-HESC-CM and EC6/Fib-CM treatment decrease the proliferation of AsPC-1 and BxPC-3 cells as evaluated by the trypan blue cell counting method. Cells were treated with T-HESC-CM (A) or EC6/Fib-CM (B) for 72 h before the determination of the cell proliferation using a trypan-blue cell counting. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control media (media containing 1% FBS). The experiment was performed twice.

4.2 PSC secretion activates intracellular Nrf2 signaling in PDAC cells

Preliminary results (was not shown) found that PSC could enhance PDAC cell viability via elevated ROS levels and Nrf2 was shown to promote ROS detoxification and tumorigenesis. This has prompted the investigation of activation of intracellular Nrf2 signaling as one of the possible mechanisms by which PSC secretion promote PDAC cell proliferation. *NRF2* and its protein expression after PSC-CM treatment were first measured using qRT-PCR and western blotting, respectively. Treatment with PSC-CM induced higher *NRF2* mRNA levels in both AsPC-1 (2.9-fold) and BxPC-3 cells (1.9-fold) where the mRNA levels of its negative regulator, *KEAP1* were downregulated in BxPC-3 cells (0.5-fold) with no significant change in AsPC-1 cells (Figure 4.5). Increased *NRF2* mRNA led to increased Nrf2 translation such that the Nrf2 protein was increased in a dose-dependent manner after PSC-CM treatment (BxPC-3: 1.7-fold, AsPC-1: 1.8-fold for 1.0 µg/µl PSC-CM) (Figure 4.5). The increased Nrf2 protein expression may lead to increased transcriptional activity; thus, Nrf2 subcellular localization upon PSC-CM treatment was subsequently examined. In particular, a greater increase of Nrf2 protein was observed in the nuclear cells (BxPC-3: 0.48-fold, AsPC-1: 0.27-fold for 1 µg/µl PSC-CM) than in the cytoplasmic (BxPC-3: 2.1-fold, AsPC-1: 2.3-fold for 1 µg/µl PSC-CM) extracts of PSC-CM-treated cells (Figure 4.6), indicating that PSC secretion induced Nrf2 nuclear translocation. Immunofluorescence staining was also used to examine Nrf2 nuclear localization, demonstrating that although Nrf2 nuclear localization was already evident in AsPC-1 control cells, greater fluorescence intensity was observed after PSC-CM treatment (Figure 4.7). Enhanced fluorescence intensity was also observed in the nucleus of PSC-CM-treated BxPC-3 cells compared to control cells, in which the staining was predominantly cytoplasmic (Figure 4.7). These data indicated that PSC secretion could induce Nrf2 activation in PDAC cells.

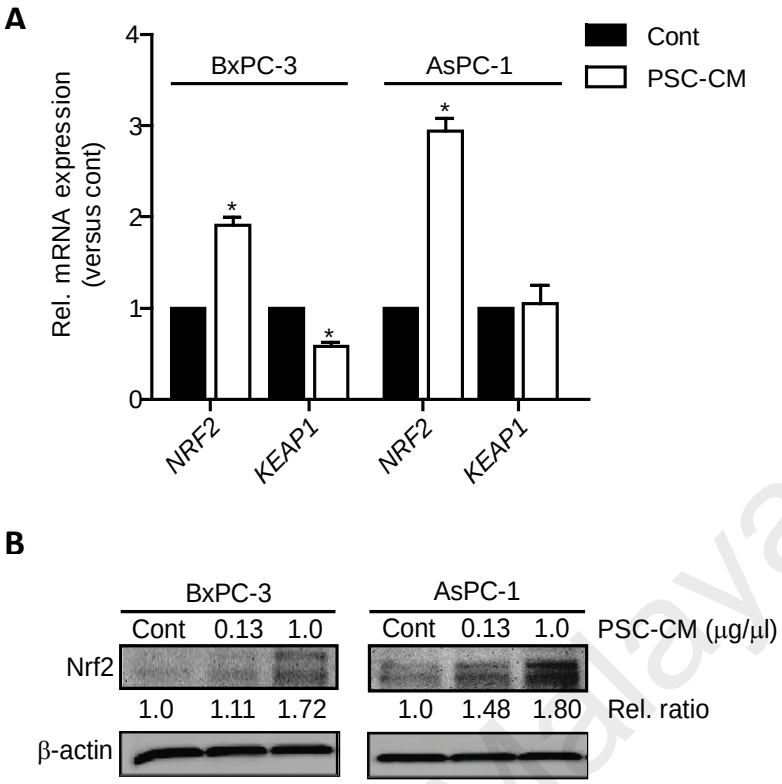

Figure 4.5: PSC-CM upregulates *NRF2* mRNA and protein levels in AsPC-1 and BxPC-3 cells. Cells were treated with PSC-CM (1 μg/μl) for 72 h before measuring *NRF2* and *KEAP1* mRNA levels with qRT-PCR (A). After 72 h, whole cell lysates were collected for Nrf2 protein levels analysis with western blotting (B). Beta actin was used as the loading control. Densitometry analysis is shown as a relative ratio against the control sample. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). Each experiment was performed thrice. The best representative result for western blotting was presented. Rel., relative; Cont, control.

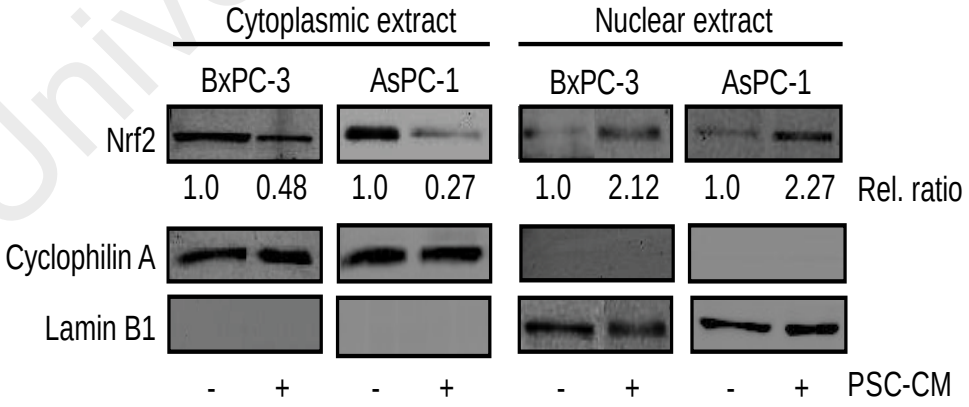

Figure 4.6: PSC-CM induces more intracellular Nrf2 nuclear protein. Nrf2 protein subcellular localization was examined in AsPC-1 and BxPC-3 cells treated with PSC-CM (1 μg/μl) for 72 h using western blotting. Cyclophilin A and lamin B1 were used as loading control for cytoplasmic and nuclear extracts, respectively. Densitometry analysis is shown as a relative ratio against the untreated cells. The experiment was performed thrice and the best representative result was presented. Rel., relative.

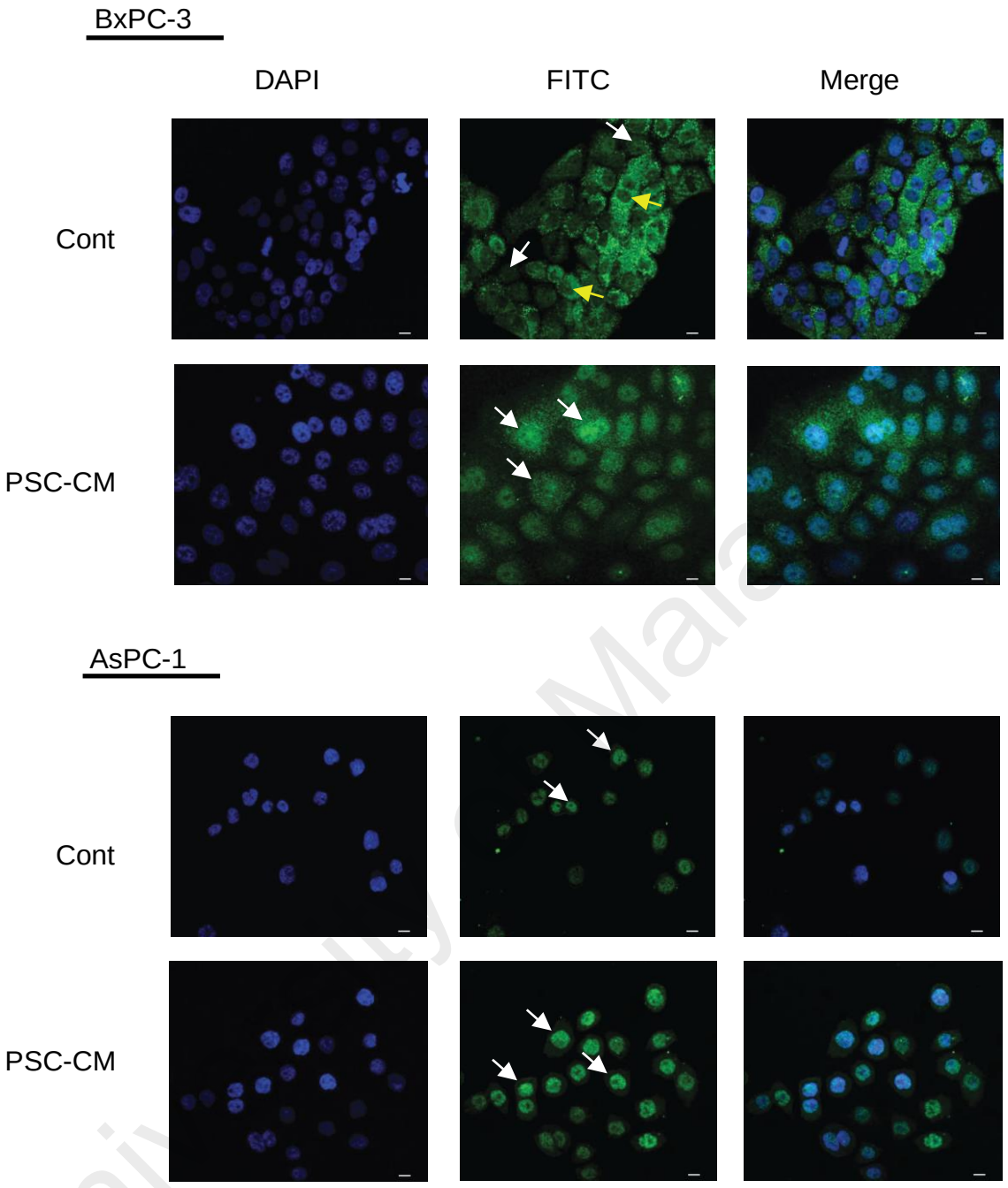

Figure 4.7: PSC-CM induces Nrf2 nuclear protein localization. BxPC-3 (top panel) and AsPC-1 (bottom panel) cells were treated with PSC-CM (1.0 µg/µl, 72 h). Nrf2 protein subcellular localization was examined using immunofluorescence staining with an anti-Nrf2 antibody (scale bar, 100 µm). The yellow arrows indicate Nrf2 cytoplasmic localization and white arrows indicate Nrf2 nuclear localization. The experiment was performed thrice and the best representative result was presented. Cont, control (untreated).

Increased Nrf2 nuclear localization may lead to upregulation of its target genes. Therefore, an ARE-promoter luciferase construct was used to examine the DNA binding activity of Nrf2 to the promoters of its target genes. After PSC-CM treatment, a higher level of Nrf2 transactivation activity was induced in AsPC-1 cells (3.5-fold) than in BxPC-3 cells (1.5-fold) ($P < 0.05$) (Figure 4.8). Despite pronounced Nrf2 activation by PSC-CM, only selective Nrf2 cytoprotective gene targets were induced (Figure 4.9), indicating that PSC secretion can affect specific Nrf2-mediated signaling pathways. In both cell types, *AKR1C1* exhibited the greatest mRNA induction, at 2.8-fold in BxPC-3 and 4.7-fold in AsPC-1. *NQO1* and *CAT* expression levels were also induced to at least 2.0-fold in BxPC-3 cells and 1.5-fold in AsPC-1 cells (Figure 4.9). However, other Nrf2 target genes including *HMOX1*, *SOD1*, *SOD2*, and *SOD3* were not significantly affected by PSC-CM treatment (Figure 4.9). Taken together, these data indicated that extracellular factors such as PSC secretion could activate Nrf2 transcriptional activity in PDAC cells and that such effects appeared to be more evident in AsPC-1 than in BxPC-3 cells.

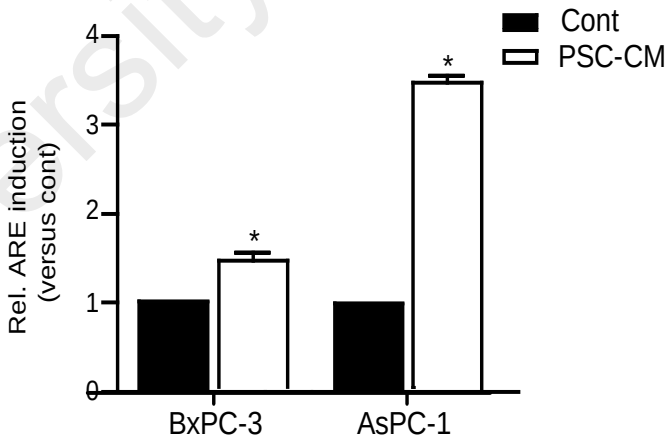

Figure 4.8: PSC-CM promotes Nrf2 transactivation activity by increasing its DNA binding activity to the ARE promoter of its downstream target genes. AsPC-1 and BxPC-3 cells were transfected with an ARE promoter reporter construct for 5 h before treatment with PSC-CM (1 µg/µl) for 72 h. ARE promoter activity was determined relative to internal *Renilla* luciferase activity and normalized using the protein content between samples. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *$P < 0.05$ indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed thrice. Rel., relative; Cont, control.

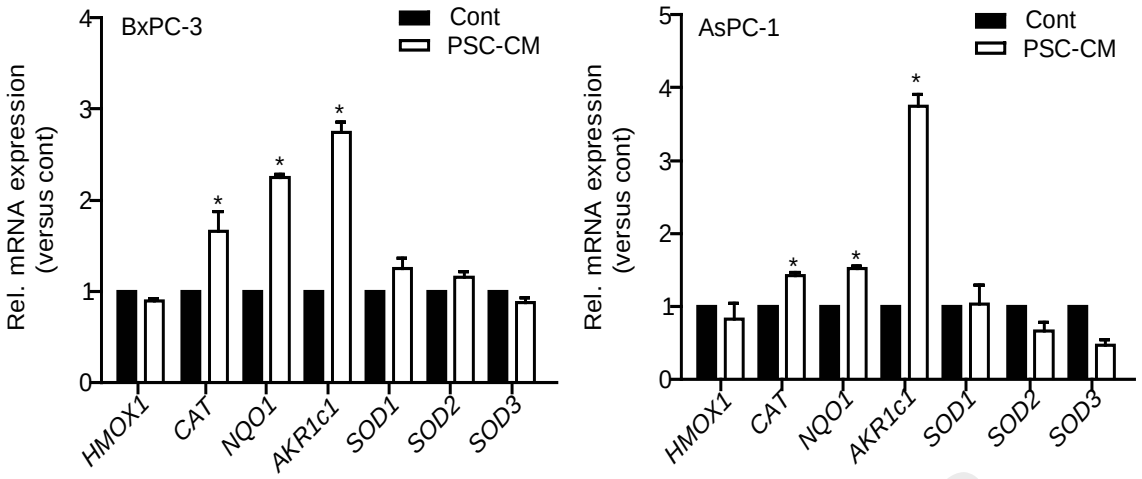

Figure 4.9: Enhanced Nrf2 transactivation activity selectively increases its antioxidant target gene expression. The mRNA expression of Nrf2-regulated genes in BxPC-3 (A) and AsPC-1 (B) cells after treatment with PSC-CM (1 µg/µl) for 72 h was determined using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed twice. Rel., relative; Cont, control.

4.3 Nrf2 activity is required for PSC-mediated PDAC cell proliferation

To determine whether Nrf2 activity is required for PSC-mediated cell proliferation, Nrf2 protein expression was downregulated using RNAi-mediated gene silencing. The efficiency of *NRF2* downregulation was examined by measuring its expression at both mRNA and protein levels using qRT-PCR and western blotting, respectively. *NRF2* mRNA was reduced to 43% and 25% in BxPC-3 and AsPC-1 cells, respectively compared to untransfected control cells (mock) (P < 0.05), with minimal changes observed in cells transfected with control siRNA (Figure 4.10A). These results suggested that the effect of Nrf2-siRNA was specific, which subsequently led to 50% and 70% reduction in Nrf2 protein expression in BxPC-3 and AsPC-1 cells, respectively (Figure 4.10B). Control siRNA was then used to compare Nrf2 target genes transcription. The transcription of the Nrf2 target genes was also markedly reduced following *NRF2* downregulation, with over 50% reduction for *AKR1C1* and *NQO1* (P < 0.05)

(Figure 4.10C). These data indicated that *NRF2* downregulation leads to the reduction of its target genes transcription.

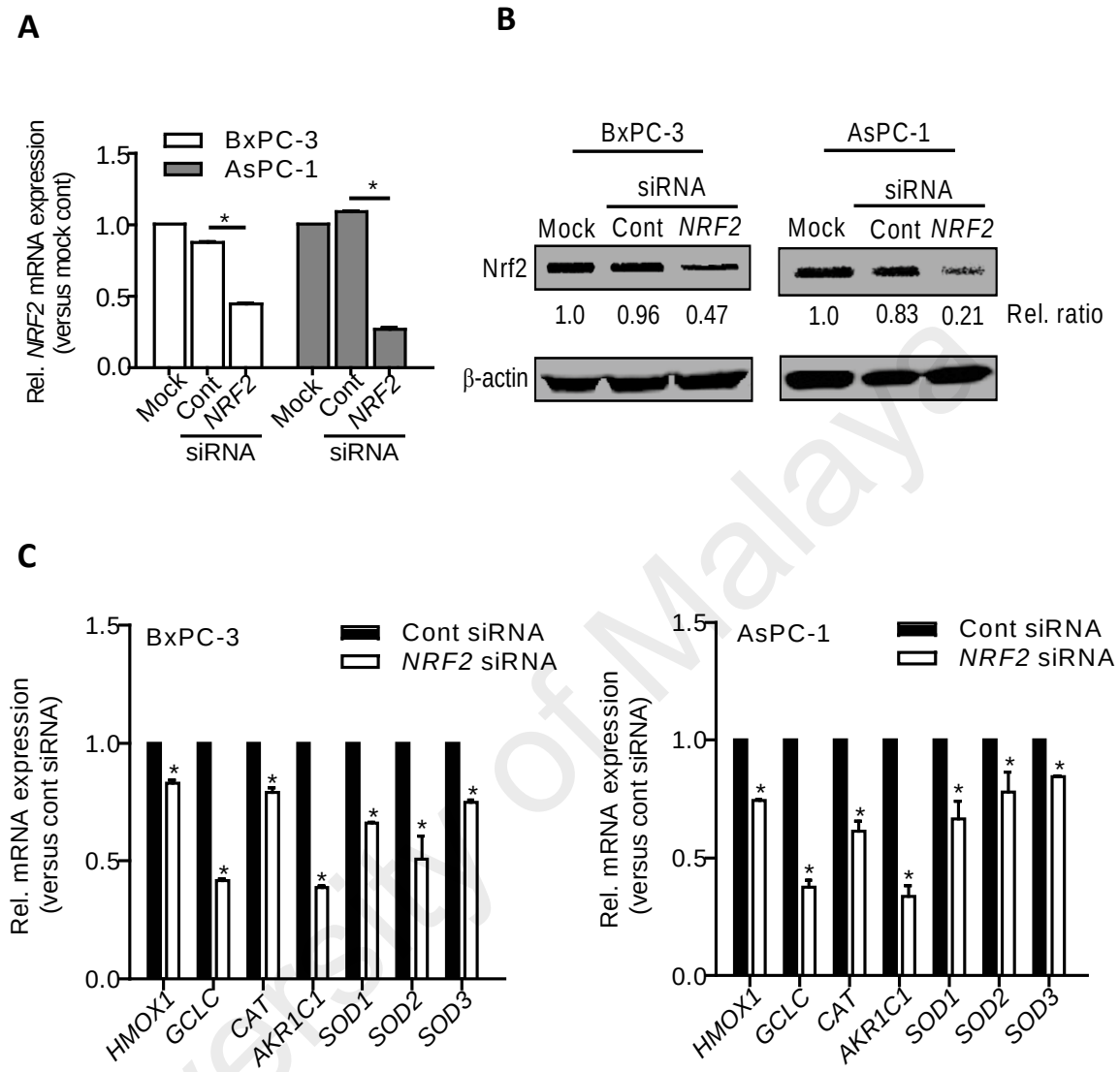

Figure 4.10: Expression of *NRF2* after RNAi-mediated gene silencing. AsPC-1 and BxPC-3 cells were transfected with control or *NRF2* siRNA (100 nM) for 72 h. *NRF2* mRNA (A) and protein levels (B) were measured with qRT-PCR and western blotting, respectively whereas qRT-PCR was used to measure its target genes mRNA (C). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicate significant differences versus control siRNA. Densitometry analysis is shown as a relative ratio against the untreated AsPC-1 and BxPC-3 cells. Each experiment was performed thrice. The best representative result for western blotting was presented. Rel., relative; Cont, control.

Next, the effects of *NRF2* knockdown on the viability and intracellular ROS levels of AsPC-1 and BxPC-3 cells were examined using MTT and DCF-DA assay, respectively. PSC secretion was then added to evaluate whether it could affect the cell viability and

ROS levels after *NRF2* knockdown. Suppressed Nrf2 protein expression led to a larger decrease in cell viability in BxPC-3 (38% inhibition) than in AsPC-1 (15% inhibition) ($P < 0.05$ when compared to control siRNA) (Figure 4.11), although greater *NRF2* knockdown was observed in the latter. PSC-CM-mediated cell proliferation was also less affected by *NRF2* downregulation in AsPC-1 compared to BxPC-3. In the presence of *NRF2* siRNA, the degree of PSC-mediated cell proliferation has decreased ~50% in BxPC-3 compared to AsPC-1 (~20%). Furthermore, the proliferation-promoting effect of PSC-CM after *NRF2* downregulation was markedly decreased in BxPC-3 cells (approximately 18% increase) while this was not observed in AsPC-1 cells (approximately 49% increase) (Figure 4.11). As expected, the intracellular ROS levels were significantly elevated to almost 5-fold in *NRF2*-silenced PDAC cells when compared to the control cells ($P < 0.05$) (Figure 4.12). Notably, a drop in intracellular ROS levels was observed upon treatment with PSC-CM in these cells (approximately 51-86% reduction) (Figure 4.12). This was likely due to the reactivation of Nrf2 signaling in these cells as an upregulation for the mRNA of *NRF2* (approximately 1.5-2-fold) and that of its target genes (*AKR1C1* and *NQO1*) (approximately 3-5-fold) was observed in *NRF2*-silenced cells treated with PSC-CM (Figure 4.13). These data suggested that *NRF2* knockdown reduced PSC-mediated PDAC cell proliferation, with the greatest effect observed in BxPC-3 cells.

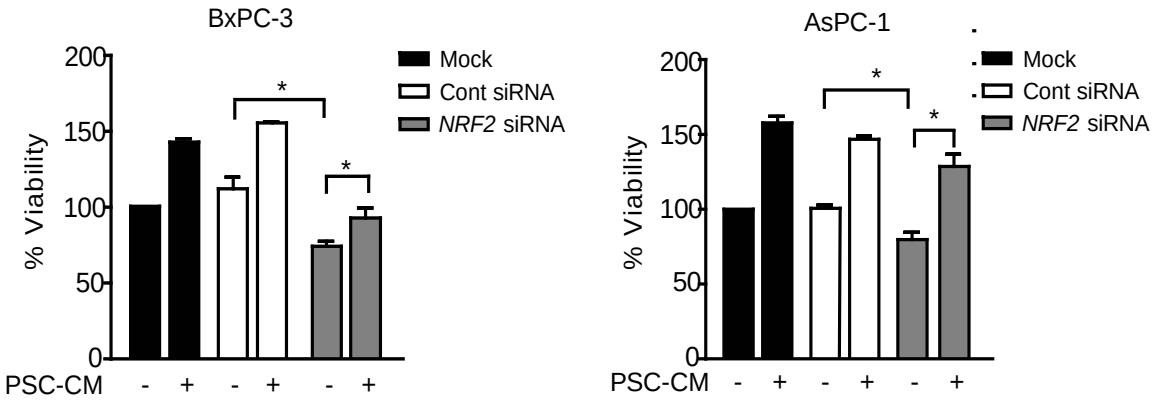

Figure 4.11: The effect of *NRF2* knockdown on PSC-mediated PDAC cell proliferation. BxPC-3 (left panel) and AsPC-1 (right panel) cells were treated with *NRF2* or control siRNA for 72 h before treatment with PSC-CM (1 µg/µl) for 72 h. Cell viability was measured with an MTT assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicate significant differences in *NRF2* siRNA versus control siRNA. The experiment was performed thrice. The best two repeated results were presented. Cont, control.

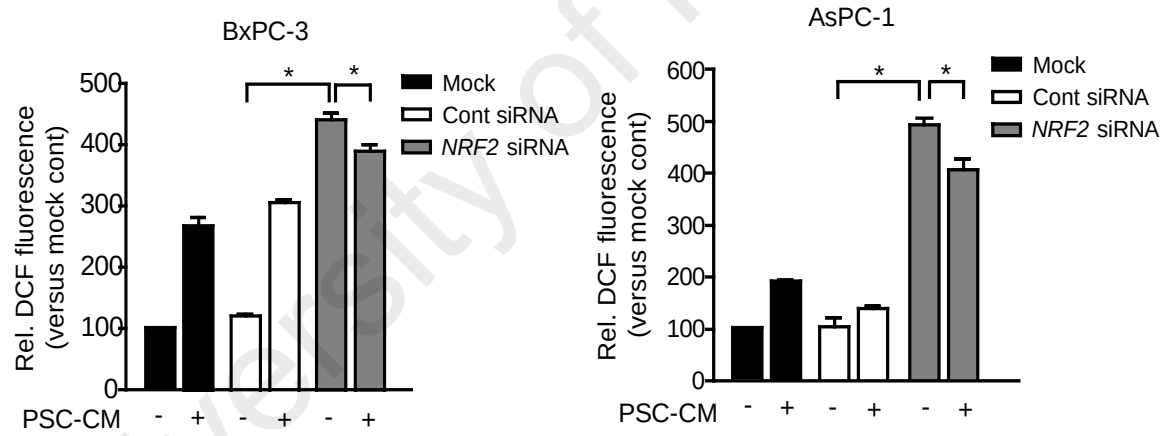

Figure 4.12: Nrf2 activity is required to mediate PSC-regulated intracellular ROS levels in PDAC cells. BxPC-3 (left panel) and AsPC-1 (right panel) cells were treated with *NRF2* or control siRNA for 72 h before treatment with PSC-CM (1 µg/µl) for 72 h. Intracellular ROS levels were measured using a DCF-DA assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicate significant differences in *NRF2* siRNA versus control siRNA. The experiment was performed thrice. The best two repeated results were presented. Rel., relative; Cont, control.

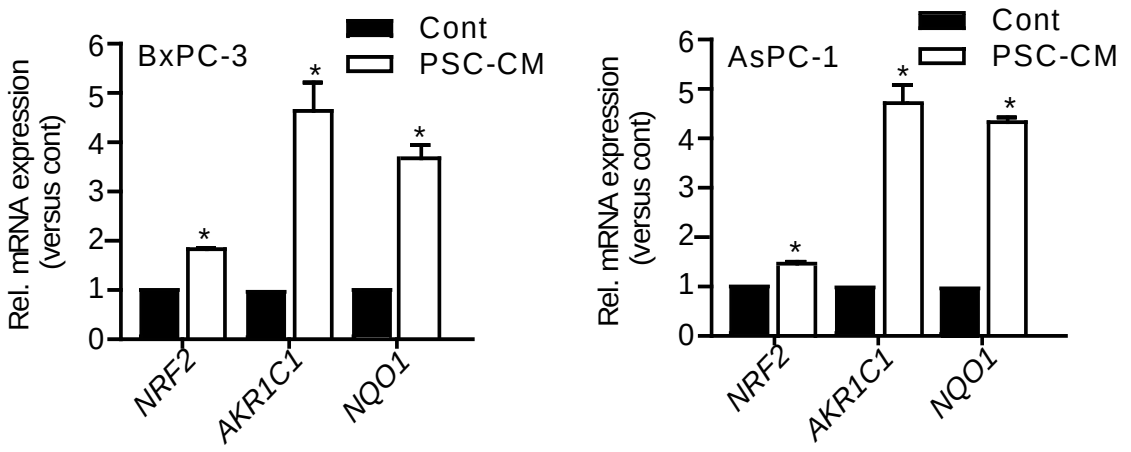

Figure 4.13: Reactivation of intracellular Nrf2 signaling in *NRF2* siRNA-transfected AsPC-1 and BxPC-3 cells after PSC-CM treatment. Cells were treated with *NRF2* siRNA for 72 h before treatment with PSC-CM (1 µg/µl) for 72 h. The cell lysates were collected and *NRF2*, *AKR1C1*, and *NQO1* mRNA expression was measured using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus *NRF2* siRNA-transfected cells treated with control medium (1% FBS). The experiment was performed twice. Rel., relative; Cont, control.

To further examine the effect of Nrf2 activity on PSC-mediated PDAC cell proliferation, an *NRF2*-expressing plasmid was transfected into AsPC-1 and BxPC-3 cells to overexpress *NRF2* followed by PSC secretion treatment. An increase in the mRNA levels of *NRF2* (approximately 5 to 6-fold) and its target genes (*AKR1C1* and *NQO1*) (~1.5-fold) was observed (Figure 4.14). *NRF2* overexpression also resulted in a significant, approximately 2-fold increase in cell viability in both BxPC-3 and AsPC-1 ($P<0.05$ when compared to the control vector) (Figure 4.15). Furthermore, enhanced cell proliferation was observed when these cells were treated with PSC-CM, with an additional 48% and 33% increase in BxPC-3 and AsPC-1 cell numbers, respectively ($P<0.05$ when compared to untreated cells) (Figure 4.15). Notably, the proliferation-promoting effect of PSC-CM in *NRF2* overexpressing cells was less evident than in control cells (Figure 4.15).

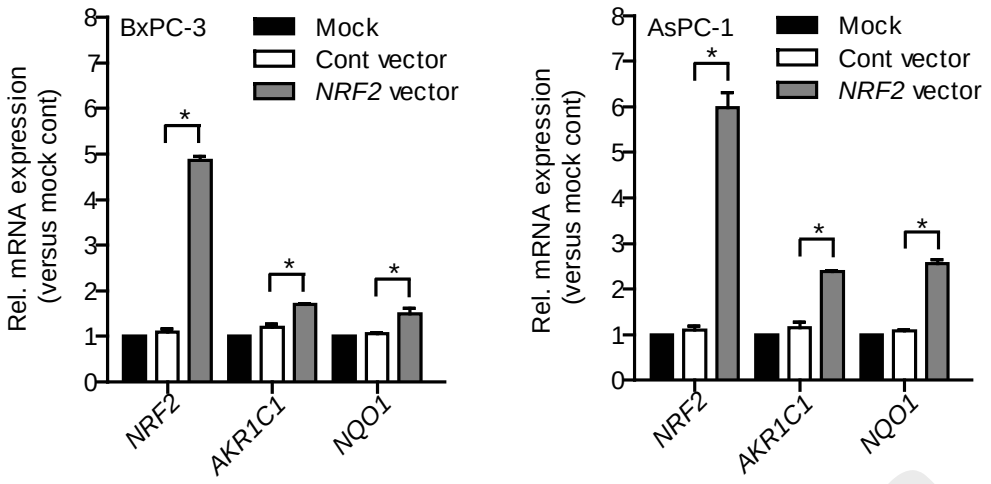

Figure 4.14: Expression of the *NRF2* gene after transfection with an *NRF2*-expressing plasmid. BxPC-3 (left panel) and AsPC-1 (right panel) cells were transfected with a control or *NRF2* expressing vector for 5 h, then *NRF2*, *AKR1C1*, and *NQO1* mRNA expression levels were measured using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control vector-transfected cells. The experiment was performed twice. Rel., relative; Cont, control.

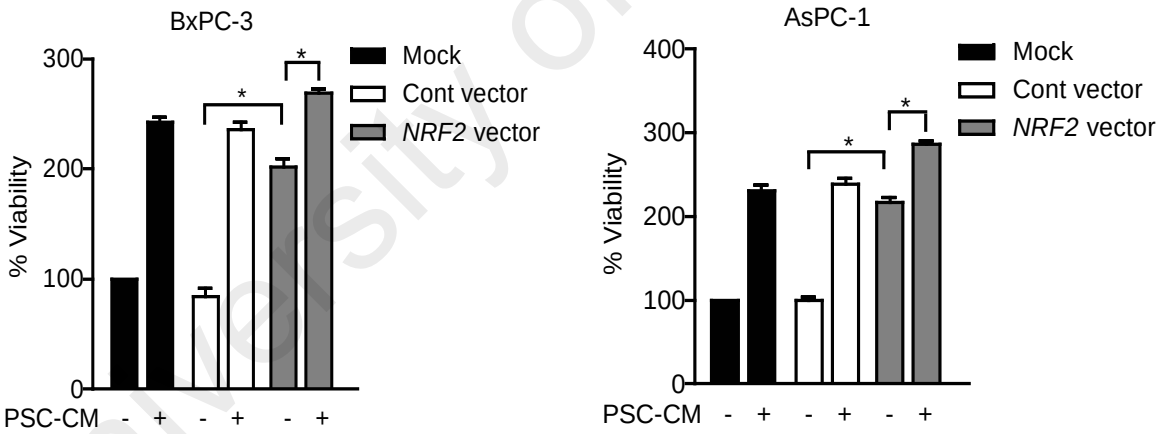

Figure 4.15: Nrf2 activation mediates the PDAC cell proliferation induced by PSC-CM. BxPC-3 (left panel) and AsPC-1 (right panel) cells were transfected with control or *NRF2* expressing vectors for 5 h, before treatment with PSC-CM (1 µg/µl) for 72 h. Cell viability was measured with an MTT assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control vector-transfected cells. The experiment was performed thrice. The best two repeated results were presented. Cont, control.

Despite sustained Nrf2 activation, only a slight drop of intracellular ROS levels (~20% reduction) was observed in Nrf2 overexpressing cells (Figure 4.16), indicating that reducing intracellular ROS levels may not be the only mechanism by which activated Nrf2 signaling promotes cell proliferation in PDAC cells. Furthermore, *NRF2* overexpression induced less ROS in PSC-CM-treated cells compared to control medium treatment, indicating *NRF2* overexpression could attenuate the ROS induction in PSC-CM treated cells. The enhanced cell proliferation and reduced intracellular ROS levels may also be explained by the moderate induction (approximately 1.5-fold) of *NRF2* mRNA and its target genes (*AKR1C1* and *NQO1*) by PSC-CM (Figure 4.17). These data suggested that constitutive Nrf2 expression further increased the PDAC cell proliferation induced by PSC secretion.

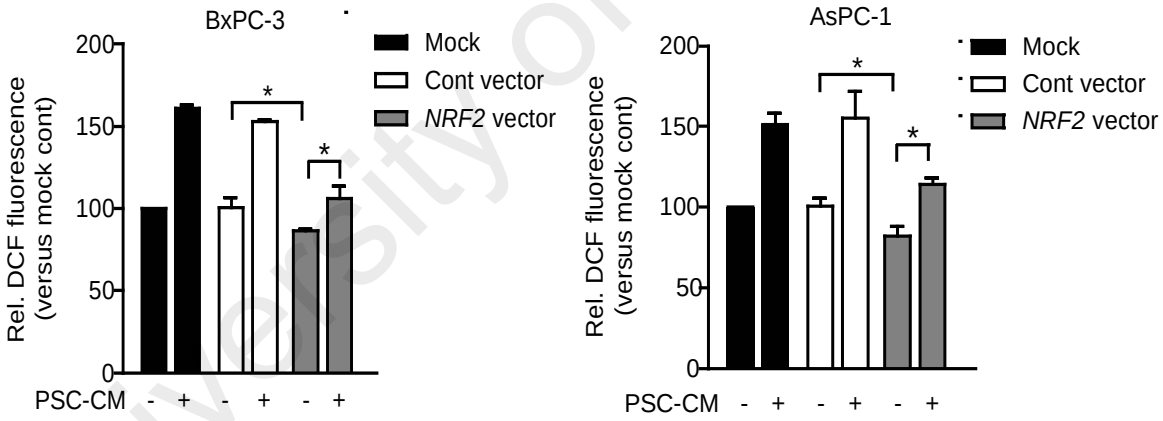

Figure 4.16: Increased Nrf2 activity regulates intracellular ROS levels to effect PSC-mediated PDAC cell proliferation. BxPC-3 (left panel) and AsPC-1 (right panel) cells were transfected with control or *NRF2* expressing vectors for 5 h, before treatment with PSC-CM (1 µg/µl) for 72 h. Intracellular ROS levels were measured using a DCF-DA assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control vector-transfected cells. The experiment was performed thrice. The best two repeated results were presented. Rel., relative; Cont, control.

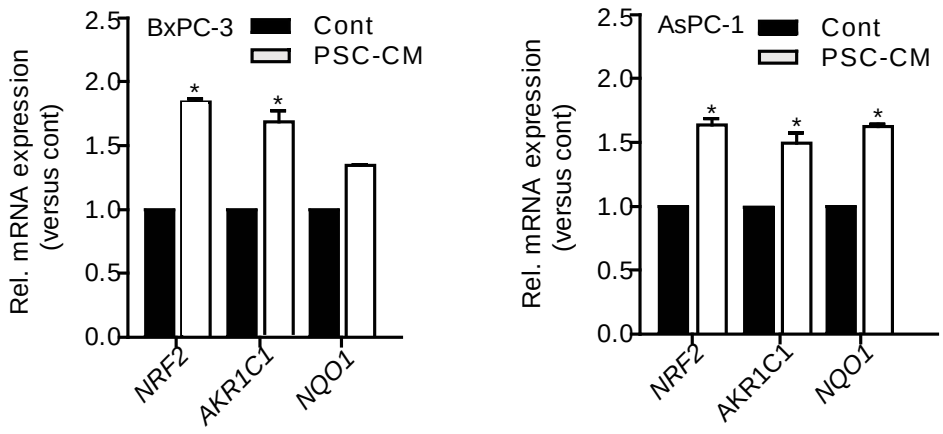

Figure 4.17: PSC-CM treatment further increases *NRF2, AKR1C1,* and *NQO1* gene expression in *NRF2* expressing vector-transfected AsPC-1 and BxPC-3 cells. Cells were transfected with a control or Nrf2 expressing vector for 5 h, then *NRF2*, *AKR1C1* and *NQO1* mRNA expression was determined using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences in *NRF2* vector-transfected cells treated with PSC-CM versus those treated with control medium (1% FBS). The experiment was performed twice. Rel., relative; Cont, control.

4.4 PSC secretion activates metabolic reprogramming via Nrf2 in PDAC cells

Next, it was examined if the proliferation-promoting effect of PSC secretion in PDAC cells occur via Nrf2-mediated metabolic reprogramming. The metabolic gene pathways that are regulated by Nrf2 include PPP, glutaminolysis, and glutathione biosynthesis. The key genes that are involved in these metabolic pathways were examined. Specifically, *G6PD*, *PGD*, *TKT*, *TALDO1*, *PPAT*, and *MTHFD2* are enzymes involved in PPP that generate de novo nucleotide and NADPH equivalents. ME1 and IDH1 enzymes are involved in glutaminolysis to produce NADPH equivalents whereas two rate-limiting enzymes, GCLC and GCLM, were measured to assess glutathione biosynthesis. NADPH and glutathione generation are used for ROS detoxification. *NRF2* knockdown significantly downregulated the mRNA of these metabolic genes in BxPC-3 and AsPC-1 cells (Figure 4.18). The greatest decreases (approximately 0.5-fold) in mRNA levels were observed for *G6PD*, *PGD*, *TKT*, *TALDO1*, *GCLC*, and *GCLC* genes

in these cells, with *ME1* also showing decreased mRNA in BxPC-3 (Figure 4.18). These data indicated that Nrf2 acts as a regulator for these metabolic genes.

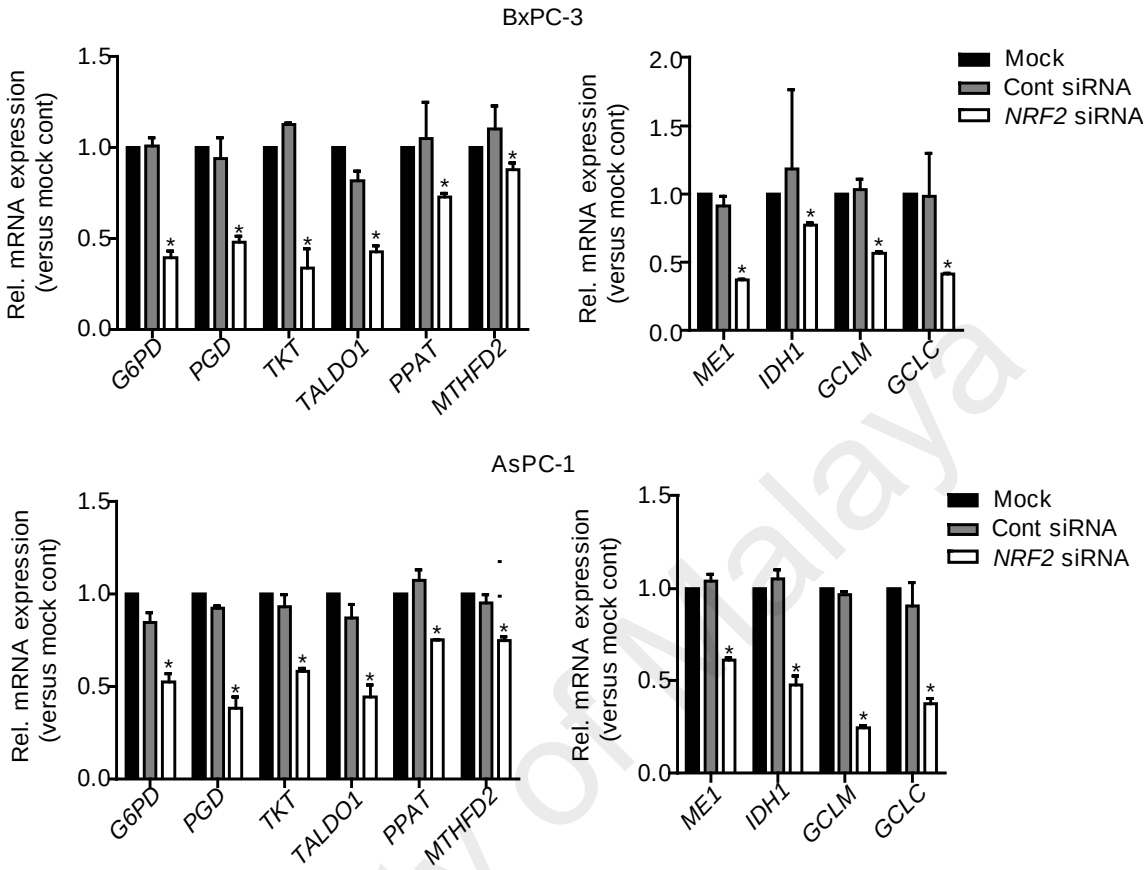

Figure 4.18: Nrf2 regulates the expression of metabolic genes that are involved in PPP, glutaminolysis, and glutathione biosynthesis. BxPC-3 (top panel) and AsPC-1 (bottom panel) cells were transfected with control or *NRF2* siRNA for 72 h. The levels of genes whose products that are involved in metabolic pathways and ROS detoxification were determined using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences in *NRF2* siRNA-transfected cells versus control siRNA-transfected cells. The experiment was performed twice. Rel., relative; Cont, control.

Transcription of genes that are involved in PPP (*G6PD*, *PGD*, *TALDO1*, and *PPAT*) were significantly induced (approximately 1.5-3-fold) in BxPC-3 and AsPC-1 cells following treatment with PSC-CM (P < 0.05 when compared to untreated cells), with the exception of *TKT* and *MTHFD2* in AsPC-1 (Figure 4.19). *ME1*, *IDH1*, *GCLC*, and *GCLM*, which encode enzymes for ROS detoxification, were also significantly upregulated (>2-fold) in these cells, except for *ME1* in AsPC-1 (P < 0.05) (Figure 4.19). These data

suggested that PSC secretion activated Nrf2-mediated metabolic genes that encode proteins involved in nucleotide synthesis and ROS detoxification in PDAC cells.

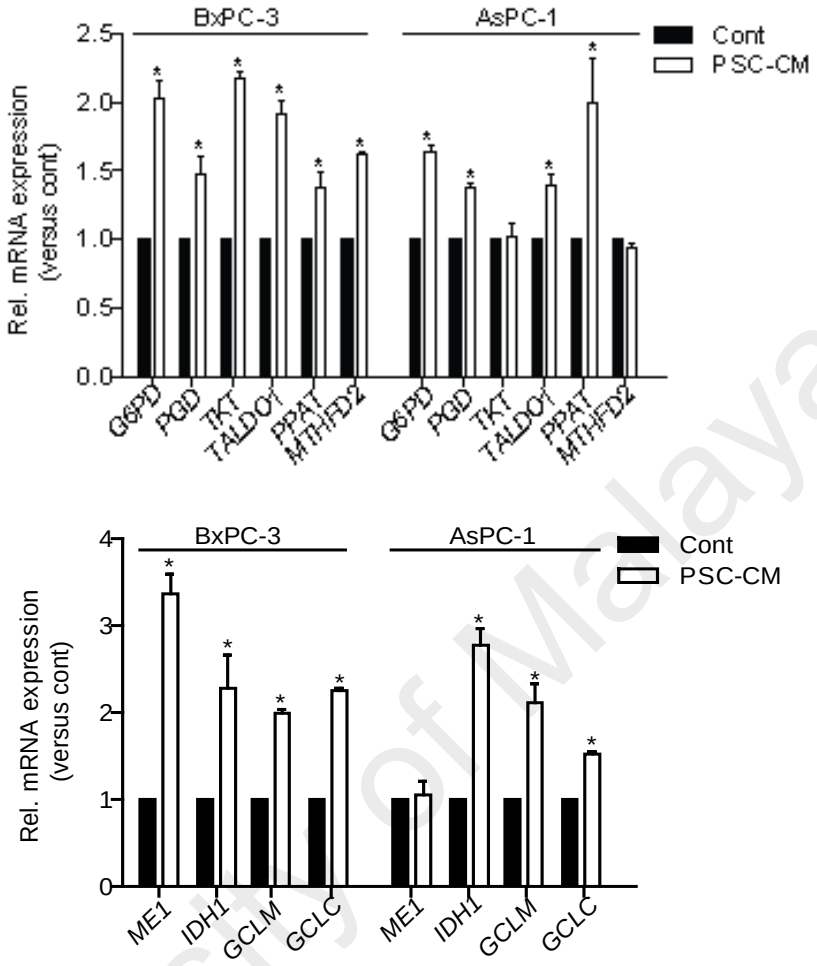

Figure 4.19: PSC-CM treatment upregulates the expression of Nrf2-mediated metabolic genes whose products are involved in PPP, glutaminolysis, and glutathione biosynthesis in AsPC-1 and BxPC-3 cells. Cells were treated with PSC-CM (1 µg/µl) for 72 h. The mRNA levels of metabolic genes were determined using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed twice. Rel., relative; Cont, control.

To further examine the role of PSC secretion in inducing Nrf2-mediated metabolic genes expression, Nrf2 was silenced using siRNA in PDAC cells before PSC-CM treatment. A further approximately 2-fold increase in the expression of these metabolic genes including *TKT*, *MTHFD2*, and *ME1* by PSC-CM treatment was observed when *NRF2* was downregulated (Figure 4.20). In particular, *PGD*, *MTHFD2*, *GCLM*, and *GCLC* transcription was enhanced to a greater degree in AsPC-1 cells than in BxPC-3

cells ($P < 0.05$), which may explain the relatively higher AsPC-1 cell proliferation induced by PSC-CM despite downregulation of *NRF2*. Notably, when *NRF2* was overexpressed, only *PPAT* and *MTHFD2* were upregulated in both cell types whereas *ME1* and *IDH1* were significantly induced in AsPC-1 upon treatment with PSC-CM (Figure 4.21). This result suggests that PDAC cells may utilize the non-oxidative arm of PPP and glutaminolysis pathways to induce further proliferation upon *NRF2* overexpression. Collectively, these findings indicated that PSC secretion could induce Nrf2-mediated metabolic pathways. Differential regulation of PSC regarding these metabolic genes was observed to promote PDAC cell proliferation consequent to dysregulated Nrf2 signaling.

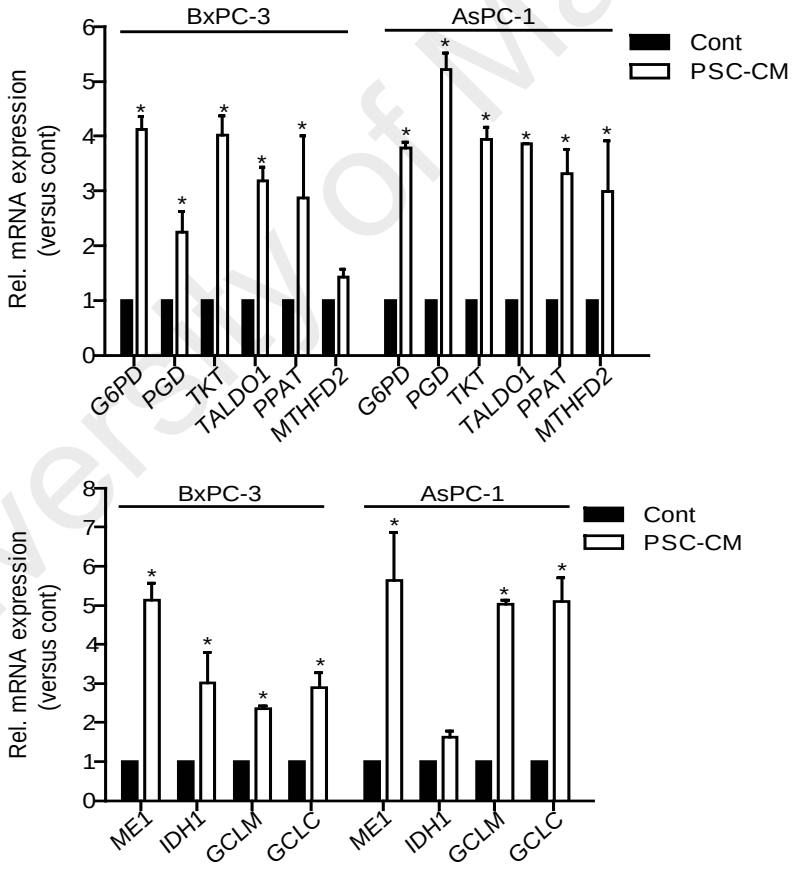

Figure 4.20: PSC-CM increases Nrf2-mediated metabolic gene expression in *NRF2*-silenced AsPC-1 and BxPC-3 cells. Cells were transfected with *NRF2* siRNA for 72 h before treatment with PSC-CM (1 µg/µl) for 72 h. The mRNA levels of metabolic genes were determined using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *$P < 0.05$ indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed twice. Rel., relative; Cont, control.

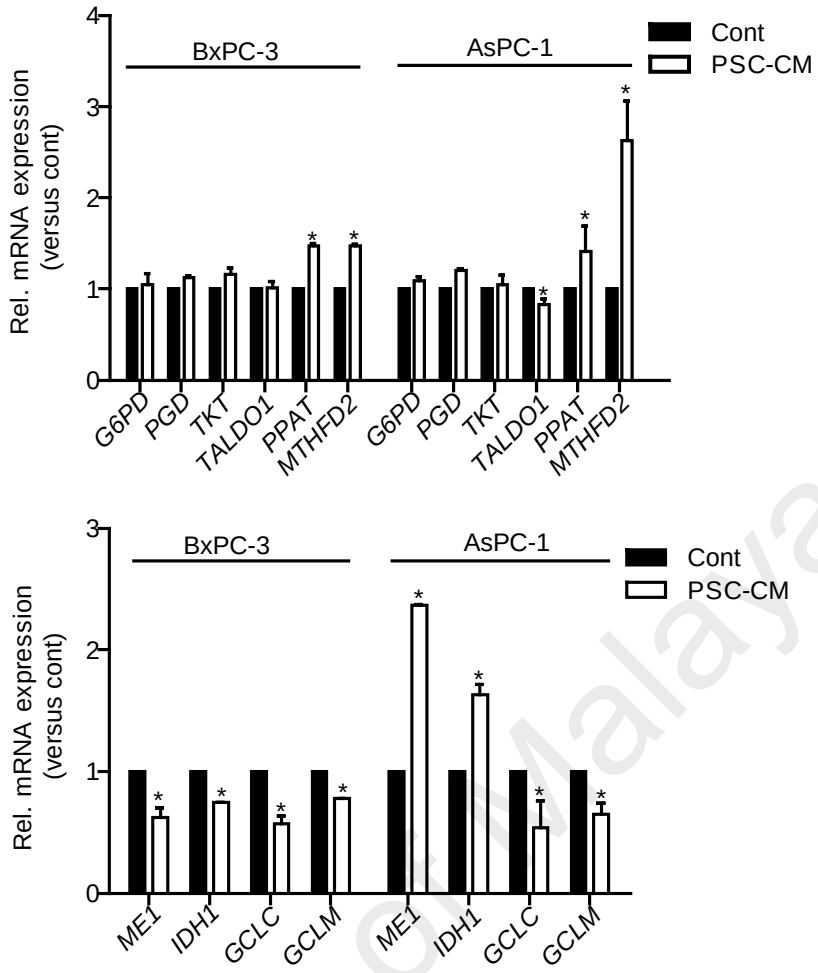

Figure 4.21: PSC-CM treatment further increases the expression of Nrf2-mediated metabolic genes whose products are involved in PPP and glutaminolysis. BxPC-3 and AsPC-1 cells were transfected with an *NRF2* cDNA plasmid for 5 h before treatment with control media or PSC-CM (1 µg/µl) for 72 h. The mRNA levels of metabolic genes were determined using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed twice. Rel., relative; Cont, control.

In addition, the effect of PSC secretion on the production of metabolites was also measured. The induction of metabolic pathways by PSC-CM led to an increased level of metabolites that are required in glycolysis, glutaminolysis, and nucleotide synthesis (Figure 4.22). Ribose 5-phosphate (R5P), a critical substrate for nucleotide synthesis, and inosine 5'-monophosphate (IMP) were significantly increased in both PSC-CM-treated PDAC cell types (P < 0.05) (Figure 4.22). In addition, the concentration of glutamate and malate, a substrate used to produce NADPH equivalents via the ME1 enzyme, was

increased at least 20-fold and 5-fold, respectively in both cells (Figure 4.22). These data indicated that PSC secretion was able to increase the production of metabolites required in glycolysis, PPP, and glutaminolysis for nucleotide synthesis and ROS detoxification in PDAC cells.

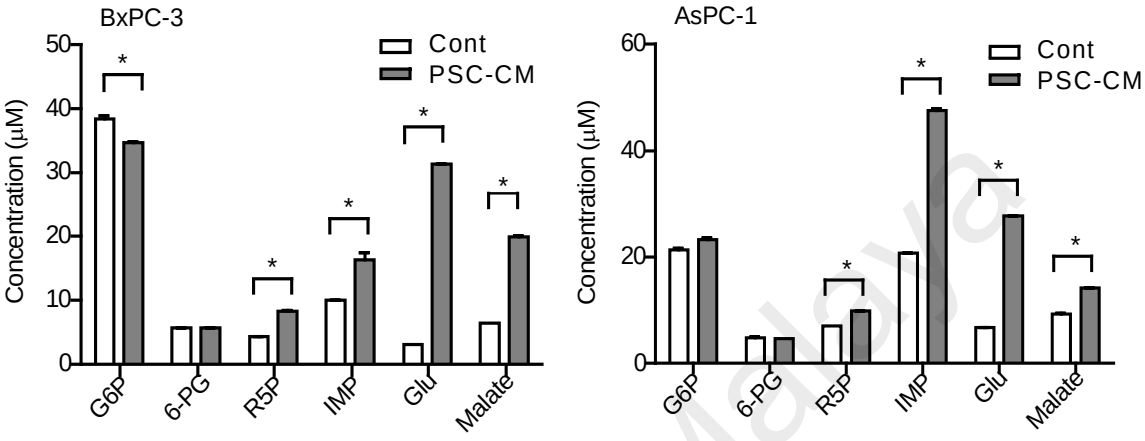

Figure 4.22: PSC-CM increases the concentration of metabolites required for purine nucleotides synthesis and ROS detoxification. Extracts from BxPC-3 (left panel) and AsPC-1 (right panel) cells treated with PSC-CM (1 μg/μl) for 72 h were subjected to UHPLC-MS/MS analysis to determine the concentration of various metabolites. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed twice. Cont, control.

To examine the role of PPP in PSC-mediated PDAC cell proliferation in more detail, G6PD expression in PDAC cells was inhibited using either a pharmacological G6PD inhibitor (DHEA) or G6PD siRNA in the presence of PSC secretion. BxPC-3 showed significant cell inhibition (approximately 95% inhibition) whereas AsPC-1 was slightly resistant to the inhibitor (approximately 80% inhibition) at 100 μM ($P < 0.05$) (Figure 4.23). A more specific downregulation of G6PD protein expression using siRNA led to approximately 70% reduction of *G6PD* mRNA (Figure 4.24), which resulted in a significant decrease in cell viability of BxPC-3 (62.6 ± 0.8%) and AsPC-1 (58.2 ± 0.9%) in comparison to control siRNA-transfected cells (Figure 4.25). These data suggested that

the activation of PPP by Nrf2 signaling was critical for PSC-mediated cell proliferation in PDAC.

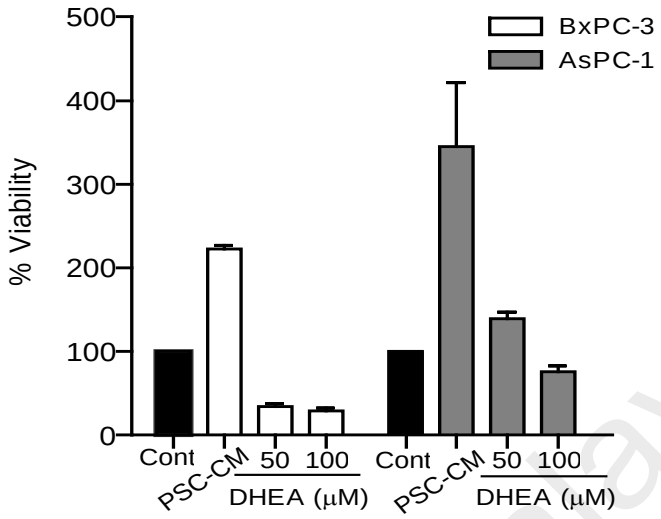

Figure 4.23: Inhibition of G6PD enzyme activity decreases the AsPC-1 and BxPC-3 cell proliferation mediated by PSC-CM. Cells were treated with PSC-CM (1 µg/µl, 72 h) with or without DHEA, before measurement of the cell viability with an MTT assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus AsPC-1 and BxPC-3 cells treated with control medium (medium containing 1% FBS). The experiment was performed twice. Cont, control.

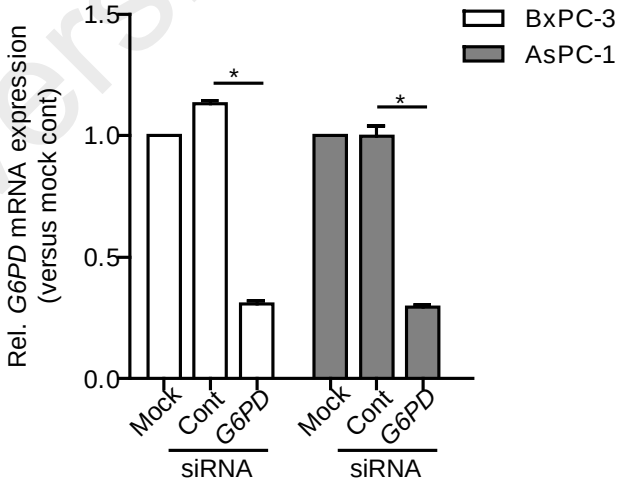

Figure 4.24: Expression of the *G6PD* gene in AsPC-1 and BxPC-3 cells after RNAi-mediated gene silencing. To silence *G6PD* gene expression, cells were transfected with control siRNA (100 nM) or *G6PD* siRNA (100 nM) for 24 h. The mRNA levels of G6PD were measured using qRT-PCR. Data shown are average of triplicates from one experiment, and he error bars represent the SD. *P < 0.05 indicates significant differences versus cells transfected with control siRNA. The experiment was performed twice. Rel., relative; Cont, control.

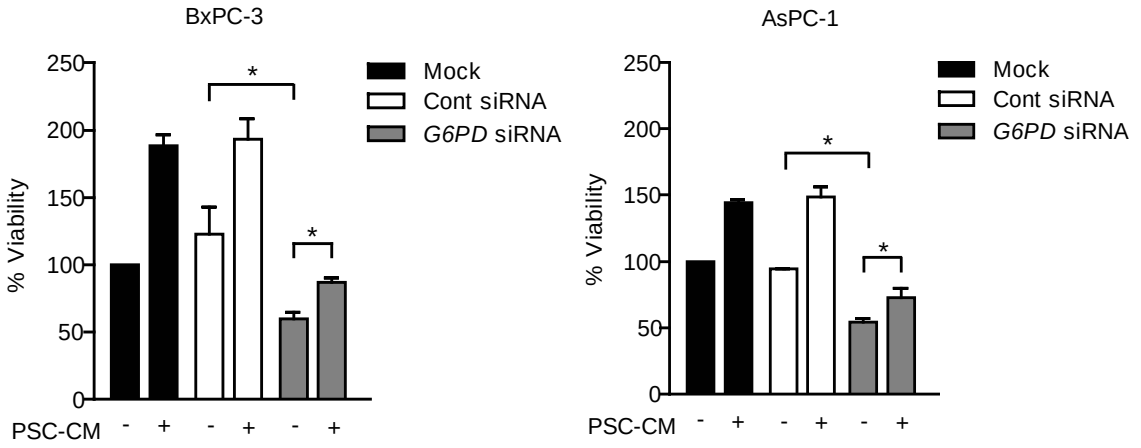

Figure 4.25: *G6PD* gene silencing decreases PDAC cell proliferation. After transfecting with *G6PD* siRNA for 72 h, BxPC-3 (left panel) and AsPC-1 (right panel) cells were treated with PSC-CM (1 µg/µl) for 72 h before the determination of cell viability with an MTT assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). The experiment was performed twice. Cont, control.

4.5 IL-6 and SDF-1α from PSC activate Nrf2 signaling

To determine the possible soluble factors in PSC secretion that may be responsible for activating Nrf2 signaling, the concentration of a panel of cytokines and growth factors was measured using ELISA kits. Among the soluble factors tested, growth-promoting oncogene alpha (GRO-α) showed the highest level (4534.89 ± 19 pg/ml) in PSC-CM, followed by stromal-derived factor-1 alpha (SDF-1α) (553.87 ± 17.68 pg/ml) and vascular endothelial growth factor (VEGF) (120.63 ± 4.94 pg/ml) (Figure 4.26A). When compared to control medium, GRO-α also represented the cytokine with the greatest fold change (32.3-fold) followed by SDF-1α (4.8-fold) and IL-6 (2.9-fold) (Figure 4.26B).

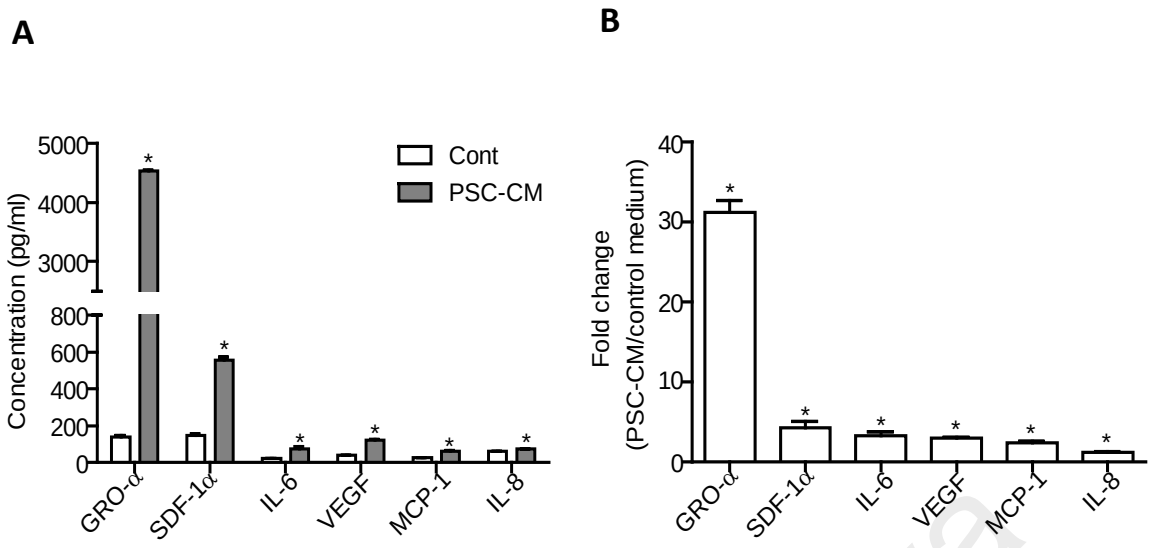

Figure 4.26: Identification and concentration measurement of soluble factors secreted by PSC. The levels of soluble factors present in control medium (medium containing 1% FBS) and PSC-CM were determined with ELISA kit, and expressed in absolute concentration (A) and in fold-change (PSC-CM versus control medium) (B). Data shown are average of triplicates from one experiment, and he error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium 1% FBS). The experiment was performed twice. Cont, control.

Next, the three secreted soluble factors, GRO-alpha, SDF-1α, and IL-6 with the highest degree of change compared to control medium were selected and their effects on cell proliferation were examined using the respective recombinant proteins and neutralizing antibodies. Treatment with recombinant protein (rh)IL-6 led to increased cell proliferation in BxPC-3 (153.5 ± 7.8 % at 200 ng/ml) but not in AsPC-1 (100.14± 3.6% at 200 ng/ml) (Figure 4.27). Notably, cell proliferation was also induced following treatment with rhSDF-1α but not with rhGRO-α (Figure 4.27). Both BxPC-3 and AsPC-1 cells responded to 100 ng/ml rhSDF-1α with increases of 151.5 ± 3.9% and 156.3 ± 2.6%, respectively (Figure 4.27).

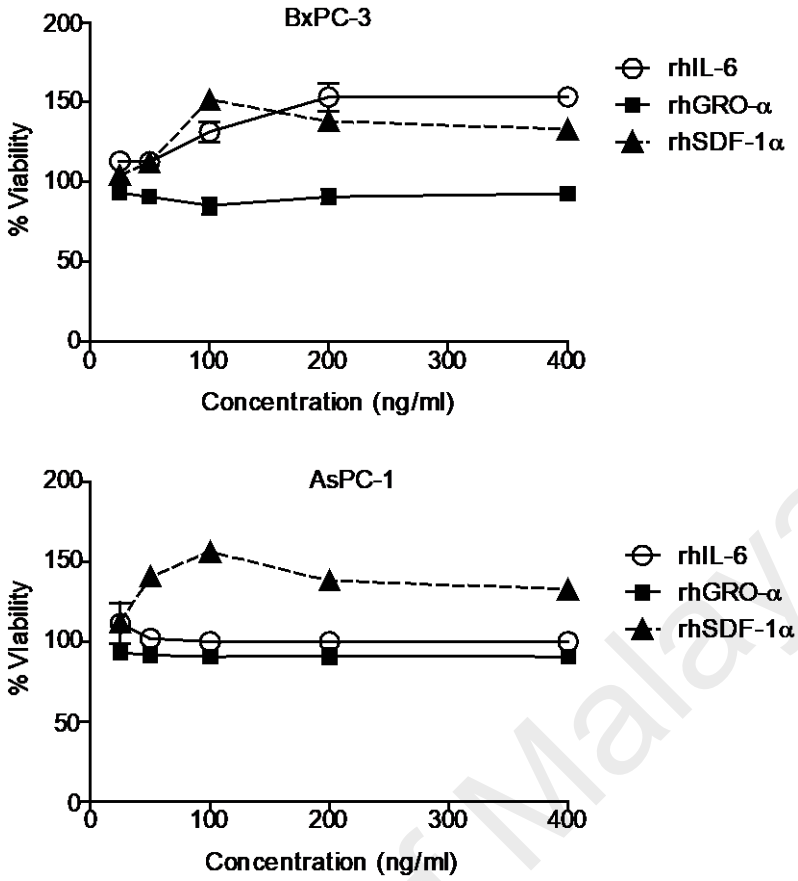

Figure 4.27: rhIL-6 and rhSDF-1α have growth-promoting effects on PDAC cells. BxPC-3 (top panel) and AsPC-1 (bottom panel) cells were treated with recombinant human proteins (rhIL-6, rhGRO-α, and rhSDF-1α) at various concentrations (0-400 ng/ml) for 72 h, before analyzing their viability with an MTT assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD, comparing with untreated AsPC-1 and BxPC-3 cells. The experiment was performed thrice. The best two repeated results were presented.

To further confirm the paracrine effect of PSC secretion on PDAC cell proliferation, IL-6 and SDF-1α in PSC-CM was neutralized using their anti-antibody. Neutralizing of IL-6 and SDF-1α resulted in significant decreases in the proliferation of BxPC-3 and AsPC-1 cells. Specifically, IL-6 neutralization led to reduced cell proliferation in BxPC-3 (54.4 ± 1.5%) and AsPC-1 (63.6 ± 1.7%) at 400 ng/ml (Figure 4.28A). Compared to IL-6, a lesser reduction in BxPC-3 cell proliferation (69.2 ± 1.2%) was induced by SDF-1α neutralization at 400 ng/ml whereas a similar reduction was observed in AsPC-1 (63.9 ± 2.7%) (Figure 4.28B).

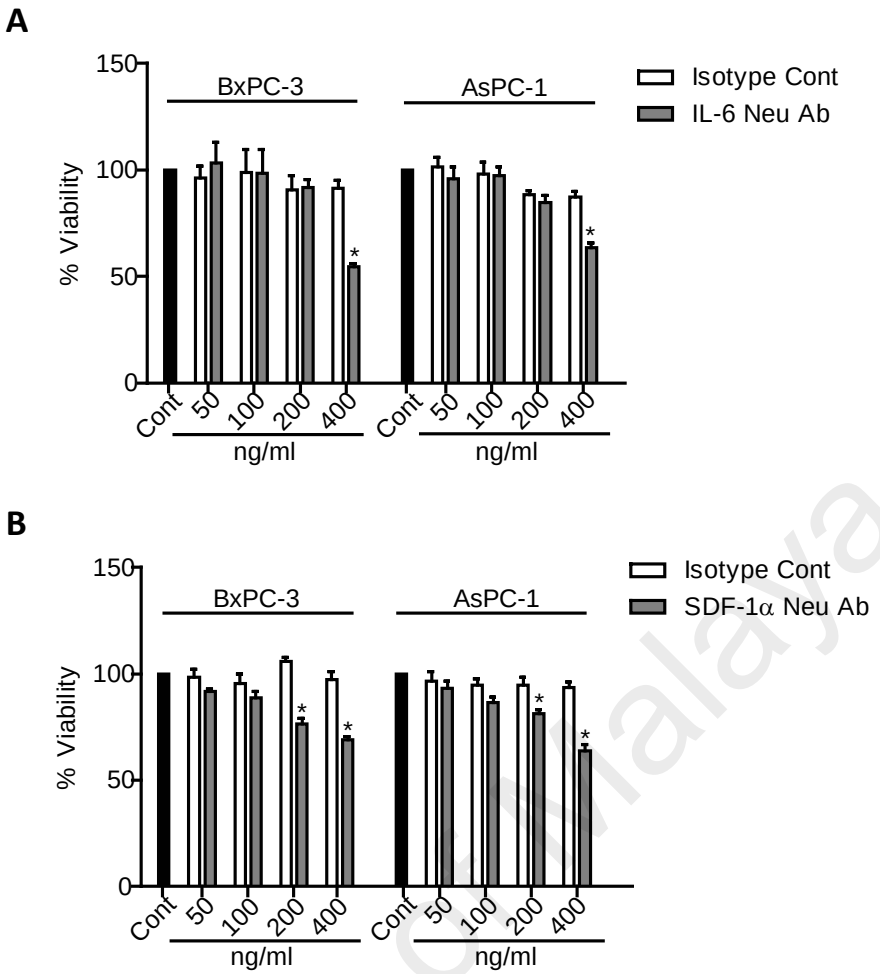

Figure 4.28: Neutralization of IL-6 and SDF-1α in PSC-CM decreases AsPC-1 and BxPC-3 cell proliferation. Cells were treated with IL-6 (A) and SDF-1α (B) neutralizing antibodies (0-400 ng/ml) in the presence of PSC-CM (1.0 µg/µl, 72 h) before the determination of cell viability with an MTT assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus AsPC-1 and BxPC-3 cells treated with PSC-CM. The experiment was performed thrice. The best two repeated results were presented. Cont, control.

To further investigate whether IL-6 is required for BxPC-3 and AsPC-1 cell proliferation, its downstream signaling molecules, JAK3 and Stat3, were inhibited by using AD412 and Stattic respectively. Upon treatment with these inhibitors, over 50% of the proliferation in these cells was inhibited. The cell proliferation decreased in a dose-dependent manner, with a greater effect being observed on AsPC-1 (AD412: 97 ± 0.3%; Stattic: 98 ± 0.1% inhibition) compared to BxPC-3 (AD412: 87 ± 0.5%; Stattic: 87 ± 0.6% inhibition) at 100 µM (Figure 4.29). These data indicated that the IL-6 and SDF-1α secreted by PSC could induce PDAC cell proliferation.

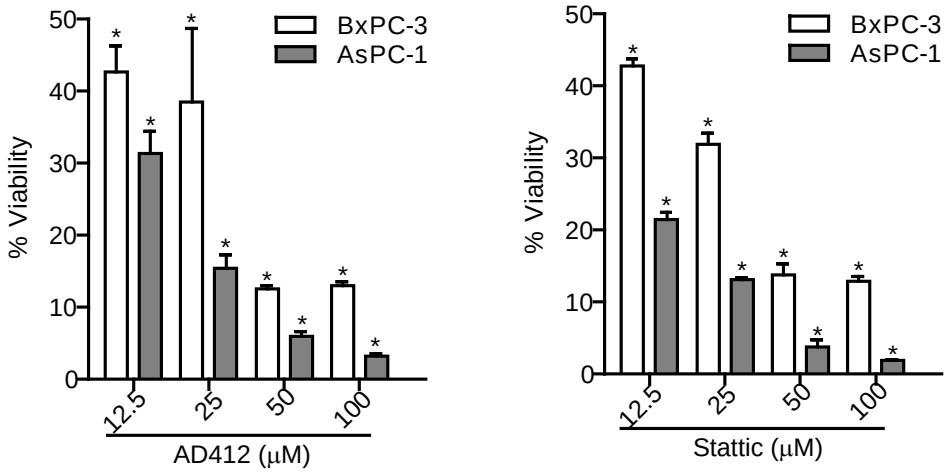

Figure 4.29: Inhibition of the JAK and Stat3 signaling induced by PSC-CM decreases AsPC-1 and BxPC-3 cell proliferation. Cells were treated with AD412 and Stattic (0-100 μM), which comprise pharmacological inhibitors for JAK3 and Stat3 signaling, respectively, in the presence of PSC-CM (1.0 μg/μl) for 72 h. An MTT assay was used to determine the cell viability. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus AsPC-1 and BxPC-3 cells treated with PSC-CM. The experiment was performed thrice. The best repeated results were presented.

To evaluate whether IL-6 and SDF-1α increase PDAC cell proliferation via Nrf2 signaling activation, the mRNA levels of *NRF2* and its target genes were measured. Nrf2 signaling was activated upon treatment with rhIL-6 and rhSDF-1α, which increased *NRF2* mRNA up to approximately 2-fold (Figure 4.30). *AKR1C1* mRNA was significantly upregulated in BxPC-3 and AsPC-1 cells after treatment with rhSDF-1α whereas upregulation was only observed in BxPC-3 cells following rhIL-6 treatment (Figure 4.30). rhIL-6 did not increase *NQO1* mRNA in either cell types, with the exception of BxPC-3 cells following treatment with rhSDF-1α (Figure 4.30). These data suggested that IL-6 and SDF-1α could independently activate Nrf2 signaling and may partly responsible for PSC-mediated PDAC cell proliferation.

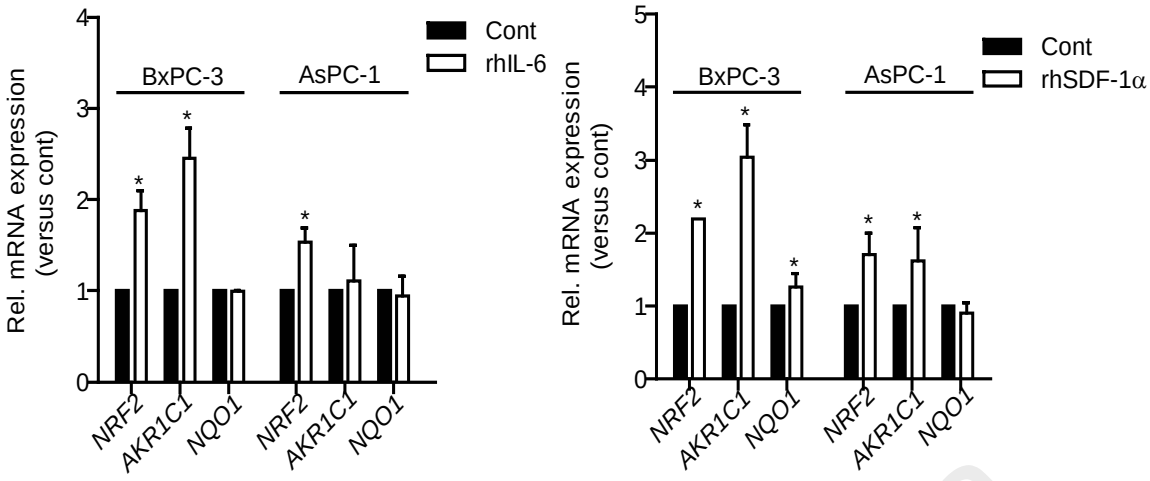

Figure 4.30: rhIL-6 and rhSDF-1α treatment increase the mRNA expression of *NRF2* and its downstream target genes in AsPC-1 and BxPC-3 cells. The levels of *NRF2* and its target genes (*AKR1C1* and *NQO1*) mRNA were measured using qRT-PCR in cells treated with rhIL-6 (100 ng/ml) and rhSDF-1α (100 ng/ml). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control medium (medium containing 1% FBS). Each experiment was performed twice. Rel., relative; Cont, control.

4.6 *IL6* and *IL6R* gene expression in PSC and PDAC cells

To determine the role of IL-6 signaling in mediating PSC-PDAC interaction, the gene expression levels of *IL6* and its receptor, *IL6R* were measured in PSC and three PDAC cell lines (AsPC-1, BxPC-3, and Panc-1). Among the PDAC cells, Panc-1 expressed the lowest level of *IL6* mRNA (0.01-fold), followed by BxPC-3 (0.31-fold) when compared to that of AsPC-1 cells. In contrast, PSC showed a high level of *IL6* mRNA expression (4.4-fold, P < 0.05) in comparison to AsPC-1 cells (Figure 4.31A). Higher *IL6R* expression, however, was detected in PDAC cells than in PSC. Panc-1 cells expressed the highest (5.8-fold) *IL6R* levels followed by BxPC-3 cells (1.46-fold), whereas PSC expressed the lowest (0.44-fold) level of *IL6R* when compared to AsPC-1 cells (Figure 4.31B). The highest *IL-6* and the lowest *IL-6R* mRNA expression in AsPC-1 cells may explain why no proliferation-promoting effect of rhIL-6 was observed in AsPC-1 cells. These data suggested that the IL-6 expressed by PSC are likely to interact in a paracrine manner with the receptor expressed by PDAC cells.

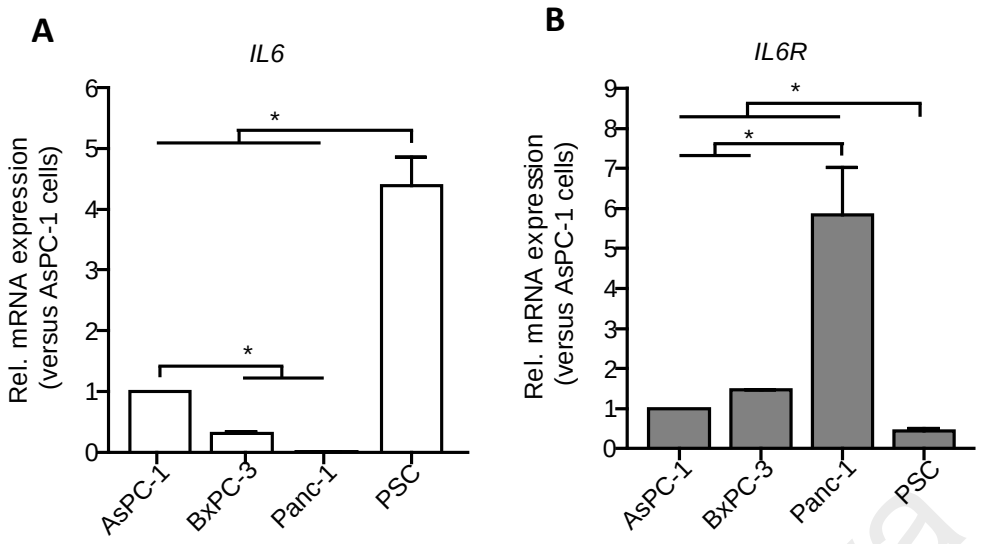

Figure 4.31: *IL6* and *ILR* gene expression profiling in PSC and PDAC cells. The mRNA levels of *IL6* and *IL-6R* in PSC and PDAC cells (AsPC-1, BxPC-3, and Panc-1) were determined by qRT-PCR. The expression of target mRNA was normalized to that of *GAPDH* mRNA. The values are expressed relative to 1 for expression in AsPC-1 cells. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus the mRNA expression in AsPC-1 cells. Each experiment was performed twice. Rel., relative.

To further assess whether the abundant IL-6 expressed by PSC may interact with PDAC cells in a paracrine manner, IL-6 expression was examined after PSC inactivation. The PSC inhibitor, ATRA, was used to induce PSC into a relatively quiescent state. After treatment with ATRA, PSC underwent a morphological change from spindle-shaped to flattened polygonal-shaped with abundant lipid droplets in the cytoplasm (Figure 4.32A). Additionally, the mRNA expression levels of *IL6* were significantly reduced (approximately 0.5-fold, P < 0.05) compared to those of untreated PSC (Figure 4.32B). The reduction in mRNA also led to reduced IL-6 protein levels in ATRA-treated PSC (Figure 4.32C), indicating that PSC activation led to higher IL-6 expression in these cells.

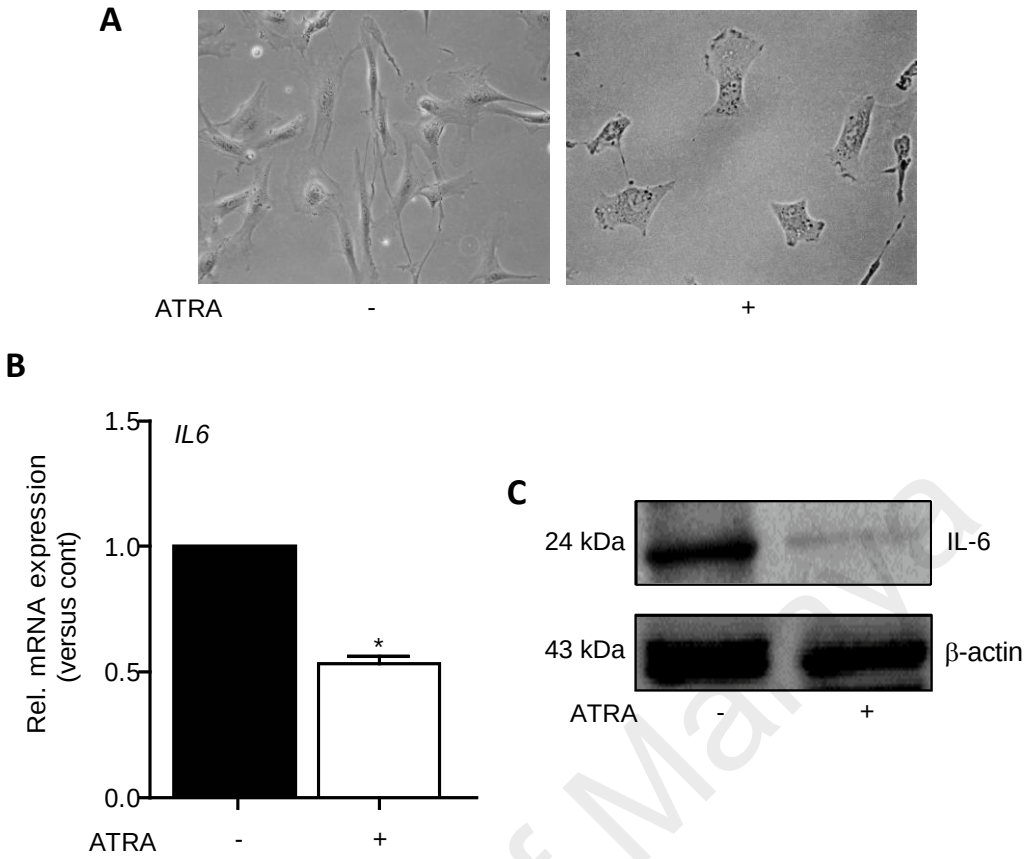

Figure 4.32: Inactivation of PSC reduces *IL6* gene and protein expression levels. PSC were treated with ATRA (20 μM) for 24 h, then the morphological change was viewed under phase contrast microscopy and photographed (A). Original magnification: 400×. *IL6* mRNA and protein expression levels were measured using qRT-PCR (B) and western blotting (C), respectively. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus PSC treated with SFM. Each experiment was performed twice. The best representative result for western blotting was presented. Rel., relative.

4.7 Nrf2 activity mediates IL-6-induced metabolic reprogramming and ROS detoxification in Panc-1 cells

To investigate whether the IL-6 secreted by PSC might promote PDAC cell proliferation via Nrf2-mediated metabolic reprogramming, the role of IL-6 in mediating the key genes in metabolic pathways was examined. Panc-1 cells were used to study the paracrine interaction of PSC with PDAC cells because it expressed the lowest *IL6* and the highest *IL6R* mRNA levels compared to AsPC-1 and BxPC-3 cells. All metabolic genes that are involved in PPP (*G6PD*, *PGD*, *TKT*, *TALDO1*, and *PPAT*) except *MTHFD2*,

glutaminolysis (*ME1* and *IDH1*), and glutathione biosynthesis (*GCLC* and *GCLM*) were significantly upregulated at least 1.5-fold ($P < 0.05$) in Panc-1 cells upon PSC-CM treatment (Figure 4.33A). A similar upregulation pattern was observed in Panc-1 cells treated with rhIL-6, with the mRNA expression of *PGD, GCLC,* and *GCLM* being slightly elevated compared to that following PSC-CM treatment, except for *ME1* (Figure 4.33A). In addition, increased metabolic gene mRNA levels (at least1.5-fold, $P < 0.05$) were also observed in BxPC-3 and AsPC-1 cells (except for *TKT* in both cell types and *ME1* in BxPC-3) after rhIL-6 treatment (Figure 4.33B), further supporting the role of IL-6 in mediating Nrf2-activated metabolic pathways.

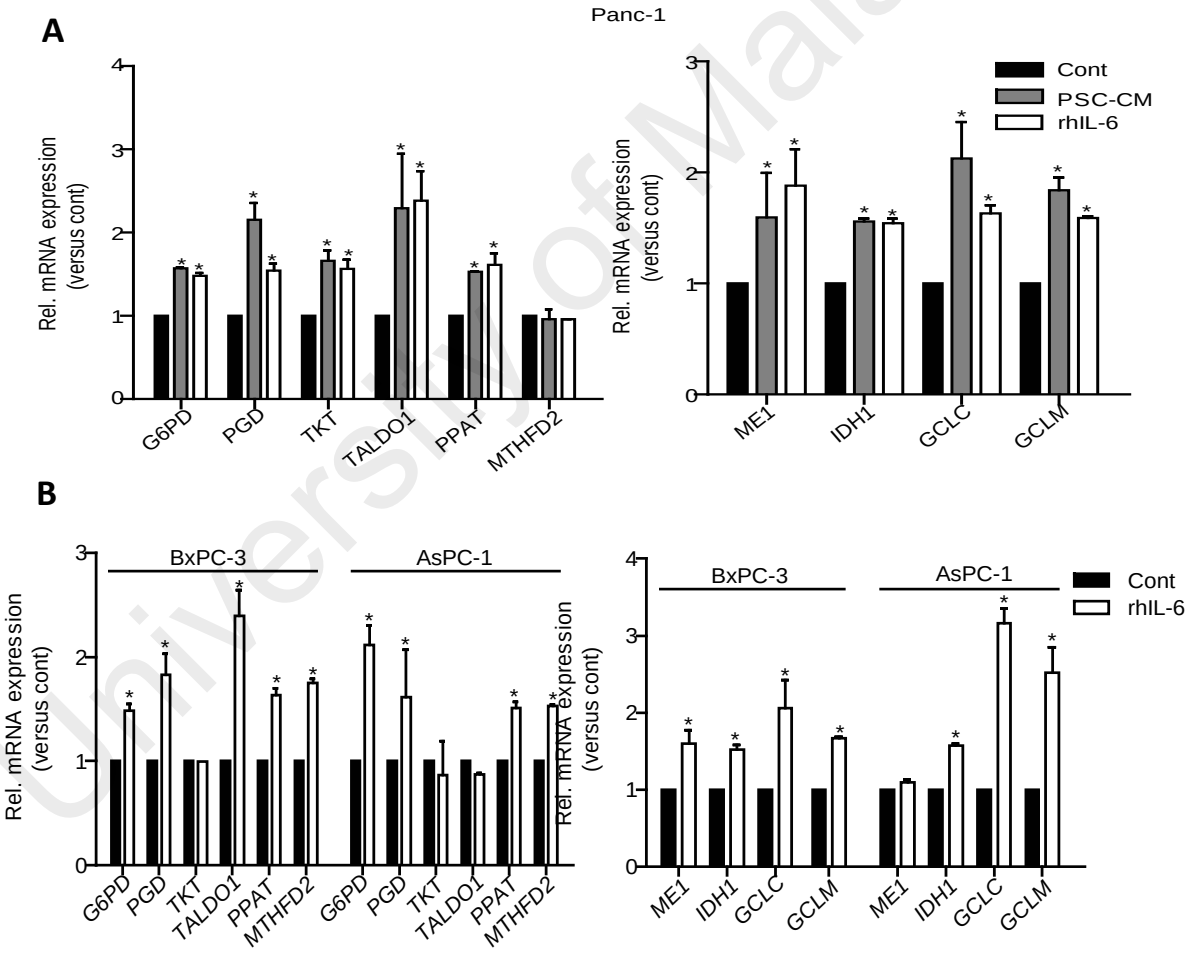

Figure 4.33: PSC-CM and rhIL-6 upregulate the expression of metabolic genes that are involved in PPP, glutaminolysis, and glutathione biosynthesis in PDAC cells. After treatment with PSC-CM (1 µg/ml) or rhIL-6 (100 ng/ml) for 24 h, the mRNA levels of Nrf2-mediated metabolic genes in Panc-1 (A), BxPC-3, and AsPC-1 (B) cells were measured using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *$P < 0.05$ indicates significant differences versus cells treated with SFM. The experiment was performed twice. Rel., relative; Cont, control.

To further determine whether IL-6 partly contributes to the upregulation of metabolic gene expression, IL-6 in PSC-CM was neutralized using an anti-IL-6 antibody and the metabolic gene expression was examined. All metabolic genes that are involved in PPP, glutaminolysis, and glutathione biosynthesis were significantly downregulated after IL-6 neutralization in PSC-CM. A larger downregulation was observed for *G6PD* and *TALDO1* (>0.5-fold) of PPP compared to that of *PGD*, *TKT*, *PPAT*, *MTHFD2*, *ME1*, *IDH1*, *GCLC*, and *GCLM* (Figure 4.34). These data indicated that IL-6 contributed to the action of PSC to activate Nrf2-mediated metabolic reprogramming.

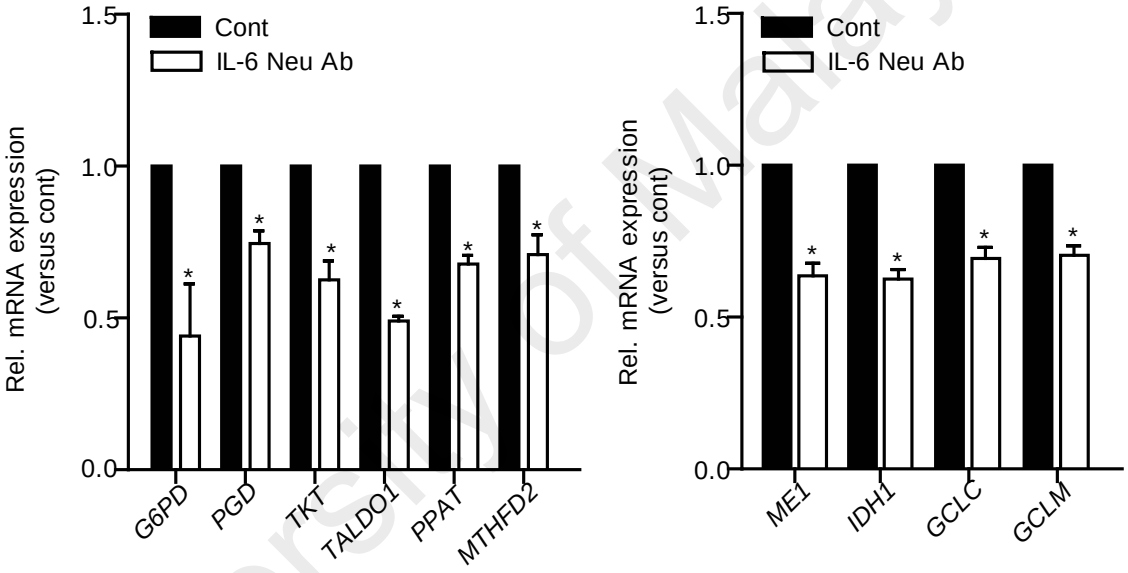

Figure 4.34: IL-6 neutralization in PSC-CM downregulates the expression of Nrf2-mediated metabolic genes that are involved in PPP, glutaminolysis, and glutathione biosynthesis in Panc-1 cells. To confirm that the effect of PSC-CM is partly due to IL-6 production, the IL-6 in PSC-CM was neutralized 24 h before measuring the mRNA levels of metabolic genes using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus treatment with PSC-CM. The experiment was performed twice. Rel., relative; Cont, control.

4.8 IL-6 secreted by PSC activates Nrf2 signaling to induce metabolic reprogramming and ROS detoxification in Panc-1 cells

To examine whether intracellular Nrf2 signaling functions to mediate PSC-secreted IL-6 induction of metabolic reprogramming for purine nucleotide synthesis and ROS detoxification, Panc-1 cells were treated with tBHQ, an Nrf2 activator, after IL-6 neutralization. The results showed that Nrf2 signaling activation markedly reversed the expression of metabolic genes caused by IL-6 neutralization in PSC-CM (Figure 4.35). All metabolic genes were significantly upregulated up to 2-fold, with greater upregulation being observed for *TKT* (approximately 4-fold) and *TALDO1* (approximately 7-fold) (Figure 4.35).

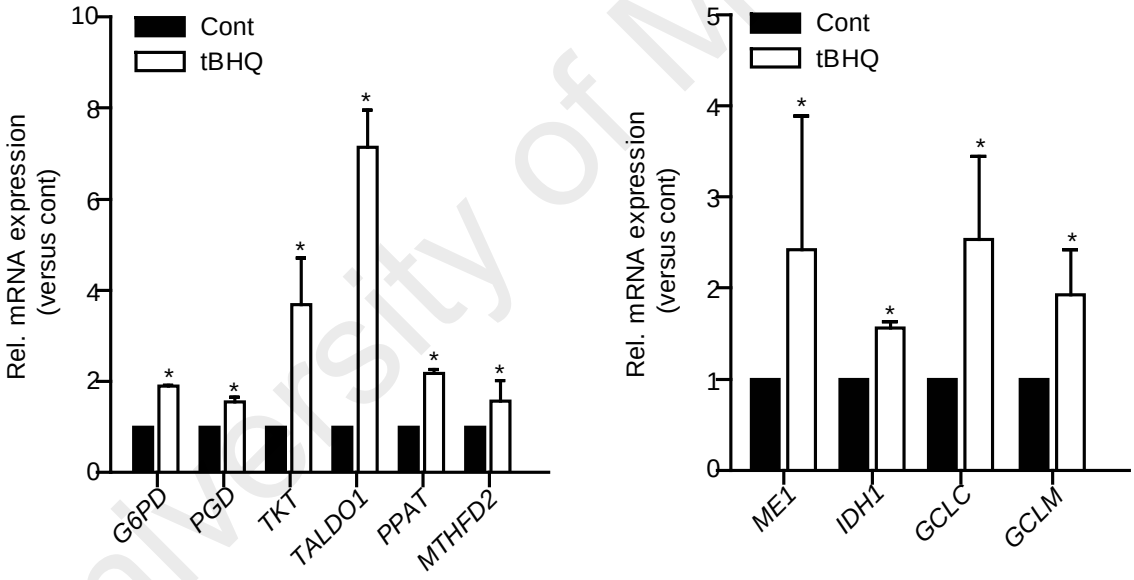

Figure 4.35: Increased Nrf2 activity increases the expression of metabolic genes that are reduced by IL-6 neutralization. Panc-1 cells were treated with tBHQ (1 µM) for 24 h in the presence of PSC-CM (1 µg/ml) and an IL-6 neutralizing antibody (4 µg/ml). qRT-PCR was used to examine the mRNA levels of metabolic genes. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus cells treated with PSC-CM together with the IL-6 neutralizing antibody. The experiment was performed twice. Rel., relative; Cont, control.

Next, the role of PSC-secreted IL-6 in ROS detoxification was also determined.by inducing oxidative stress in Panc-1 cells with H_2O_2 followed by rhIL-6 treatment. tBHQ was used as positive antioxidant control. Treatment with rhIL-6 alone significantly

reduced the intracellular ROS levels (68 ± 2.9% inhibition, $P < 0.05$) induced by H_2O_2 whereas tBHQ restored the intracellular ROS almost to basal levels (Figure 4.36). Treatment of Panc-1 cells with rhIL-6 alone significantly induced *NRF2* mRNA (Figure 4.37A). Furthermore, the application of rhIL-6 treatment to Panc-1 cells treated with H_2O_2 induced a significant upregulation of the mRNA expression of *NRF2* and its target genes (*AKR1C1* and *NQO1*) ($P<0.05$) (Figure 4.37B), indicating that the antioxidant activity of IL-6 partly consisted of Nrf2 signaling activation. These results suggested that the IL-6 secreted by PSC may activate Nrf2 signaling to induce metabolic reprogramming in Panc-1 cells.

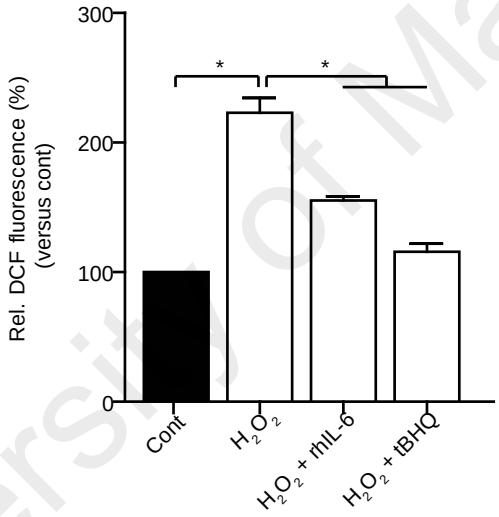

Figure 4.36: rhIL-6 reduces the intracellular ROS levels induced by H_2O_2. Oxidative stress was induced with H_2O_2 (600 µM) for 2 h in the presence of rhIL-6 (100 ng/ml); tBHQ (50 µM) was used as positive control. The intracellular ROS levels were measured with using a DCF-DA assay. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *$P < 0.05$ indicates significant differences versus SFM treatment. The experiment was performed twice. Rel., relative.

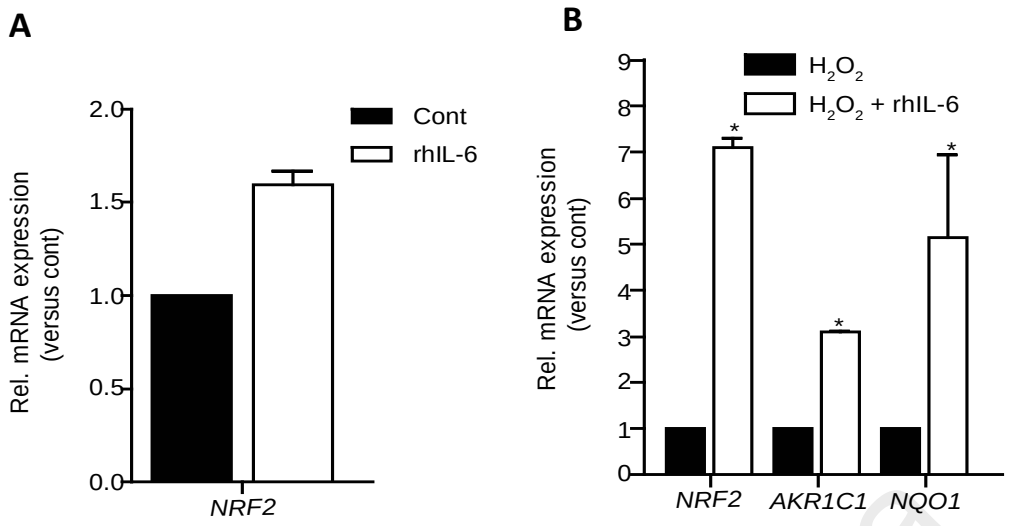

Figure 4.37: IL-6 increases *NRF2* gene expression and exerts antioxidant activity by inducing Nrf2 signaling. Panc-1 cells were treated with rhIL-6 (100 ng/ml) alone (A) or with H_2O_2 (600 µM) for 2 h in the presence of rhIL-6 (100 ng/ml) (B). The mRNA levels of *NRF2* and its antioxidant target genes (*AKR1C1* and *NQO1*) were measured using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus cells treated with rhIL-6 (left panel) or H_2O_2 (right panel). The experiment was performed twice. Rel., relative; Cont, control.

4.9 PSC-secreted IL-6 induces migration and EMT phenotypes in Panc-1 cells

To further elucidate the role of IL-6 secreted by PSC in PDAC progression, its effect on motility and invasion capacity was examined by assessing EMT induction in Panc-1 cells. The morphological and phenotypic changes (migration and invasion capability) and gene expression of EMT-related markers and transcription factors were examined. Panc-1 cells acquired spindle shape and more scattered morphology with less cell-cell adhesion after treatment with PSC-CM (Figure 4.38). Similar results were obtained in Panc-1 cells treated with rhIL-6 (Figure 4.38). When the IL-6 in PSC-CM was neutralized, Panc-1 cells exhibited morphologic characteristics similar to control (SFM), featuring a cobblestone-like shape, less scattering, and tight cell-cell adhesion (Figure 4.38). These data suggested that the IL-6 secreted by PSC induced Panc-1 cells to acquire mesenchymal-like morphology.

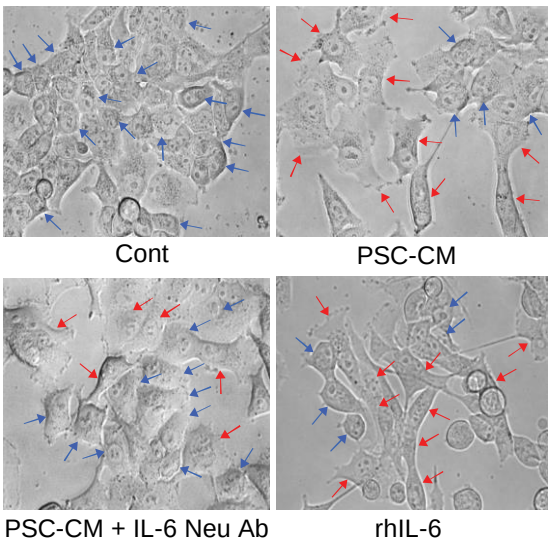

Figure 4.38: IL-6 secreted by PSC induces an EMT-like morphology in Panc-1 cells. The morphological change after treatment SFM (control) or PSC-CM (1 µg/ml) with or without IL-6 neutralizing antibody (4 µg/ml) or rhIL-6 (100 ng/ml) alone for 24 h was viewed under phase contrast and photographed with an inverted light microscope. Original magnification: 400×. Epithelial and epithelial-like cells were shown by blue arrow while mesenchymal-like cells were shown by yellow arrow. The experiment was performed twice and the best representative result was presented**.** Cont, control.

The morphology of Panc-1 cells after PSC-CM and rhIL-6 treatment was similar to that of mesenchymal cells, indirectly indicating that the IL-6 secreted by PSC could increase the migration and invasion capacity in Panc-1 cells. To test this conjecture, Panc-1 cell motility was examined using scratch wound healing and *in vitro* transwell migration assays whereas the invasion capacity was examined using an *in vitro* transwell invasion assay. PSC-CM and rhIL-6 treatment showed similar effect on cell motility by markedly inducing wound closure (approximately 23-24-fold) in Panc-1 cells compared to control (SFM) ($P < 0.05$) (Figure 4.39). IL-6 neutralization reduced the wound closure ability caused by PSC-CM (6.3-fold compared to SFM) (Figure 4.39). Treatment of PSC-CM increased the migration capacity of Panc-1 cells to 4.5-fold whereas rhIL-6 treatment increased the number of migrated cells to 3.2-fold ($P<0.05$), respectively (Figure 4.40). Upon neutralization of the IL-6 in PSC-CM using an anti-IL-6 antibody,

the number of migrated cells was significantly reduced (2-fold, $P < 0.05$) in comparison to that following PSC-CM treatment (Figure 4.40).

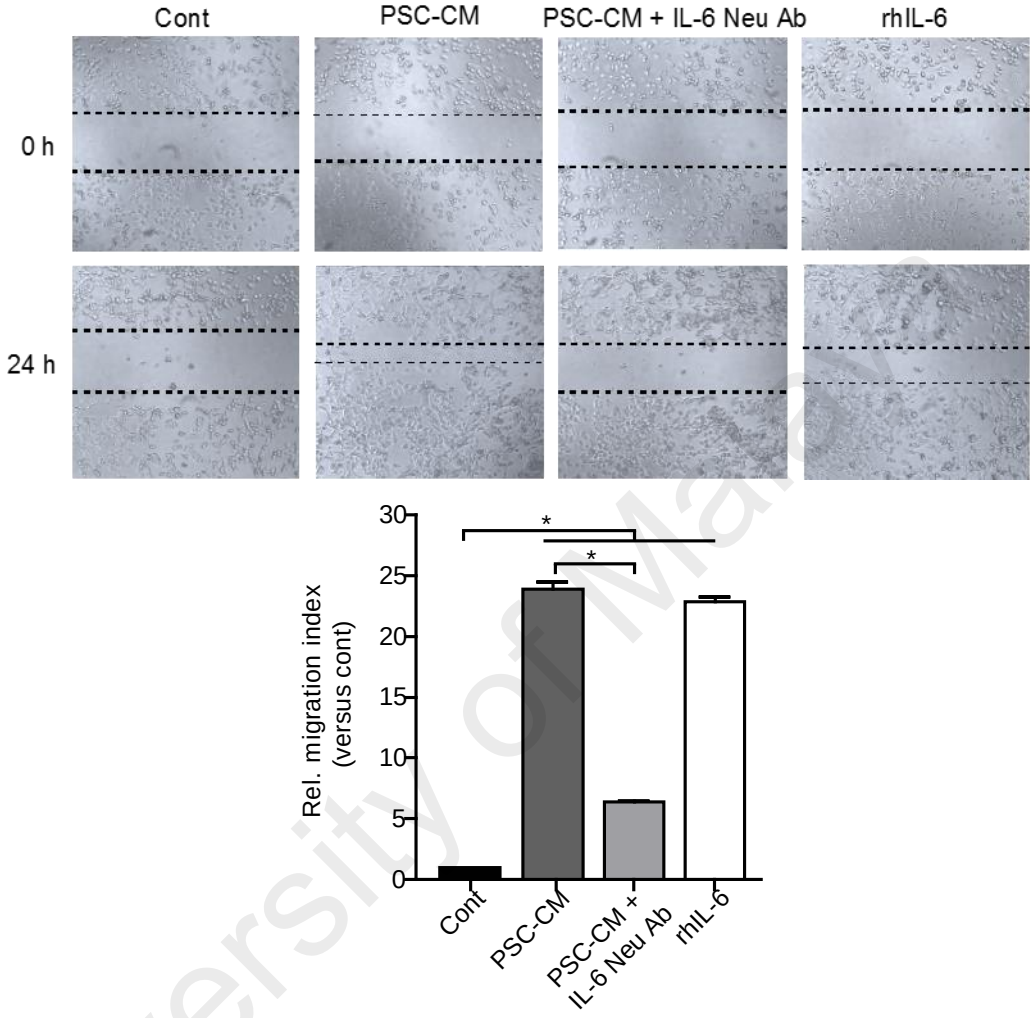

Figure 4.39: IL-6 secreted by PSC increases Panc-1 cell motility. A scratch wound healing assay was used to assess cell motility after 24 h treatment with PSC-CM (1 µg/ml) with or without the IL-6 neutralizing antibody (4 µg/ml) or rhIL-6 (100 ng/ml) alone. The wound closure was viewed under bright field and photographed with an inverted light microscope (top panel). Original magnification: 40×. The distance of wound closure covered by the migrating cells after treatment relative to that stimulated by the control cells was indicated as the migration index (bottom panel). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *$P < 0.05$ indicates significant differences versus SFM treatment. The experiment was performed twice and the best representative was presented. Rel., relative; Cont, control.

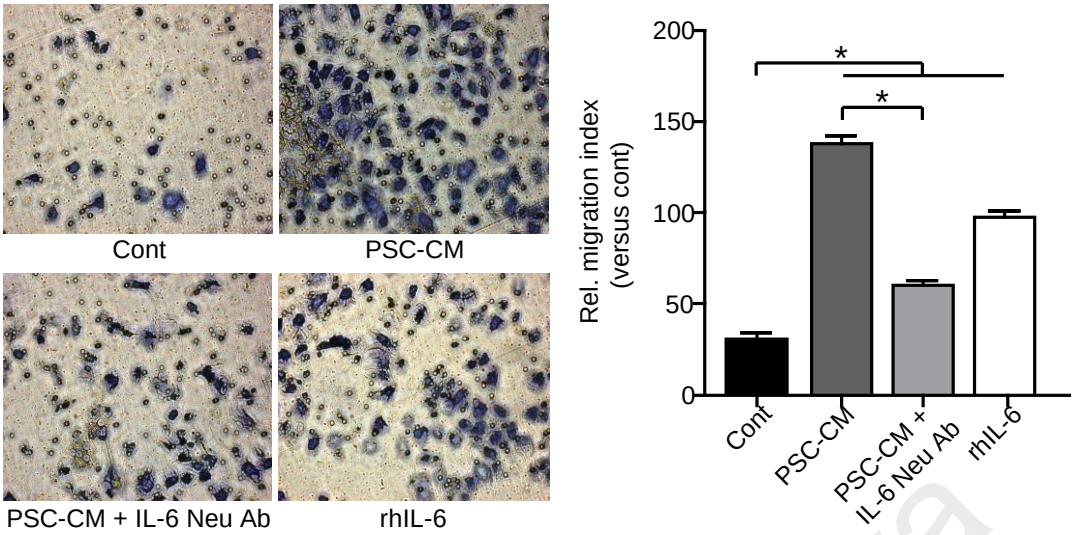

Figure 4.40: IL-6 secreted by PSC promotes Panc-1 cell migration. A transwell migration assay was used to assess cellular motility. After 20 h incubation with PSC-CM (1 µg/ml) with or without an IL-6 neutralizing antibody (4 µg/ml) or rhIL-6 (100 ng/ml) alone, the migrated cells on the lower surface of the filter were stained and counted (left panel). The bar graph shows the number of migrated cells for each category of cells (right panel). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus SFM treatment. The experiment was performed twice and the best representative result was presented. Cont, control.

Treatment with PSC-CM for 24 h significantly increased the invasion capacity of Panc-1 cells by approximately 6-fold (P < 0.05) (Figure 4.41). A similar result was observed in Panc-1 cells after rhIL-6 treatment, which increased the number of invaded cells to 5.1-fold compared to the control (SFM). This effect was specific to IL-6 action, because the addition of an IL-6 neutralizing antibody to the cells reduced the number of invaded cells by 2.6-fold (Figure 4.41). Taken together, these results indicated that the IL-6 secreted by PSC can induce EMT-like morphology and phenotypes.

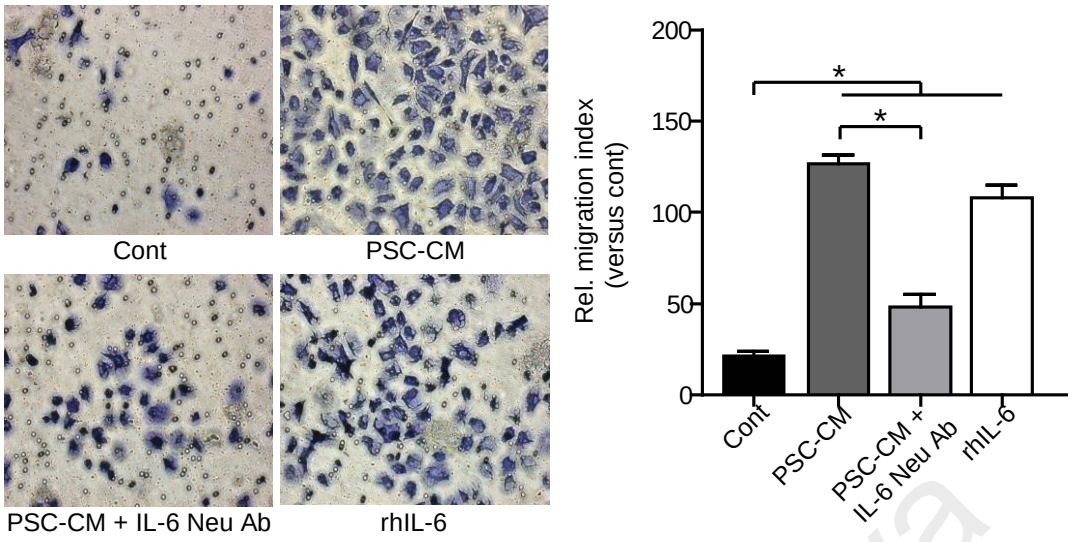

Figure 4.41: IL-6 secreted by PSC promotes Panc-1 cell invasion. A transwell invasion assay was used to assess the cellular invasion ability. After 20 h incubation with PSC-CM (1 µg/ml) with or without an IL-6 neutralizing antibody (4 µg/ml) or rhIL-6 (100 ng/ml) alone, the invaded cells on the lower surface of the filter were stained and counted (left panel). The bar graph shows the number of invaded cells for each category of cells (right panel). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus SFM treatment. The experiment was performed twice and the best representative result was presented. Cont, control.

4.10 IL-6 and JAK/Stat3 signaling induces EMT gene expression in Panc-1 cells

As the IL-6 secreted by PSC was able to induce EMT phenotypes in Panc-1 cells, its effect on EMT-related gene expression was also studied. The mRNA levels of *CDH1*, which encodes an epithelial-like marker, E-cadherin was significantly downregulated after PSC-CM treatment (0.26-fold, P < 0.05) (Figure 4.42). Downregulation of *CDH1* mRNA (0.19-fold, P < 0.05) was also observed following rhIL-6 treatment (Figure 4.42). However, upregulation of mRNA expression was observed for the mesenchymal-like markers (*CDH2*, *VIM*, *FN1*, and *COL1A1*) and EMT-related transcription factors (*TWIST2*, *SNAIL*, and *SLUG*) except for *SIP1* (Figure 4.42).

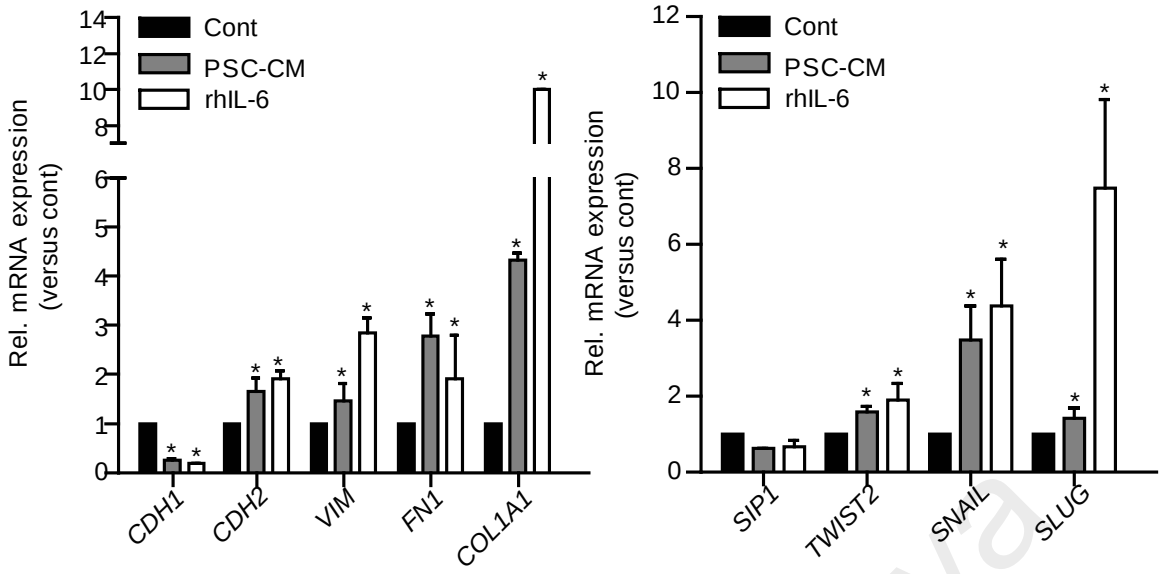

Figure 4.42: PSC-CM and rhIL-6 induce EMT by regulating EMT-related gene expression in Panc-1 cells. The mRNA levels of EMT-related markers and transcription factor genes was examined using qRT-PCR after 24 h treatment with PSC-CM (1 µg/ml) or rhIL-6 (100 ng/ml). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus Panc-1 cells treated with SFM. The experiment was performed twice. Rel., relative; Cont, control.

Upon neutralization of the IL-6 in PSC-CM, the *CDH1* gene was significantly upregulated (2.6-fold, P < 0.05) and the *CDH2*, *VIM*, *FN1*, *COL1A1*, TWIST2, SNAIL, and SLUG were significantly downregulated (approximately 0.5-fold, P < 0.05) (Figure 4.43). Notably, *SIP1* was not affected. These results indicated that the IL-6 secreted by PSC enabled Panc-1 cells to acquire EMT phenotypes by inducing EMT gene expression.

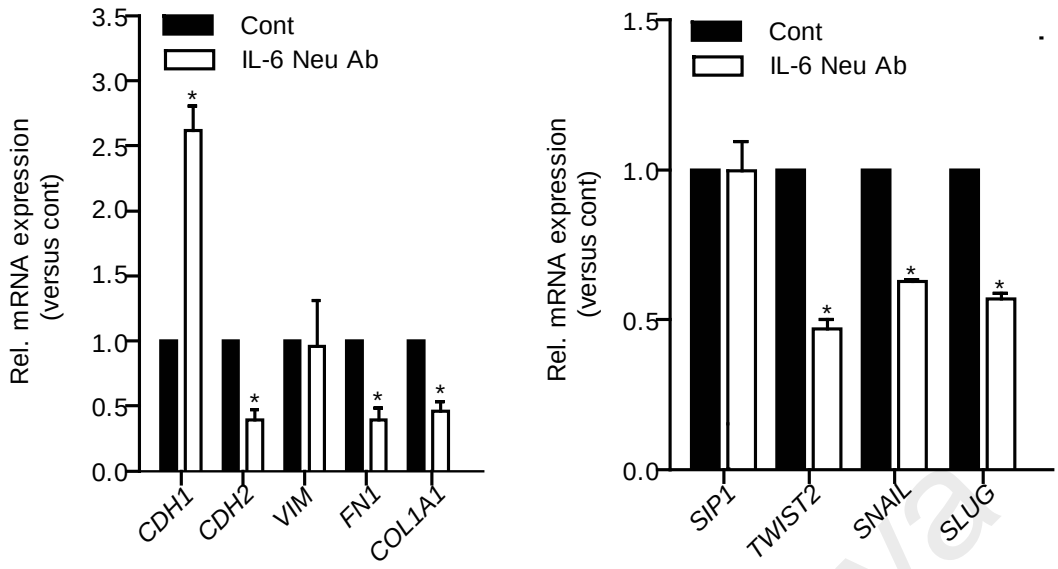

Figure 4.43: IL-6 neutralization in PSC secretion reduces EMT-related gene expression in Panc-1 cells. To confirm the effect of IL-6, PSC-secreted IL-6 was neutralized for 24 h before measuring the mRNA levels of EMT-related marker and transcription factor genes using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus cells treated with PSC-CM. The experiment was performed twice. Rel., relative.; Cont, control.

To evaluate the mechanism exerted by IL-6 to induce EMT in Panc-1 cells, the contribution of its downstream JAK/Stat3 signaling pathway on EMT induction was examined. Phospho-Stat3 protein expression was increased (approximately 2-fold) following treatment with PSC-CM and rhIL-6 compared to that in cells treated with control (SFM) (Figure 4.44). IL-6 neutralization decreased the phospho-Stat3 protein expression induced by PSC-CM (Figure 4.44), indicating that the increased phospho-Stat3 protein induced by PSC is contributed by IL-6. CYT-387 and Stattic were then used to inhibit JAK1/2 and Stat3 signaling, respectively before examining their effect on EMT-related gene expression in Panc-1 cells. Inhibition of JAK1/2 and Stat3 signaling abrogated the EMT induced by PSC-CM, during which *CDH1* mRNA levels were significantly upregulated (approximately 5-fold, P < 0.05) (Figure 4.45). In contrast, a significant downregulation was observed for *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *TWIST2*, *SNAIL*, and *SLUG* (>0.5-fold, P < 0.05) (Figure 4.45). Stattic showed a greater inhibition

of EMT induction compared to CYT-387 as indicated by the expression of EMT-related marker and transcription factor genes. Taken together, these findings suggested that the IL-6 derived from PSC activates JAK/Stat3 signaling to induce EMT in Panc-1 cells by regulating EMT-related gene expression.

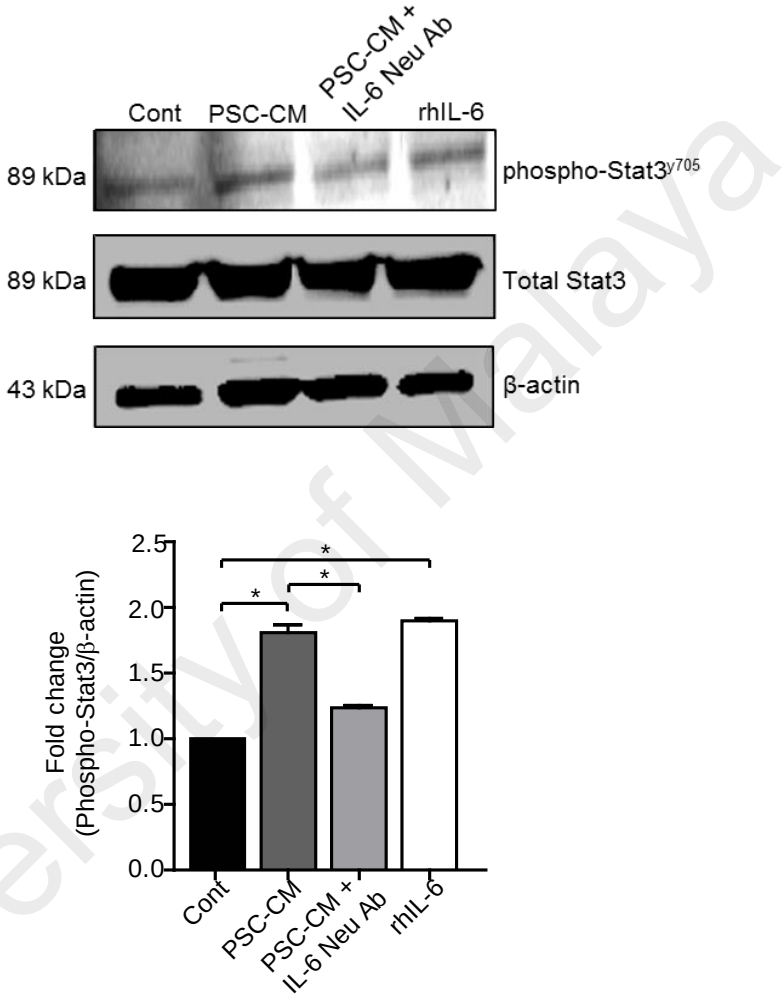

Figure 4.44: IL-6 secreted by PSC increases phosphorylated Stat3 protein in Panc-1 cells. The protein levels of phospho-Stat3 was measured with western blotting, after treatment for 24 h with PSC-CM (1 µg/ml) with or without IL-6 neutralizing antibody (4 µg/ml) (top panel). SFM treatment was used as control. The densitometry value of phospho-Stat3 was normalized to beta-actin and relative to the control (bottom panel). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus SFM treatment. The experiment was performed thrice and the best representative result was presented. Cont, control.

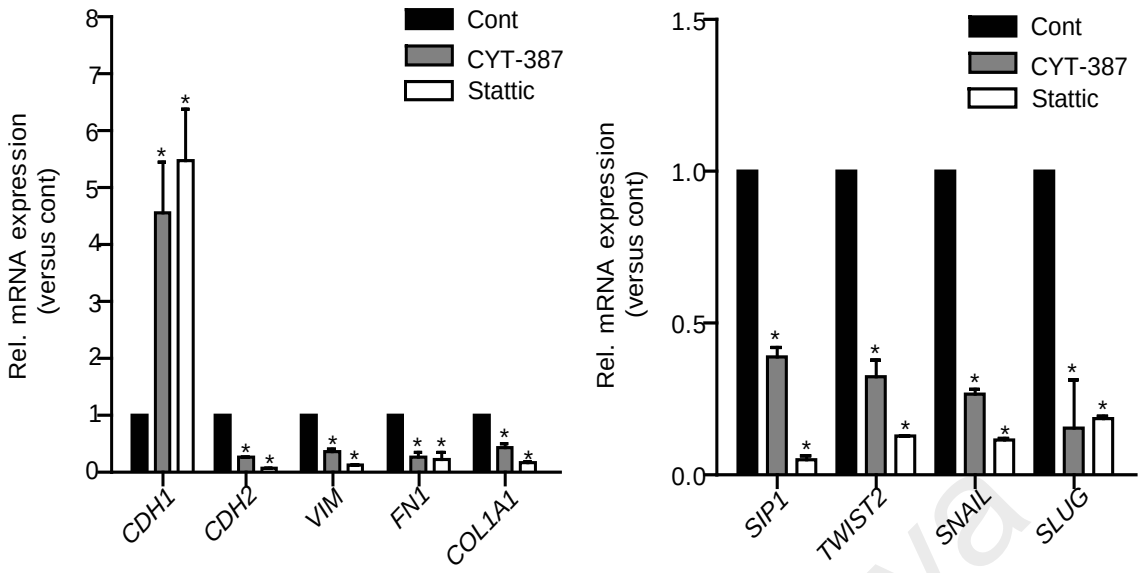

Figure 4.45: Inhibition of the JAK and Stat3 signaling induced by PSC-CM decreases EMT-related gene expression in Panc-1 cells. Cells were treated with CYT-387 (12.5 µM) and Stattic (12.5 µM) in the presence of PSC-CM (1 µg/ml) for 72 h. The mRNA levels of EMT-related marker (left panel) and transcription factor (right panel) genes were measured using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus Panc-1 cells treated with PSC-CM. The experiment was performed thrice. Rel., relative; Cont, control.

4.11 JAK/Stat3 signaling regulates Nrf2 activity to mediate IL-6-induced EMT in Panc-1 cells

The role of intracellular Nrf2 signaling in mediating IL-6-induced migration and EMT in Panc-1 cells was then determined. To test this objective, tBHQ was used to enhance Nrf2 signaling in cells with suppressed IL-6 signaling. tBHQ treatment alone induced Panc-1 cells to exhibit a mesenchymal cell morphology, with a cobblestone-like shape, increased scattering, and tight cell-cell adhesion. Upon blockage of IL-6 signaling, Panc-1 cells acquired a cobblestone-like shape and demonstrated tight cell-cell adhesion (Figure 4.46). However, the cell morphology changed to spindle shape and the cell-cell adhesion was loose after tBHQ treatment (Figure 4.46), indicating that increased Nrf2 activity was able to induce Panc-1 cells to acquire a mesenchymal-like morphology.

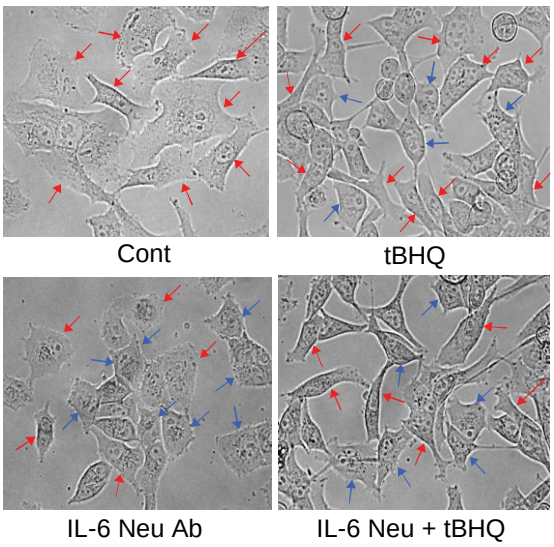

Figure 4.46: Nrf2 activity mediates the EMT-like morphology in Panc-1 cells induced by the IL-6 secreted by PSC. Following the neutralization of IL-6 in PSC-CM (1 µg/ml) (control) with or without tBHQ (1 µM) for 24 h, the cell morphology was viewed under phase contrast and photographed using an inverted light microscope. Original magnification: 400×. Epithelial and epithelial-like cells were shown by blue arrow while mesenchymal-like cells were shown by red arrow. The experiment was performed twice and the best representative result was presented. Cont, control.

The addition of tBHQ induced motility in Panc-1 cells as it enhanced wound closure to almost a similar level as that of the control (PSC-CM) (Figure 4.47). IL-6 neutralization significantly reduced (>0.5-fold, $P < 0.05$) the wound closure induced by PSC-CM. When tBHQ was added to the cells treated with the IL-6 neutralizing antibody, wound closure was significantly enhanced (2-fold, $P < 0.05$) through the increase in cell motility (Figure 4.47).

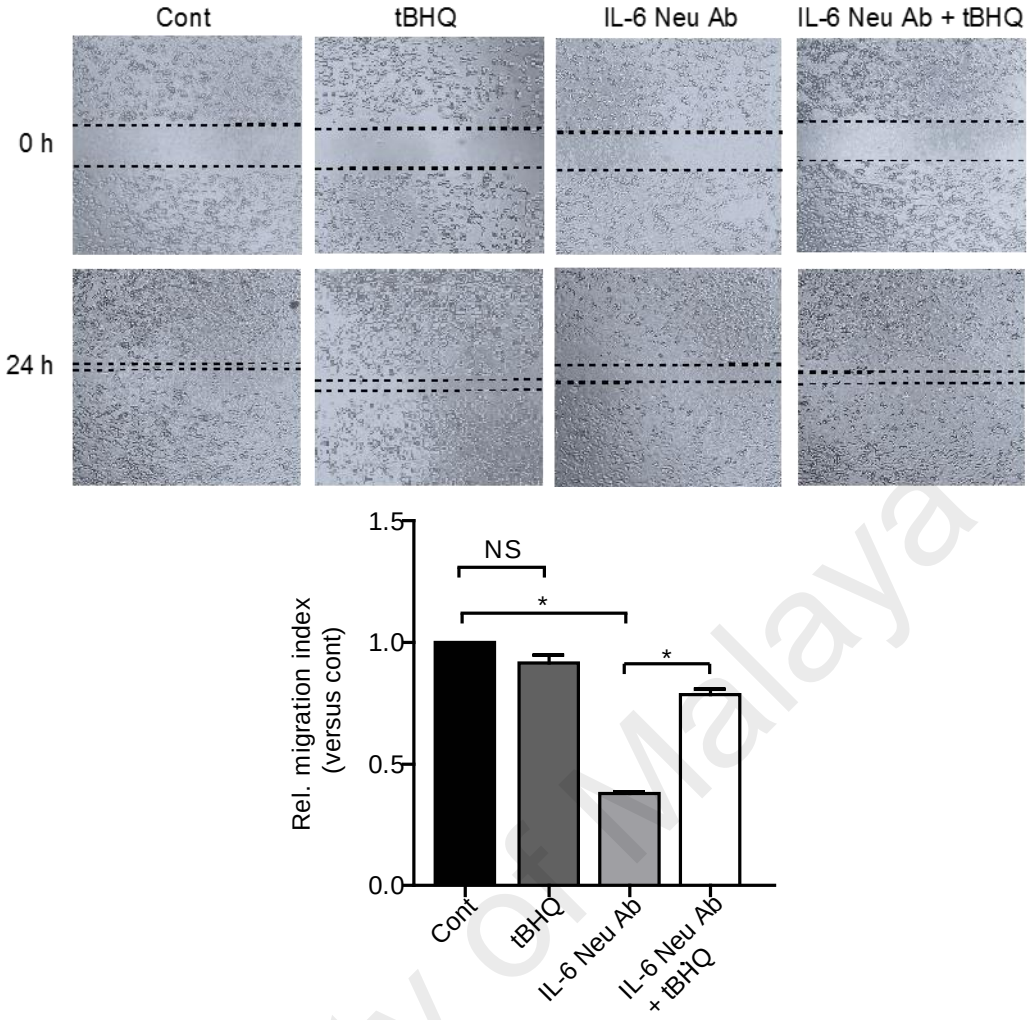

Figure 4.47: Nrf2 activity mediates the Panc-1 cell motility induced by the IL-6 from PSC. A scratch wound healing assay was used to assess cell motility after 24 h neutralization of IL-6 in PSC-CM (1 µg/ml) with or without tBHQ (1 µM). The wound closure was viewed under bright field and photographed with an inverted light microscope (top panel). Original magnification: 40×. The distance of wound closure covered by migrating cells after treatment relative to that stimulated by control cells was indicated as the migration index (bottom panel). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus Panc-1 cells treated with PSC-CM. Otherwise, NS, not significant. The experiment was performed twice and the best representative result was presented. Rel., relative; Cont, control.

Furthermore, Nrf2 signaling activation has a promoting effect on cell migration and invasion. The number of migrated cells induced by tBHQ was less than the control migrated cells (Figure 4.48). When the IL-6 in PSC-CM was neutralized, the number of migrated cells was significantly reduced (approximately 0.5-fold, P < 0.05) (Figure 4.48). The addition of tBHQ to the cells treated with an IL-6 neutralizing antibody increased the

migratory capacity (by 1.3-fold) (Figure 4.48). Similar effects were observed in the transwell invasion assay. The invasion capacity induced by tBHQ was less than that of control-treated cells (Figure 4.49). IL-6 neutralizing antibody significantly reduced the number of invaded cells induced by the control treatment (approximately 0.5-fold, $P < 0.05$) (Figure 4.49). Subsequent tBHQ treatment increased the number of invaded cells (1.5-fold) that had traversed the Matrigel-coated layer compared to those treated with the IL-6 neutralizing antibody alone (Figure 4.49). These data suggested that the IL-6-induced EMT phenotypes in Panc-1 cells were partly due to Nrf2 activity.

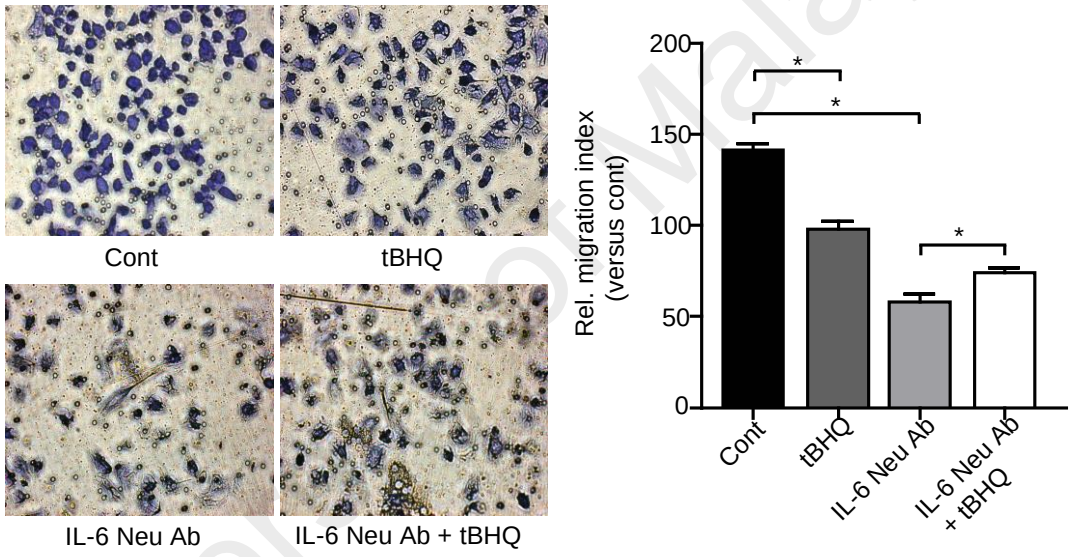

Figure 4.48: Nrf2 activity mediates the Panc-1 cell migration induced by the IL-6 from PSC. A transwell migration assay was used to assess cell motility. After 20 h incubation with an IL-6 neutralizing antibody (4 µg/ml) with or without tBHQ (1 µM), the migrated cells on the lower surface of the filter were stained and counted (left panel). The bar graphs show the number of migrated cells for each category of cells (right panel). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *$P < 0.05$ indicates significant differences versus Panc-1 cells treated with PSC-CM. The experiment was performed twice and the best representative result was presented. Cont, control.

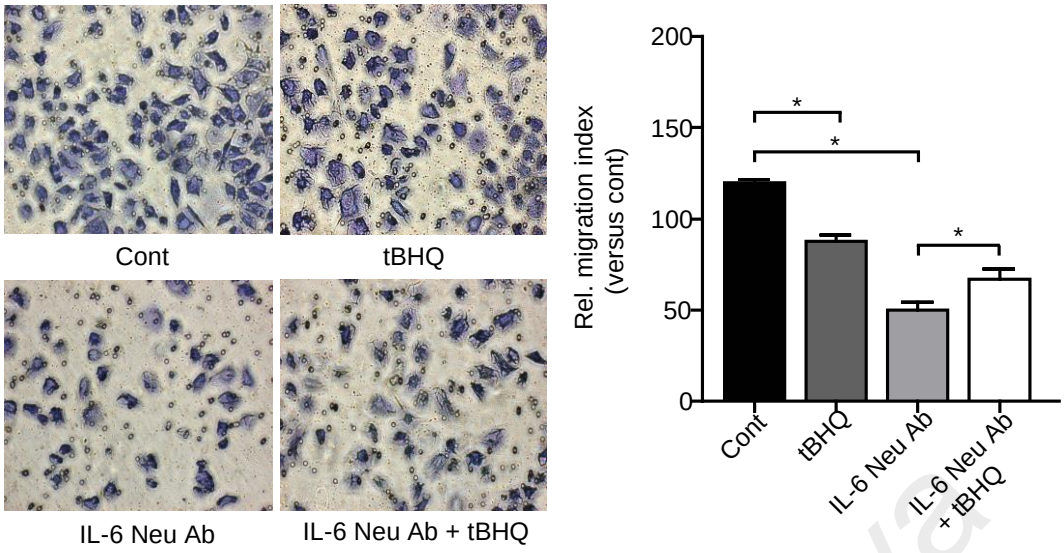

Figure 4.49: Nrf2 activity mediates the Panc-1 cell invasion induced by the IL-6 from PSC. A transwell invasion assay was used to assess cellular motility and invasion ability. After 20 h incubation with an IL-6 neutralizing antibody (4 µg/ml) with or without tBHQ (1 µM), the invading cells on the lower surface of the filter were stained and counted (top). The bar graphs show the number of invaded cells for each category of cells (bottom). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus Panc-1 cells treated with PSC-CM. The experiment was performed twice and the best representative result was presented. Cont, control.

In addition, the gene expression of EMT-related markers and transcription factors after tBHQ treatment was measured. The results showed that tBHQ treatment significantly downregulated *CDH1* mRNA levels (0.6-fold) whereas it upregulated the mRNA levels of *CDH2* (1.5-fold), *FN1* (1.6-fold), *TWIST2* (2.9-fold), *SNAIL* (1.9-fold), and *SLUG* (1.8-fold) (Figure 4.50).

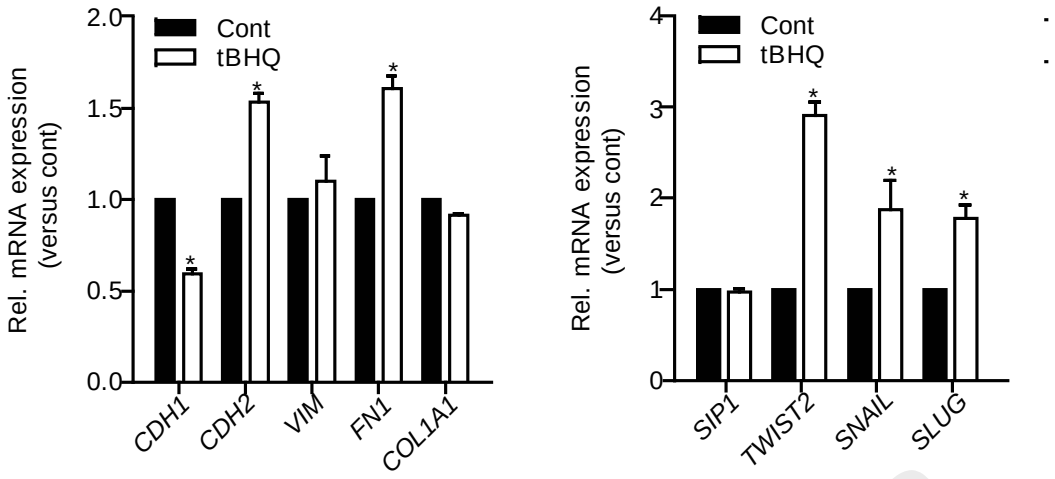

Figure 4.50: Nrf2 activity mediates the EMT-related gene expression in Panc-1 cells induced by the IL-6 from PSC. The mRNA levels of EMT-related marker and transcription factor genes were examined using qRT-PCR after 24 h treatment with tBHQ (1 µM) in the presence of PSC-CM (1 µg/ml) and an IL-6 neutralizing antibody (4 µg/ml). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus Panc-1 cells treated with PSC-CM in the presence of IL-6 Neu antibody. The experiment was performed twice. Rel., relative; Cont, control.

To determine whether tBHQ-activated intracellular Nrf2 signaling was essential to induce EMT in Panc-1 cells, *NRF2* gene expression was downregulated before tBHQ treatment (Figure 4.51). *NRF2* mRNA was reduced to 16% in Panc-1 cells as compared to mock treatment ($P < 0.05$), with minimal changes in cells transfected with control siRNA (Figure 4.51). tBHQ treatment was not able to significantly alter the mRNA expression of *NRF2* or EMT-related genes significantly compared to control siRNA-transfected Panc-1 cells (Figure 4.52), indicating that the Nrf2 downregulation was specific. These results suggested that Nrf2 regulated EMT-related gene expression to mediate the migration and EMT induced by IL-6.

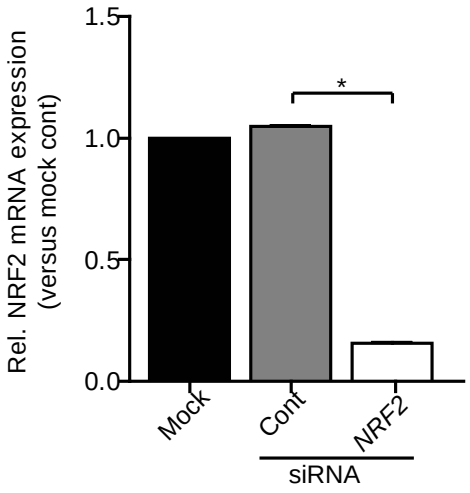

Figure 4.51: Expression of *NRF2* after RNAi-mediated gene silencing in Panc-1 cells. To silence *NRF2* gene expression, cells were transfected with control siRNA (100 nM) or *NRF2* siRNA (100 nM) for 24 h. The mRNA levels of *NRF2* were measured using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences cells transfected with control siRNA. The experiment was performed twice. Rel., relative; Cont, control.

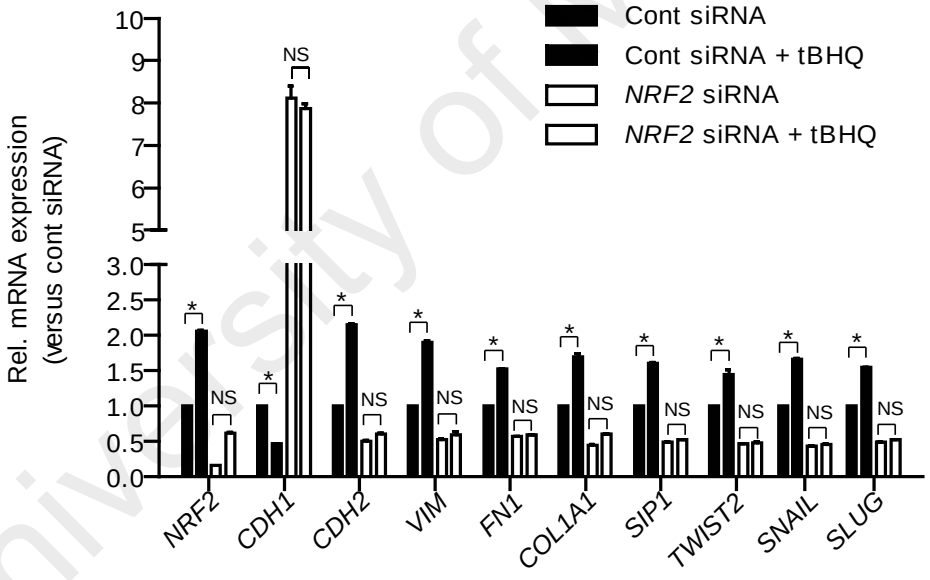

Figure 4.52: Effect of tBHQ on *NRF2* and EMT-related gene expression in *NRF2*-silenced Panc-1 cells. After silencing *NRF2*, cells were treated with tBHQ (1 μM) for 24 h. The mRNA levels of EMT-related maker and transcription factor genes were measured using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. The experiment was performed twice. Rel., relative.

To further examine the mechanism of IL-6-induced EMT in Panc-1 cells, the effect of IL-6-activated JAK/Stat3 signaling on Nrf2 activity was investigated. To test this, JAK/Stat3 signaling was first inhibited before measuring the mRNA expression of

NRF2 and its target genes. The results showed that the mRNA levels of *NRF2* were significantly downregulated (>0.04-fold, $P < 0.05$) after JAK1/2 and Stat3 signaling inhibition. Additionally, two target genes (*AKR1C1* and *NQO1*) of Nrf2 were also significantly reduced (>0.05-fold, $P < 0.05$) upon treatment with CYT-387 and Stattic (Figure 4.53). The inhibition caused by Stattic was greater compared to that by CYT-387. These data indicated that the IL-6-induced JAK/Stat3 signaling was able to regulate Nrf2 signaling in Panc-1 cells.

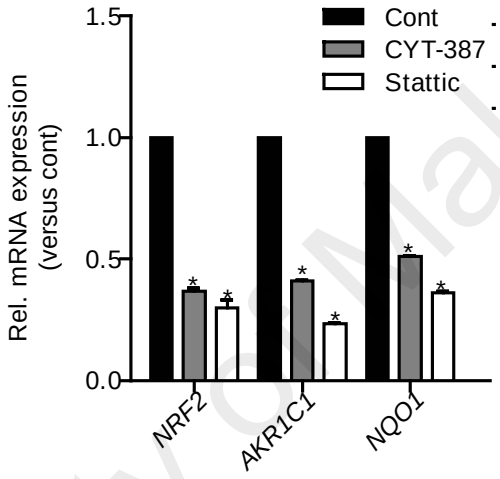

Figure 4.53: Inhibition of JAK and Stat3 signaling decreases the mRNA expression of *NRF2* and its target genes. Panc-1 cells were treated with CYT-387 (100 µM) and Stattic (100 µM) in the presence of PSC-CM (1 µg/ml) for 72 h, the mRNA levels of *NRF2* and its target genes, *AKR1C1* and *NQO1* were measured using qRT-PCR. Data shown are average of triplicates from one experiment, and the error bars represent the SD. *$P < 0.05$ indicates significant differences versus cells treated with PSC-CM. The experiment was performed twice. Rel., relative; Cont, control.

Next, Nrf2 and Stat3 signaling were simultaneously inhibited before examining the effect on the mRNA levels of EMT-related marker and transcription factor genes. *NRF2* gene expression was first downregulated using siRNA-mediated technique. *NRF2* knockdown upregulated the mRNA levels of *CDH1* (6.3-fold) and downregulated the mRNA levels of *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *TWIST2*, *SNAIL*, and *SLUG* (approximately 0.1-0.5-fold) (Figure 4.54A). Stattic treatment further enhanced the inhibition effect of *NRF2* knockdown, as evidenced by greater upregulation of *CDH1*

(1.6-fold) and downregulation of *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *TWIST2*, *SNAIL*, and *SLUG* (approximately 0.4-0.6-fold) (Figure 4.54B). These data indicated that the downstream signaling of IL-6, particularly of Stat3 could regulate Nrf2 signaling to mediate EMT gene expression in Panc-1 cells.

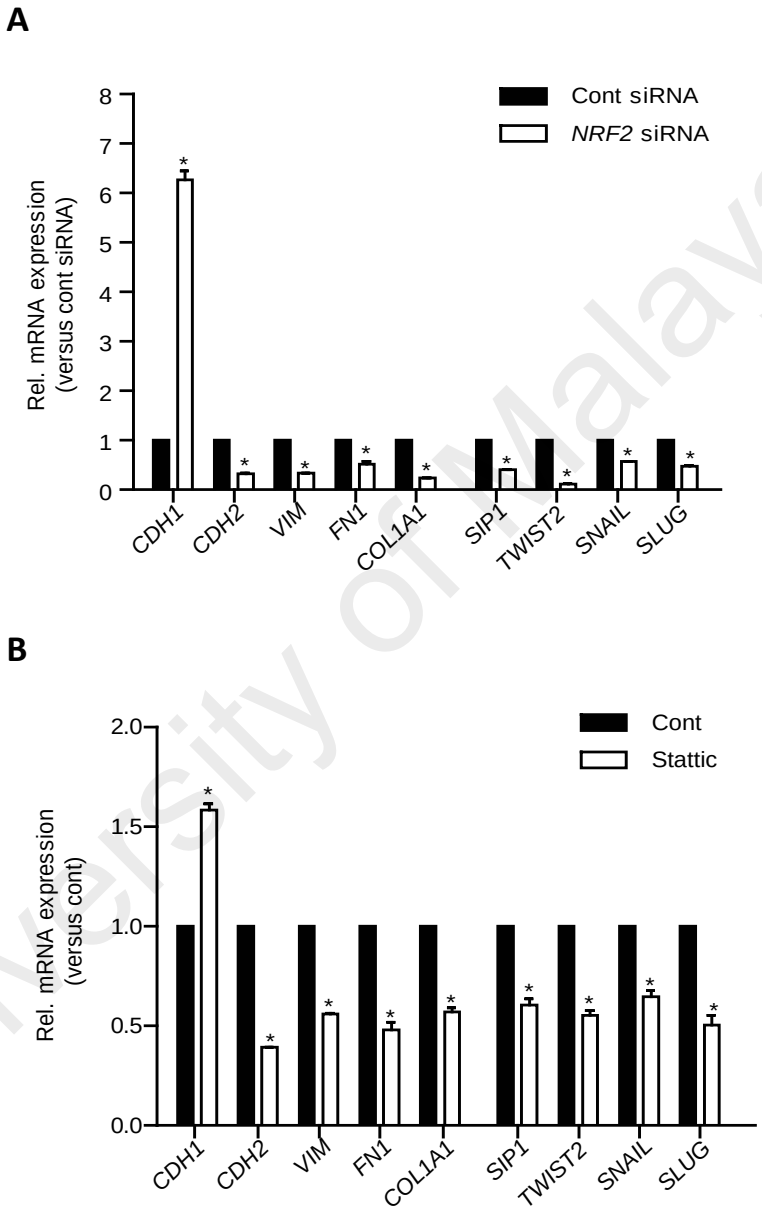

Figure 4.54: Inhibition of Stat3 signaling enhances the inhibitory effect of *NRF2* knockdown on the expression of EMT-related genes. After silencing Nrf2 for 24 h, the mRNA levels of EMT-related marker and transcription factor genes were measured using qRT-PCR (A). *NRF2*-silenced Panc-1 cells were then treated with Stattic (25 µM) for 72 h before measuring the expression of EMT-related marker and transcription factor genes (B). Data shown are average of triplicates from one experiment, and the error bars represent the SD. *P < 0.05 indicates significant differences versus control siRNA. The experiment was performed twice. Rel., relative.

CHAPTER 5: DISCUSSION

The role of PSC in promoting PDAC cell proliferation and invasiveness has been recognized recently (Hwang et al., 2008; Masamune, Kikuta, Watanabe, Satoh, Hirota, et al., 2008), but the underlying mechanisms are unclear. Nrf2, a highly expressed transcription factor in PDAC, provides a cytoprotective role against oxidative stress by regulating ARE-driven genes. Nrf2 is also reported to regulate metabolic reprograming and EMT in cancer cells. However, limited scientific evidence is yet available regarding the precise roles of Nrf2 in mediating cell proliferation and EMT in PDAC. In this study, the roles of PSC secretory factors in PDAC cell proliferation and EMT were investigated, particularly from the aspect of Nrf2 signaling (Figure 5.1). PSC secretion induced PDAC cell proliferation by activating antioxidant and metabolic programs via Nrf2 transcriptional activity. Specifically, IL-6 promoted Nrf2 transcriptional activity to induce genes that are involved in PPP, glutaminolysis, and glutathione biosynthesis, to effect ROS detoxification and purine nucleotide synthesis. This action creates a more reducing intracellular environment that favors PDAC cell proliferation. Furthermore, this study showed that IL-6 secreted by PSC also induced EMT phenotypes in PDAC Panc-1 cells. Subsequent mechanistic investigation revealed that the induction of EMT occurs via Stat3-activated Nrf2 signaling. Nrf2 depletion in turn downregulated the expression of EMT-related genes; this effect was enhanced by the inhibition of Stat3 signaling. Thus, this study delineated the roles of PSC secretion, in particular clarifying the function of IL-6 in activating PDAC intracellular redox signaling to induce cell proliferation via Nrf2-mediated metabolic reprogramming. Additionally, the IL-6 secreted by PSC could also induce the EMT program via the Stat3/Nrf2 signaling pathway to promote PDAC cell motility and invasiveness.

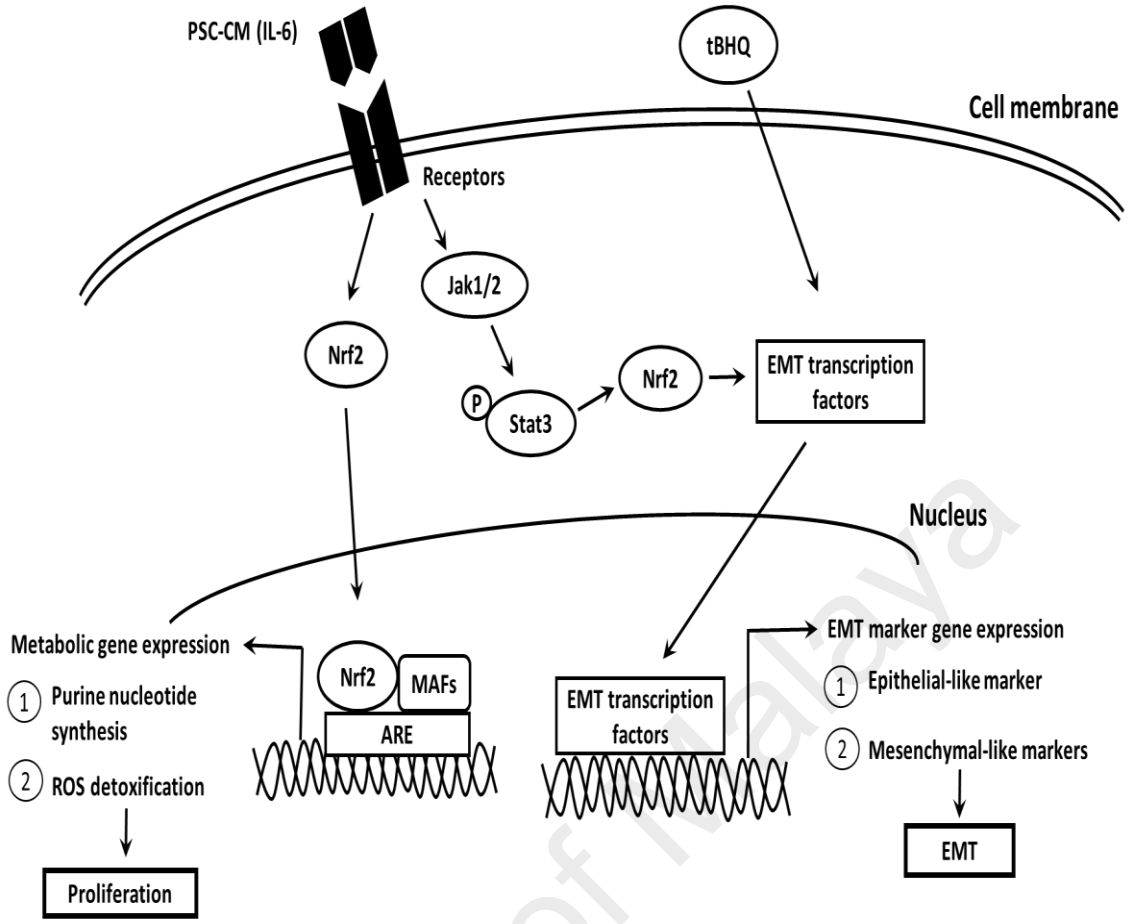

Figure 5.1: Schematic diagram illustrating the action of PSC-secreted IL-6 on Nrf2-mediated metabolic reprogramming for PDAC cell proliferation and EMT induction via the JAK/Stat3/Nrf2 pathway. The IL-6 secreted by PSC interacts with PDAC cells by binding to IL-6 receptors, leading to Nrf2 activation. Activated cytoplasmic Nrf2 protein translocates into the nucleus. In the nucleus, Nrf2 dimerizes with members of the masculoaproneurotic fibrosarcoma (Maf) protein family to facilitate the binding of Nrf2 to ARE in the promoter of its metabolic target genes, driving their expression to promote PDAC cell proliferation by facilitating nucleotide synthesis and ROS detoxification. Upon binding to its receptors, IL-6 also activates JAK/Sta3 signaling, which in turn induces intracellular Nrf2 signaling. Activated Nrf2 stimulates EMT-related transcription factors to drive the expression of EMT-related marker genes to induce EMT in PDAC cells.

5.1 Roles of PSC in PDAC progression

Unlike many adenocarcinomas, PDAC is often characterized by severe stromal reaction (Feig et al., 2012). This is mainly due to the conversion of quiescent PSC to their activated state by acute and chronic inflammation within the pancreas. Activated PSC release abundant cytokines and growth factors, which are known to stimulate PDAC progression *in vitro* (Erkan et al., 2012). Using an indirect co-culture system, the present

study showed that PSC secretion, specifically IL-6, has a role in promoting the proliferation of PDAC cells (AsPC-1, BxPC-3, and Panc-1) and could induce their migration and invasion capacity. Although the current study was limited to the use of only a PSC line isolated from a patient with PDAC undergoing surgery, Hwang et al. (2008) also showed that the secretion derived from an isolated PSC line also dose-dependently increased PDAC cell (BxPC-3 and Panc-1) proliferation. In addition, treatment of Panc-1 cells with secretion from different PSC lines similarly reduced the cytotoxicity and increased the cell viability after gemcitabine treatment (H. Zhang et al., 2015). These findings indicate that PSC secretion has a role in promoting PDAC cell proliferation.

Early distant metastasis involving the migration and invasion is one of the major factors responsible for the poor outcome of PDAC. Notably, PSC co-migrate with PDAC cells during the dissemination step in metastasis to establish a metastatic niche for the tumor cells (Z. Xu et al., 2010). The data presented in this thesis provides *in vitro* evidence that PSC secretion can promote PDAC cell motility and invasiveness by inducing the migration and invasion capacity of PDAC cells. These findings are supported by other studies that a greater tumor size and increased incidence of metastasis were observed when PSC were co-injected with PDAC tumor cells in mouse models (Hwang et al., 2008; Vonlaufen, Joshi, et al., 2008). These effects are likely due to the resultant increased migratory capacity and invasiveness of PDAC cells. Furthermore, Guo et al. (2014) also reported that the IL-6 secreted by PSC has the capacity to induce the growth and invasive properties of PDAC cells, as IL-6 neutralization reduced the cell viability, invasion, and colony formation of MiaPaCa-2 cells. Together, these findings indicate that PSC secretion plays an importance role in PDAC metastasis.

In addition to cell proliferation and invasion, PSC also have a role in hypoxia and angiogenesis, which are two prominent features in PDAC (Koong et al., 2000; Olive et al., 2009). Hypoxia stabilizes the HIF-1α transcription factor to increase the expression

of genes associated with angiogenesis (Akakura et al., 2001). Additionally, PSC secrete VEGF to stimulate tube formation in human microvascular endothelial cells (Z. Xu et al., 2010). This finding was supported by *in vivo* evidence that co-injection of PSC with MiaPaCa-2 cells significantly enhanced tumor angiogenesis by upregulating the endothelial cell marker CD31 as shown in an orthotropic mouse model (Z. Xu et al., 2010). Accordingly, PSC are likely to have an active role in modulating oxidative stress pathways to support a growth-permissive tumor microenvironment allowing the PDAC tumor cells to thrive in such extreme conditions.

5.2 Nrf2 activation in PDAC cell proliferation

PDAC requires high ROS levels to promote survival (Teoh et al., 2007); however, the role of oxidative stress in PDAC progression remains debatable. A recent study has reported that low intracellular ROS levels are essential for PDAC carcinogenesis in a $KRAS^{G12D}$-driven mouse model, which are probably achieved through the induction of an Nrf2-mediated antioxidant program (DeNicola et al., 2011). Consistent with this, Nrf2 activation and its high expression levels are often exhibited by human PDAC tumors and cell lines (Lister et al., 2011). Elevated Nrf2 activity has also been observed in other cancers wherein it has been linked to cellular proliferation and the development of drug resistance phenotype (Shibata, Kokubu, et al., 2008; Shibata, Ohta, et al., 2008; Stacy et al., 2006). These findings indicate that Nrf2 activity has a role in PDAC cancer progression.

Persistent Nrf2 activation represents an important mechanism by which PDAC tissue may sense and adapt to oxidative stress. However, a dysregulated Nrf2/Keap1 system is rarely caused by somatic mutation in *NRF2* and *KEAP1* genes in PDAC (Lister et al., 2011). Instead, Nrf2 activation can be observed in colonic tissue that has been exposed to oxidative stress during chronic inflammation. For example, colonic tissue

dissected from mice deficient in a multifunctional stress response gene (immediate early response-E3, *IER3* knockout mice) exhibited greater Nrf2 activity than that from *IER3*$^{+/+}$ mice (Stachel et al., 2014). The present study further showed that stromal cells in the tumor microenvironment could create a dysregulated Nrf2/Keap1 system to cause persistent Nrf2 activation. In particular, PSC secretion, specifically IL-6, was found to activate the Nrf2 signaling pathway. Nrf2 signaling activation plays an important role in PSC-mediated cell proliferation by regulating the genes that are involved in metabolic pathways, providing purine nucleotide synthesis and ROS detoxification. This observation was supported by a study demonstrating that exogenous TGF-β1 was capable of inducing Nrf2 to promote the invasiveness of a PDAC cell line (Colo357) (Arfmann-Knubel et al., 2015). Therefore, the data presented in the current study suggest that an external factor such as the paracrine effect of PSC in the tumor microenvironment may lead to Nrf2 activation, which promotes PDAC cell proliferation.

It is well established that Nrf2 constitutes the main regulator of cellular redox homeostasis by upregulating the products of ARE-bearing genes products to confer a beneficial defense cellular mechanism (Murakami & Motohashi, 2015). Accordingly, it was not surprising to observe the upregulation of Nrf2 antioxidant genes such as *AKR1C1*, *NQO1*, and *CAT* in PDAC cells after their exposure to PSC secretion as was shown in the present study. However, the induction of these genes alone could not explain the observed increased cell proliferation mediated by PSC. Highly proliferative cells, such as in cancer, would additionally require high metabolic activities (Mitsuishi, Taguchi, et al., 2012). Notably, Nrf2 signaling activation has been reported to represent a mechanism, by which cancer cells acquire high metabolic activities. For example, lung cancer cells (A459) with existing high Nrf2 levels support their proliferation by inducing Nrf2-mediated metabolic pathways under the sustained activation of the PI3K/Akt pathway (Mitsuishi, Taguchi, et al., 2012). It was discovered in the present study that PDAC cells also utilize a similar

mechanism to induce their proliferation. Specifically, PSC secretion was able to induce metabolic reprogramming by activating Nrf2 metabolic target genes and increasing the concentration of metabolites (R5P and IMP) that are involved in PPP, glutaminolysis, and glutathione biosynthesis. Thus, it is likely that the increased synthesis of purine nucleotide, a major product of PPP activation, led to the increased PDAC cell proliferation.

In addition, Nrf2-mediated metabolic reprogramming also provides an efficient ROS detoxification system to reduce the accumulation of ROS in cancer cells with high metabolic activity (Ishimoto et al., 2011). NADPH equivalents generated via ROS detoxification enzymes, such as G6PD and PGD in PPP; ME1, and IDH1 in glutaminolysis; and GCLC and GCLM in glutathione biosynthesis are critical for the proper function of cellular redox homeostasis (Stanton, 2012). The data presented in the thesis also showed that Nrf2 was partly responsible for these metabolic genes induction. It should be noted that other transcription factors can also regulate their expression. For example, specificity protein transcription factor was reported to activate cancer cell metabolism by regulating several enzymes (Archer, 2011). The data in the present study showed that the inhibition of G6PD enzyme activity achieved using either a pharmacological inhibitor or by RNAi-mediated gene silencing abrogated PSC-mediated PDAC cell proliferation. Furthermore, complete knockout of *G6PD* in embryonic stem cells has been shown to eliminate NADPH production, resulting in a higher risk of cell death by a potent oxidant (Pandolfi et al., 1995). Thus, PSC utilize Nrf2-mediated metabolic reprogramming as a mechanism to promote PDAC cell proliferation.

5.3 Nrf2 activation in PDAC motility and invasiveness

Nrf2 also plays a role in inducing PDAC cell motility and invasiveness. One of the underlying mechanisms for this is EMT, which leads to the acquisition of motility and invasive capability of PDAC cells. However, the role of EMT in motility and invasiveness

is limited to *in vitro* as recent reports showed that EMT suppression had no effect on the emergence of invasive, systemic dissemination, and metastasis of tumors including PDAC as studied using mouse models (K. R. Fischer et al., 2015; Zheng et al., 2015). Nonetheless, EMT plays a pivotal role in recurrent cancer metastasis after chemotherapy (K. R. Fischer et al., 2015). EMT can be induced by Nrf2 activity. For example, blockage of Nrf2 suppressed the migration and invasion of esophageal squamous cancer cells that were induced under hypoxic condition (Shen et al., 2014). The expression of Nrf2 has also been correlated with lymph node metastasis as evidenced in the pathological sections of patients with esophageal squamous cancer (Shen et al., 2014). Additionally, inhibition of the Nrf2-mediated signaling pathway was found to suppress EMT in human tongue squamous cancer cells (Pan et al., 2015). In PDAC, the activation of Nrf2 signaling using its activator, tBHQ was shown to increase the protein levels of mesenchymal vimentin and Slug and to reduce the epithelial E-cadherin levels in normal human pancreatic ductal epithelial (HPDE) and cancer cells (Colo357) (Arfmann-Knubel et al., 2015). In Colo357 cells, Nrf2 signaling could also be induced by exogenous TGF-β1; however, this effect was not observed in immortal HPDE cells. Indirectly, this finding indicates that the interaction with stromal cells in the tumor microenvironment may mediate the mechanism of Nrf2-mediated EMT in PDAC. This hypothesis is further strengthened by the observation in the current study that the PSC secretion was capable of stimulating EMT phenotypes and related marker gene expression by activating Nrf2 signaling in Panc-1 cells. Together, these findings suggest the importance role of Nrf2 in PSC-mediated EMT in PDAC.

5.4 IL-6 signaling requires Nrf2 activation for PDAC cell proliferation and invasiveness

Many studies have demonstrated that IL-6 signaling plays an important role in PDAC progression. For example, the inhibition of IL-6 signaling resulted in delayed PDAC progression from PanINs and reduced the primary tumor growth and recurrences *in vivo* (Goumas et al., 2015; Y. Zhang et al., 2013). The IL-6 derived from the stromal cells also contributes to the tumor progression. In fact, IL-6 receptor expression was found in many human PDAC tissues and was positively associated with poor outcome in resected PDAC (Denley et al., 2013; Masui et al., 2002). However, IL-6 receptor is rarely expressed in AsPC-1 cells (Block et al., 2012; Masui et al., 2002) which explains the absence of proliferation-promoting effect of IL-6 on these cells as shown in the present study. This observation suggests that these cells did not depend on autocrine signaling. In addition to that, the present study further showed that the paracrine effect of IL-6 produced by PSC was able to induce PDAC cell proliferation and invasiveness via EMT. This finding is supported by the demonstration that the inactivation of PSC by ATRA with reduced differentiation markers expression led to downregulation of *IL6* expression from CAFs, subsequently inhibited the migration and EMT of AsPC-1 and Panc-1 cells (Ali et al., 2015; Guan et al., 2014). Furthermore, Guan et al. (2014) demonstrated that the IL-6 derived from cancer cells was not able to rescue the EMT attenuated by retinoic acid, providing an insight into the different functions of IL-6 autocrine and paracrine signaling in promoting tumor progression (Guan et al., 2014). Indirectly, these result indicate the specific effect of paracrine IL-6 signaling in mediating PSC-induced PDAC cell proliferation and EMT.

The present study further showed that IL-6 activates Nrf2 signaling to induce PDAC cell proliferation and EMT. The activation of Nrf2 by IL-6 may possibly through JAK/Stat3 signaling. Inhibition of IL-6/Stat3 pathway induced by PSC secretion

suppressed the migration and expression of EMT-related markers in Panc-1 cells as presented in the study. This is consistent with previous demonstrations that JAK/Stat3 activation by IL-6 could regulate the migration and metastasis of PDAC cells (Hamada, Masamune, Yoshida, Takikawa, & Shimosegawa, 2016; Y. Zhang et al., 2013). Furthermore, JAKs have been shown to activate the multiple serine/threonine sites in Nrf2 (Fahmi et al., 2013). Notably, the present study also revealed that Stat3 could regulate the expression of *NRF2* and its target genes (*AKR1C1* and *NQO1*) and that the inhibition of Stat3 signaling further enhanced the inhibitory effect of *NRF2* knockdown on EMT-related gene expression. Stat3 has been reported as a potential interactor of Nrf2 based on the domain-motif interactions (Turei et al., 2013). The positive interaction between Stat3 and Nrf2 signaling has also been reported in diabetic disease. For example, Stat3/Nrf2 signaling induced by isoflurane postconditioning was shown to confer cardioprotection in type 1 diabetic rats compared to non-diabetic rats (Y. Wang, Li, Fang, Xia, & IRWIN, 2016). This evidence possibly explains IL-6 could activate Nrf2 signaling by inducing JAK/Stat3 signaling. With the discovery of this novel finding, *in vivo* model such as IL-6-or Nrf2-decifient are needed to strengthen and validate the role of IL-6/Nrf2 signaling pathway in PDAC cell proliferation and motility *in vitro*.

In addition, the data presented in this study showed that SDF-1α derived from PSC can also induce PDAC cell proliferation and the expression of *NRF2* and its target genes. The SDF-1α/CXCR4 axis has been implicated to function in the proliferation, migration, invasion, and metastasis of PDAC cells (Gao et al., 2010; Matsuo et al., 2009; Shakir et al., 2015). Nrf2 can directly bind to the promoter of the *CXCR4* receptor gene and increase its transcription (Tsai et al., 2013). More recently, a study has demonstrated that SDF-1α could interact with IL-6 to mediate the effects of PSC on gemcitabine chemoresistance (H. Zhang et al., 2015). rhSDF-1α increased IL-6 expression and secretion in Panc-1 cells, and blockage of IL-6 signaling abrogated the protective effect

of rhSDF-1α against gemcitabine-induced apoptosis in Panc-1 cells (H. Zhang et al., 2015). These findings suggest the synergistic role between IL-6 and SDF-1α in PSC-mediated chemoresistance. Thus, it may be possible that the IL-6 and SDF-1α secreted by PSC may cooperatively or synergistically mediate the action of PSC on PDAC cell proliferation, motility and invasion capacity. In turn, their mechanistic action on these phenotypes may be due to the activation of Nrf2 signaling. However, further investigation is required to confirm this hypothesis.

5.5 Therapeutic implications of IL-6 and Nrf2 in PDAC

PSC provide a growth-permissive microenvironment to facilitate PDAC cell growth and distant metastasis by producing a stromal reaction. One way to deplete the effects of PSC activation on PDAC tumor progression is to inhibit the interaction of the secreted soluble factors from PSC with their respective receptors on PDAC cells. The present study showed that the IL-6 secreted by PSC play a major role in both cell proliferation and metastasis in PDAC. The action of IL-6 requires the activation of Nrf2 signaling through the Stat3 signaling pathway. Therefore, devising strategies to target the IL-6/Stat3/Nrf2 signaling pathway may provide novel therapeutic options to improve the poor prognosis of patients with PDAC.

To date, the potential effects of an anti-IL-6 antibody or inhibitors of IL-6 signaling in PDAC have been tested in only a few murine models. For example, a reduction of the number of PanINs was shown in a genetically engineered model (GEM) using ten-week-old iKras mice after treatment with an anti-IL-6 antibody (Y. Zhang et al., 2013). Additionally, Holmer et al. (2014) have shown that the inhibition of both the trans-signaling and classic signaling of IL-6 using sgp130Fc and tocilizumab, respectively, significantly reduced the tumor growth in an orthotopic model using SCID/bg mice. However, it should be considered whether a complete blockade of IL-6 signaling or rather

the specific inhibition of trans-signaling would be necessary for effectively targeting IL-6 signaling. IL-6 trans-signaling is a potent agonist for the gp130 receptor; thus, it can induce signaling in cells lacking IL-6R. Most murine model studies have suggested that IL-6 trans-signaling represents the main signaling mechanism that drives tumor growth. Furthermore, Stat3/Socs3 pathway activation by IL-6 trans-signaling has been shown to be required in addition to oncogenic $KRAS^{G12D}$ to promote PanIN progression and PDAC (Lesina et al., 2011). These effects were reduced when IL-6 trans-signaling or Stat3 was inhibited (Lesina et al., 2011). Inhibition of trans-signaling can be achieved by the sgp130Fc derivative FE 999301, which is currently being tested in a phase I clinical trial (Holmer et al., 2014). Thus, blockage of IL-6 trans-signaling could be a superior strategy if the possible side effects, such as increased risk of bacterial infection could be minimized (Sodenkamp et al., 2012). Therefore, the inhibition of IL-6 may serve as a new targeted PDAC treatment option and the evaluation of appropriate agents in clinical trials is recommended.

In view of the role of Nrf2 in tumor carcinogenesis, a strategy toward selectively inhibiting Nrf2 may also hold a potential therapeutic promise. A growing number of Nrf2 inhibitor compounds have been proposed, all of which are derived from natural sources. These include retinoic acid, brusatol, luteotlin, and trigonelline, among which luteolin and trigonelline have already been studied in PDAC (A. J. Hayes et al., 2015). Luteolin (3',4',5',7-tetrahydroxyflavone), a flavonoid that has been identified as a potent Nrf2 inhibitor, was shown to suppress Nrf2 target genes expression (X. Tang et al., 2011). Additionally, it also reduced PDAC cell proliferation and augmented the apoptotic effect of gemcitabine when administered in combination (Johnson, Dia, Wallig, & Gonzalez de Mejia, 2015). However, the effect induced by luteolin on Nrf2 is not specific as it could also target other signaling pathways related to the cell cycle, angiogenesis, and inflammation (Cai et al., 2012).

In comparison to luteolin, the inhibitory effect of trigonelline (1-methylpyridinium-3-carboxylate), a major alkaloid in coffee bean extract, on Nrf2 signaling in PDAC was more specific. It inhibited Nrf2 nuclear translocation and induced the sensitivity of PDAC cells to chemotherapeutic agents (Arlt et al., 2013). Arlt et al. (2013) also revealed that the suppressed Nrf2 activity in PDAC cells caused by trigonelline reduced the proteasomal activity. However, the greatest inhibitory effect of trigonelline on Nrf2/ARE signaling was limited as doses higher than a specific submaxillary level did not increase the inhibitory effect (Arlt et al., 2013). Therefore, despite being complicated by non-specific Nrf2 targeting and dosing concentration, Nrf2 inhibitors provide a useful tool to explore the mechanisms and the effects of Nrf2 inhibition in pre-clinical settings.

However, several challenges need to be considered prior to the application of Nrf2 targeted therapies safely and effectively to patients with PDAC. In particular, it is not clear whether delivering anticancer drugs in combination with a Nrf2 inhibitor may cause intolerance or considerable side effects, which may suppress the cytoprotective system of Nrf2 in normal cells that has been shown to protect against the tumor formation induced by chemical carcinogens. For example, *NRF2* knockout mice are more likely than wild-type mice to develop neoplasia and progress to tumor after exposure to chemical carcinogens (J. H. Lee et al., 2013). The mechanism by which the host Nrf2 protects against chemical carcinogen-induced tumorigenesis may involve reducing the DNA damage induced by high intracellular ROS levels (J. H. Lee et al., 2013).

Another difficulty in developing clinical Nrf2 targeted therapies lies in the limited studies available related to the effects of modulating Nrf2/ARE signaling on the interaction between tumor metastasis and host. It is unknown whether Nrf2/ARE signaling inhibition may alter the biological behaviour of metastasis. Tumor formation induced by chemotherapeutic agents was shown to increase cell migratory and metastatic

capabilities in a xenograft mouse model by inducing an Nrf2 antioxidant program (H. Wang et al., 2016). Conversely, studies on Nrf2-deficient mice have provided evidence showing that the host Nrf2 has protective role against cancer metastasis, as these mice exhibited a higher number of metastatic nodules than wild-type mice (Satoh et al., 2010). These findings indicated the differential roles of the Nrf2-driven cytoprotective system between normal and tumor tissues. Therefore, further investigations into the mechanistic role of Nrf2 in PDAC metastasis and into the application of IL-6 and Nrf2-targeted therapies in pre-clinical settings are required before these strategies could be subjected to clinical trials.

CHAPTER 6: CONCLUSION

6.1 Overview

This study showed that PSC secretion, specifically IL-6 can be a contributing factor in promoting PDAC progression through cell proliferation and motility via Nrf2 signaling activation. Using the *in vitro* model, the data presented showed that PSC secretion could promote the proliferation of PDAC cells. Furthermore, the data showed that PSC could constitute one of the contributing factors to cause dysregulation of the Nrf2/Keap1 system, resulting in persistent Nrf2 activation. PSC secretion induced *NRF2* gene and protein expression, nuclear translocation, and transcriptional activity, which led to the induction of its antioxidant target gene expression. In the absence of PSC, higher nuclear Nrf2 was observed in AsPC-1 cells than in BxPC-3 cells. *NRF2* downregulation significantly reduced PDAC cell proliferation, with a greater impact on BxPC-3 cells although greater *NRF2* knockdown was observed in AsPC-1 cells. Notably, *NRF2* overexpression resulted in a significant increase in cell proliferation, indicating that Nrf2 downstream signaling may be consistently activated in PDAC even in the absence of PSC. When PSC secretion was added, the increased PDAC cell proliferation was further enhanced. These data suggest that PSC-derived soluble factors induce PDAC cell proliferation via the activation of Nrf2 signaling.

PSC-induced PDAC cell proliferation occurs via Nrf2-mediated metabolic reprogramming. *NRF2* knockdown led to the downregulation of all the metabolic genes involved in PPP, glutaminolysis, and glutathione biosynthesis. Transcription of the majority of these genes was significantly induced following treatment with PSC secretion. Increased metabolic gene expression led to increased levels of the metabolites that are required in PPP and glutaminolysis; thus, permitting nucleotide synthesis and ROS detoxification. Furthermore, the inhibition of G6PD activity led to a significant decrease in PDAC cell proliferation that was only marginally increased with the application of PSC

secretion. These data suggest that the modulation of metabolic pathways by Nrf2 signaling is critical for PSC-induced PDAC cell proliferation.

In identification of the key soluble factors from PSC that mediate these phenotypes, GRO-α, IL-6, and SDF-1α were highly expressed in PSC secretion. rhGRO-α did not increase PDAC cell proliferation compared to IL-6 and SDF-1α. Furthermore, blockage of IL-6 and SDF-1α in PSC secretion via neutralizing antibodies resulted in a significant decrease in PDAC cell proliferation, with IL-6 blockage resulting in over 50% reduction. Consequently, IL-6 and IL-6 receptor gene expression was determined in PSC and PDAC cells. Panc-1 cells were chosen as a key model to investigate the role of IL-6 in PSC-PDAC interactions because it expressed the lowest *IL6* and the highest *IL6R* receptor levels compared to other PDAC cells. Significant reduction in Panc-1 cell proliferation and Nrf2-induced metabolic genes was observed after the neutralization of IL-6 activity. IL-6 neutralization also caused PDAC cells to exhibit a polygonal shape, scattering, and less cell-cell adhesion following PSC secretion treatment, all of which were restored when tBHQ was added. Concurrently, the mRNA levels of *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *SNAIL*, *SLUG*, and *TWIST2* genes, but not of the epithelial marker *CDH1*, were upregulated. This effect was reversed when the IL-6 in PSC secretion was neutralized. *NRF2* mRNA was upregulated in IL-6-treated PDAC cells, indicating that IL-6 mediates PSC-induced EMT and metabolic genes via Nrf2. Furthermore, the inhibition of Stat3 signaling upregulated *CDH1* whereas it downregulated *CDH2*, *VIM*, *FN1*, *COL1A1*, *SIP1*, *SNAIL*, *SLUG*, and *TWIST2*, as well as *NRF2* and its target genes (*AKR1C1* and *NQO1*). Stat3 inhibition further enhanced the inhibitory effect of *NRF2* knockdown on EMT-related gene expression, indicating that IL-6 induces EMT by activating Nrf2 via Stat3 signaling.

In conclusion, this study provides the *in vitro* evidence that the effects of IL-6 secreted by PSC, one of the prominent stromal cells in PDAC microenvironment, on

PDAC cell proliferation, motility and invasion capacity are mediated by Nrf2 signaling activation. This activation induces metabolic reprogramming to confer purine nucleotide synthesis and ROS detoxification, which are favorable for PDAC cell proliferation, and also promotes EMT phenotypes via the Stat3/Nrf2 signaling pathway. Therefore, understanding the function of PSC-secreted IL-6 and its downstream Stat3/Nrf2 signaling pathways may aid in the identification of potential therapeutic targets to improve the prognosis of PDAC.

6.2 Suggestions for future studies

6.2.1 Proto-oncogenes regulation by PSC in activating Nrf2 for PDAC progression

The data presented in the current study showed differential Nrf2 nuclear protein expression in *KRAS* mutant AsPC-1 cells and *KRAS* wild-type BxPC-3 cells even in the absence of PSC. Furthermore, *NRF2* downregulation resulted in a greater reduction in BxPC-3 cell proliferation although more efficient *NRF2* knockdown was observed in AsPC-1 cells. Conversely, *NRF2* overexpression alone increased the proliferation of both AsPC-1 and BxPC-3 cell types. These findings indirectly deduce that proto-oncogenes may consistently induced Nrf2 signaling pathway in PDAC cells. Activation of these proto-oncogenes has been found to constitute an important metabolic switch regulator to promote tumor progression (Sousa & Kimmelman, 2014). In this regard, it might be informative to investigate whether PSC could regulate these proto-oncogenes to activate Nrf2-mediated signaling pathways to promote PDAC progression, particularly cell proliferation, motility and invasion.

PDAC is known to harbour a high frequency of *KRAS* mutation (90%). Recent studies have demonstrated that the activity of $KRAS^{G12D}$ is required for all stages of carcinogenesis including inception, progression, and metastasis, as $KRAS^{G12D}$ inactivation using genetic approaches invariably reversed the ongoing carcinogenic process (Collins,

Bednar, et al., 2012; Collins, Brisset, et al., 2012). Oncogenic *KRAS* supports biomass synthesis (e.g., protein and nucleotides) for PDAC cell proliferation by redirecting glucose toward anabolic pathways such as PPP while maintaining a low level of intracellular ROS (Ying et al., 2012). Kras activation in PPP leads to increased transcription of two enzymes of the non-oxidative arm, ribose 5-phosphate isomerase A (RpiA) and ribulose-5-phosphate-3-epimerase, which results in increased flux into the non-oxidative arm. Thus, oncogenic *KRAS* utilizes glutaminolysis to maintain redox balance. In turn, glutaminolysis has been shown to be Kras-dependent, as oncogenic *KRAS* drives the expression of glutamic-oxaloacetic transaminase(GOT)1 and ME1, which are indispensable for PDAC cell proliferation by producing NADPH to combat oxidative stress (Son et al., 2013). The expression of Kras at physiological levels has been shown to activate the Nrf2-mediated antioxidant program to decrease cellular ROS levels, thereby protecting PDAC cells against the detrimental effects of oxidative stress. In addition, the present study also showed that Nrf2 could regulate several enzymes in PPP and glutaminolysis for nucleotide synthesis and ROS detoxification. It was also shown that oncogenic *KRAS* mutation can induce EMT to promote PDAC invasiveness and metastasis. However, it is not clear whether oncogenic Kras-induced Nrf2 signaling plays a role in EMT in PDAC. Notably, oncogenic *KRAS* expression can be activated by IL-6 as indicated by the reduction of the onset of PanINs, proliferation, maintenance, and progression in iKras;$IL6^{(-/-)}$ mice similar to that observed in iKras* mice (Y. Zhang et al., 2013). These evidences suggest that IL-6 from PSC may utilize oncogenic *KRAS* to induce Nrf2 signaling for the stimulation of PDAC cell proliferation and metastasis. However, further studies are required to confirm this hypothesis.

Tumor suppressor p53 is also mutated in most PDAC tumors, and its frequency is associated with tumor progression. For example, deletion or mutation of p53 accelerated the development of PDAC tumors in *KRAS*-driven GEM models (Hingorani et al., 2005;

Pellegata et al., 1994; Perez-Mancera, Guerra, Barbacid, & Tuveson, 2012). Wild-type p53 has also been reported as a unique regulator of PPP. For example, it can enhance glycolysis and oxidative phosphorylation, thus serving as a regulator of apoptosis. Furthermore, wild-type p53 can also inhibit PPP by inhibiting G6PD activity, subsequently reducing the NADPH production for ROS detoxification and macromolecules formation (Jiang et al., 2011; Mitsuishi, Motohashi, & Yamamoto, 2012). Conversely, tumor-associated *TP53* mutant increases NADPH production because it does not affect G6PD activity (Mitsuishi, Motohashi, et al., 2012). This indicates that *TP53*-mutated PDAC may substantially increase the Nrf2-mediated induction of PPP at the transcriptional level. In addition, p53 has also been reported to regulate EMT through miRNA expression to promote PDAC metastasis (Chang et al., 2011; Dong et al., 2013). However, to our knowledge no studies have investigated the mediation of Nrf2 signaling in p53-mediated EMT in PDAC. Alternatively, IL-6 may itself interact with p53 mutant to promote PanIN progression, as blockage of IL-6 signaling using an anti-IL-6 antibody reduced the number of PanIN formation in Pdx1-Cre;KrasLSL-G12D/+;p53fl/+;Rosa26LSL-YFP/+ (KPCY) mice, which harbour both *KRAS* and *TP53* mutations. Therefore, additional investigations are required to study the roles of PSC, specifically of IL-6 may activate p53 mutant pathway to promote PDAC cell proliferation and metast via Nrf2 signaling.

The Myc oncogene is known to act as an activator of PPP, inducing the genes that are involved in nucleotide synthesis including thymidylate synthase for pyrimidine metabolism and inosine monophosphate dehydrogenase 1 and 2 for purine metabolism. Myc is a downstream effector of Kras that is used to regulate the non-oxidative arm of PPP, as shown by studies wherein the *KRAS*^{G12D}*-MYC* axis could induce *NRF2* gene expression and protein activity in PDAC (DeNicola et al., 2011; Ying et al., 2012). It has also been shown that Myc acts as a Nrf2-interacting protein to regulate Phase II genes via

electrophile responsive elements (Levy & Forman, 2010). Thus, Myc may depend on Nrf2 activity to regulate the non-oxidative arm of PPP to promote PDAC cell proliferation. Myc has also been reported to regulate the metabolism of glucose, glutamine, and the tricarboxylic acid (TCA) cycle (Cho, Cho, Lee, & Kang, 2010; Gerriets & Rathmell, 2012; Levine & Puzio-Kuter, 2010; Mannava et al., 2008). Myc antioxidant activity is also effected by its induction of the Nrf2-mediated antioxidant program to promote ROS detoxification (DeNicola et al., 2011). Furthermore, Myc plays a role in promoting PDAC metastasis by inducing EMT in PDAC stem cells through miRNA regulation. For example, it has been shown that the overexpression of miR-200 inhibited the migration and invasion of cancer stem cells derived from Panc-1, and also resulted in the downregulation of mesenchymal genes (*CDH2* and *VIM*) and upregulation of the epithelial *CDH1* gene (Y. Lu et al., 2014). Notably, it was reported that IL-6 induces Myc expression via Stat3 phosphorylation as observed in human myeloma cells (Shamsasenjan et al., 2009). However, it is unknown whether IL-6 from PSC might activate Myc to induce PDAC progression via Nrf2 signaling. Therefore, studying the interaction of Nrf2 with tumor-associated proteins in mediating metabolic reprogramming and EMT may provide further insights on PSC-mediated PDAC progression.

6.2.2 Application of *in vivo* models to examine the roles of PSC-mediated PDAC progression

The current results are limited to the two-dimensional (2D) nature of *in vitro* experiments. Therefore, another potential future study would be to use a three-dimensional (3D) model to validate the results obtained, as the cell morphology and behaviour of tumor and stromal cells in two-dimensional (2D) models of cancer can differ significantly from those in 3D models (Kenny, Krausz, Yamada, & Lengyel, 2007; Yamada & Cukierman, 2007). Thus, the direct 3D co-culture of PSC and PDAC cells to

replicate the situation in the human tumor with its prominent stromal reaction, should be used to strengthen the *in vitro* evidence. In addition, a good *in vivo* model is also required, in which the tumors should develop in anatomically relevant sites, and not surrounded by physiologically irrelevant microenvironment as this would affect the characteristics of the cancer cells. Accordingly, many investigators utilize orthotopic xenograft models to investigate PSC-PDAC interactions and have shown that PSC can enhance the stromal reaction to affect tumor growth, invasion, and metastasis (Arumugam et al., 2011; Bailey et al., 2008; Feldmann et al., 2007; Haas et al., 2009; Hehlgans et al., 2009; Hwang et al., 2008; Vonlaufen, Joshi, et al., 2008; Z. Xu et al., 2010). For example, the co-injection of PSC at varying amounts with PDAC cells into the pancreas of nude mice yielded tumor formation, growth, and distant metastatic sites. Notably, the presence of PSC increased the incidence of tumor formation when limiting numbers of PDAC cells were injected (Hwang et al., 2008). In addition, a study incorporating a GEM model with LSL-*KRAS*^{G12D}/+; LSL-*Trp53R172H*/+; Pdx-1-Cre reported that the inactivation of PSC following treatment with ATRA led to a significant reduction of tumor growth and invasion (Froeling et al., 2011). These findings suggest that using *in vivo* models to develop a PDAC tumor together with GEM expressing the *KRAS* mutation or other oncogenes may allow the establishment of an accurate representation of the effects of PSC on PDAC progression.

Besides, Zhang et al. (2013) developed the *IL6*-knockout mice with conditional *KRAS*^{G12D} mutation. They found that IL-6 coupled with oncogenic *KRAS* could activate Nrf2-mediated ROS detoxification program, which is required for PanIN development and PDAC progression. More recently, *NRF2*-knockout mice with conditional *KRAS*^{G12D} and *TP53* mutations was developed, in which the number of PanINs and invasive cancer were less frequent in *NRF2*-knockout mice compared to control mice (Hamada, Masamune, Toguchi, Yamamoto, & Shimosegawa, 2016; "NRF2-Mediated Translation

Promotes Pancreatic Cancer Maintenance," 2016). These in turn may be useful in the exploration of the relationship between IL-6 and Nrf2 signaling mechanisms in promoting PDAC cell proliferation through metabolic changes and in our understanding of the role of the IL-6/Stat3/Nrf2 signaling pathway in promoting EMT for PDAC.

6.2.3 Interaction of PSC with other stromal cells for PDAC progression

In the current study, the roles of PSC in driving PDAC progression were investigated. However, PSC comprise only one of the major stromal cells in the PDAC tumor microenvironment. In fact, the interplay of PSC with the stromal cells in the tumor microenvironment has been shown to play a role in PDAC carcinogenesis (Bayne et al., 2012; Cedeno-Laurent & Dimitroff, 2012; D. Tang et al., 2012). PSC may interact with the stromal cells under hypoxia conditions, which represent a prominent feature in PDAC. PSC serve as a contributing factor to create the hypoxic microenvironment by producing an extensive deposition of ECM proteins to destroy the normal parenchymal architecture and compress the fine capillary network, thereby limiting the oxygen diffusion. It has been reported that endothelial progenitor cells protect PSC from severe hypoxia by activating Nrf2 signaling and the expression of its target gene (*HMOX-1*) via the PI3K/Akt pathway (Zhou et al., 2013). Conversely, Nrf2 knockdown increased apoptosis, impaired cell proliferation, and inhibited the expression of HIF-1α. In addition, Nrf2 suppression was able to reduce angiogenesis by lowering the accumulation of the HIF-1α protein and limiting the expression of *VEGF* under the hypoxic condition (Zhou et al., 2013).

In addition to endothelial cells, monocytes/macrophages have also been shown to activate PSC in PDAC. In the hypoxia environment, tumor-derived chemokines, such as VEGF can be induced to attract monocytes/macrophages (Murdoch, Giannoudis, & Lewis, 2004). Li et al. (2016) have revealed that HIF-1α recruited macrophages by means

of C-C motif chemokine ligand 2 (CCL2) secretion under hypoxic condition. The recruited macrophages subsequently accelerated the activation of PSC, suggesting that HIF-1α may promote that inflammation and fibrosis of PDAC through CCL2 secretion (N. Li et al., 2016). Furthermore, macrophages have been considered as anti-tumorigenic because of their production of high amount of ROS. However, it was discovered that macrophages contribute to PDAC initiation. This may be explained by the high antioxidant capacity in PDAC cells that express oncogenic Kras (Liou & Storz, 2015), wherein the oncogenic *KRAS* induces the Nrf2 antioxidant program to counteract the detrimental effect of high ROS levels produced by macrophages (Liou & Storz, 2015). Notably, this effect acts in opposition to the function of PSC, which create a relatively reduced environment by activating Nrf2 signaling to promote PDAC progression, as shown by in the data presented in this thesis. These evidences suggest that the effects of the interaction between PSC and the stromal cells in promoting PDAC progression may occur via Nrf2 signaling. However, further investigations of such aspects will facilitate a more comprehensive understanding of PSC-mediated PDAC progression and Nrf2 signaling involvement.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A. Conferences

1. International Conference on Advances in Medical Science 2013

   16-18th April 2013

   Hotel Impiana Kuala Lumpur, Malaysia

   Oral presentation

2. Pharmacology & Physiology International Scientific Congress 2014

   22-24th August 2014

   Putra World Trade Centre, Kuala Lumpur, Malaysia

   Poster presentation

   Award: Best poster presenter

3. The IUPHAR World Conference on the Pharmacology of Natural and Traditional Medicine 2015

   22-24th July 2015

   Yong Loo Lin School of Medicine, National University of Singapore, Singapore

   Poster presentation

4. Digital Pathology Congress: Asia

   22-23th August 2016

   Renaissance Kuala Lumpur Hotel, Malaysia

   Poster presentation

B. Publications

1. Wu, Y. S., Looi, C. Y., Subramaniam, K. S., Masamune, A., Chung, I. (2016). Soluble factors from stellate cells induce pancreatic cancer cell proliferation via Nrf2-activated metabolic reprogramming and ROS detoxification. *Oncotarget*, *7*(24), 36719-36732. doi:10.18632/oncotarget.9165.

2. Wu, Y. S., Chung, I., Wong, W. F., Sim, M. S., Masamune, A., Looi, C. Y. (2016). Paracrine IL-6 signaling mediates the effect of pancreatic stellate cells on epithelial-mesenchymal transition via Stat3/Nrf2 pathway in pancreatic cancer cells. *BBA- General Subjects*, doi:10.1016/j.bbagen.2016.10.006