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Targeting retinoid receptors with dysregulated microNRAs in pancreatic duct adenocarcinoma

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To my parents, for their unconditional love, endless support and encouragement

Declaration

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I hereby declare, that the submitted thesis entitled

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I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university

München, 10.2018 Yin, Shuai

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ABBREVIATIONS

3’UTR	3’ untranslated region
ATRA	All-trans retinoic acid
Bp	Base pair
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CSCs	Cancer Stem Cells
CAF	Cancer-associated Fibroblasts
cDNA	Complementary DNA
dNTPs	Deoxynucleotide Triphosphates
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's modified Eagle's medium
EMT	Epithelial-Mesenchymal Transition
EDTA	Ethylene-diamine Tetraacetic Acid Disodium Salt
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
g	Gram

g	Gravity
h	Hours
HPDE	Human Pancreatic Duct Epithelial
HCl	Hydrogen chloride
mRNA	Messenger RNA
MTA	Methyltransferase
µg	Micro gram
miR	microRNA
miRNA	microRNA
mg	Milli gram
m	Minutes
MAPK	Mitogen-activated protein kinase
TEMED	N,N,N’,N’-Tetramethylenediamine
ng	Nano gram
nM	Nano Molar
nt	Nucleotides
PDAC	Pancreatic Duct Adenocarcinoma
PanINs	Pancreatic Intraepithelial Neoplasias
PSCs	Pancreatic Stellate Cells

PBS	Phosphate Bffered Saline
PI3K	Phosphatidylinositol 3-kinase
PAGE	Polyacrylarnide Gel Electrophoresis
PCR	Polymerase chain reaction
PVDF	Polyvinylidene Difluoride
Pre-miR	Precursor microRNA
qPCR	Quantitative PCR
qRT-PCR	Quantitative RT-PCR
RB	retinoblastoma
RA	Retinoic Acid
RARα / RARA	Retinoic acid receptor alpha
RARβ / RARB	Retinoic acid receptor beta
RARγ	Retinoic acid receptor gama
RARE	Retinoic acid response element
RXRα / RXRA	Retinoic X receptor alpha
RXRβ / RXRB	Retinoic X receptor beta
RXRγ	Retinoic X receptor gama
RR	Retinoid receptors
RT-PCR	Reverse transcriptase- PCR

RNA	Ribonucleic Acid
RISC	RNA induced silencing complex
RT	Room temperature
STAT3	Signal transducer and activator of transcription 3
SDS	Sodium Dodecyl Sulphate
TAE	Tris-acetate-EDTA
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

Summary

Retinoid (i.e., vitamin A, all-trans retinoic acid and other synthetic derivatives of vitamin A) induce cell differentiation and growth via the interaction of retinoid receptors in various normal and malignant cells. In addition to the success of all-trans-retinoic acid -based differentiation therapy in acute promyelocytic leukemia, emerging attempt in the combination of RA therapy and traditional chemotherapy has been studied in various solid cancer. Retinoid receptors (RARA, RARB, RXRA, and RXRB) and retinoic acid were reported to be down-regulated in pancreatic duct adenocarcinoma (PDAC) compared to normal pancreas. Moreover, the reduced expression of retinoid receptors correlates with the expression of these markers involved in the cell differentiation and the epithelial-mesenchymal transition. Further analysis revealed that the expressions of RARA and RXRB are associated with the overall survival in PDAC patients. Reduced level of retinoid and their receptors is associated with worse survival outcomes in PDAC patients. Yet, the mechanism of the down-regulation of retinoid receptors is not well defined. miRNA, as a class of small non-coding RNAs, can incorporate into the RNA-induced silencing complex and post-transcriptionally regulate the mRNA expression typically by binding to the 3' untranslated region (3'-UTR) of the complementary mRNA sequence. The aim of this study was to find out whether selected dysregulated miRNAs in PDAC are responsible for the decreased level of retinoid receptors. For this purpose, we first performed a bioinformatics research to reveal conserved target sequences for deregulated miRNAs within the 3'UTR region of retinoid receptor mRNA. Next, we investigated the expression of selected retinoid receptors and miRNAs in BxPC-3, DanG, MiaPaCa-2, Panc-1 cell lines and the Human Pancreatic Duct Epithelial (HPDE) cell line. Further, we investigated the expression level by manipulating the expression of selected miRNAs. We demonstrated that none of these miRNAs can target the selected retinoid receptors *in vitro*. Therefore, we conclude that dysregulated miR-138, miR-206, miR-613, miR-9 and miR-27a/b are not involved in the regulation of the expression of retinoid receptors in PDAC patients.

1. Introduction

1.1 Cancer

1.1.1 Cancer statistics

Cancer is a complex disease based on progressive accumulations of genetic aberrations and epigenetic changes of cells that escape from normal cellular control. Up to date, cancer constitutes an enormous burden of human health worldwide, among one of the most leading causes of disease-related death in both developing and developed countries .

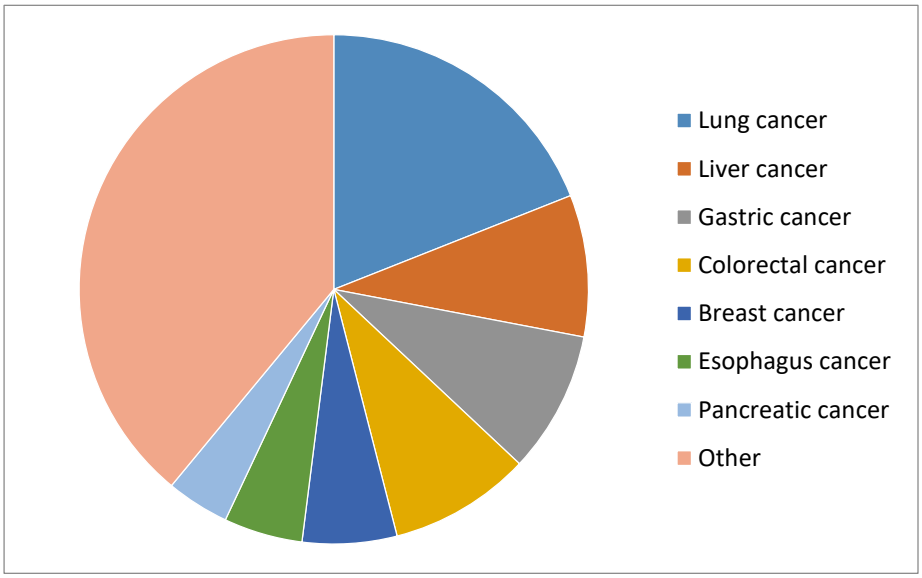

Figure 1. Constitute of global cancer deaths. (Source: GLOBOCAN 2012)

In 2012, nearly 14.1 million new cancer cases were diagnosed. More than 8.2 million cases died from cancer, despite the amount of newly diagnosed cases of cancer still climbing yearly due to the growth and aging of the population. It is estimated in 2030, 23.6 million new cancer cases

are to be diagnosed (Jemal et al., 2011). In less developed countries, rapidly increasing population and the adoption of lifestyle containing cancer-related risk, such as alcohol abuse, smoking, poor diet, physical inactivity, has further increased the incidence of cancer. Among these newly diagnosed cases, lung cancer is the most common cancer in men while breast cancer is most common in women in developing countries. In more developed countries, cancer with the highest incidence among men and women are prostate cancer and lung cancer, respectively. Cancer death in developing countries accounts for 65% of worldwide death, less than the percentage of their population. Percentages of cancer-related death worldwide (for both sexes) are shown in Figure 1.

Although cancer occurs based on genetic aberrations, understanding the chaos of cancer cell physiology is crucial to the illumination of the complexity of cancer. Over the past three decades, abundant new knowledge of cancer research has been gained for the better understanding of cancer and eventually the preparation for novel therapeutic strategy. Decades ago, pathologists have defined the pathological type of cancer in term of the cell type. The category of different cancer types is indicated in the following section.

1.1.2 Types of Cancer

Tumors are classified into two broad categories depending on their aggressiveness: the benign and malignant tumor. The majority of primary tumors in humans are benign and innocuous, except in rare cases where the progression of these localized masses oppress important adjacent tissues and organs, or secrete an excessive hormone which causes a pathological status. For instance, benign brain tumor could press on adjoining structures when proliferating large enough, thus causing dysfunction of important nerve (Cook & Freedman, 2011). Another example is that pheochromocytoma can induce paroxysmal elevated blood pressure by high-level secretion of

catecholamines (Mann, 2008). Generally, deaths caused by benign tumors are much rarer compared to that by malignant tumors (Ostrom et al., 2016).

Malignant tumors that derived from epithelial cells are called carcinomas (e.g. cancer). Moreover, these carcinomas that only originate from glandular epithelial are called adenocarcinoma, while those arise from squamous epithelium in tissues like skin and the esophagus are termed as squamous cell carcinomas. Malignant tumors that arising from non-epithelial cells can be categorized into three groups. The first group is termed as sarcomas, representing those tumor cells derived from osteoblasts, myocytes, adipocytes and fibroblast. The second group of non-epithelial cancers is derived from those cells in the hematopoietic system like bone marrow，resulting in an abnormal production of non-functional blood cell or lymphatic cell. The term leukemia refers to that the malignant cells developed from circulating hematopoietic cell, while lymphomas represent that those lymphoid lineages originated solid malignant tumor are likely found in lymph nodes. The third group of non-epithelial malignant tumors includes glioma, astrocytoma and ependymoma which developed from either central or puerperal nervous system. There are also other specific malignant tumor types, such as melanoma and malignant teratoma, belong to none of the group mentioned above (Herrington, 2014).

1.1.3 The Biology of cancer

During the past several decades, rapid advances and numerous knowledge in cancer research have provided new insight into cancer biology. Among the most famous point views, Weinberg *et al* have raised the famous viewpoint that there are 10 crucial alterations in cancer cells which conclude malignant property of cancer: (i) sustaining proliferative signaling, (ii) evading growth suppressors, (iii) avoiding immune destruction, (iv) enabling replicative immortality, (v) tumor-promoting inflammation, (vi) activating invasion and metastasis, (vii) inducing Angiogenesis, (viii)

genome instability and mutation, (ix) resisting cell death, (x) deregulating cellular energetics (Figure 2). The essence of these hallmarks of cancer cell will be described in following section respectively (Hanahan & Weinberg, 2011).

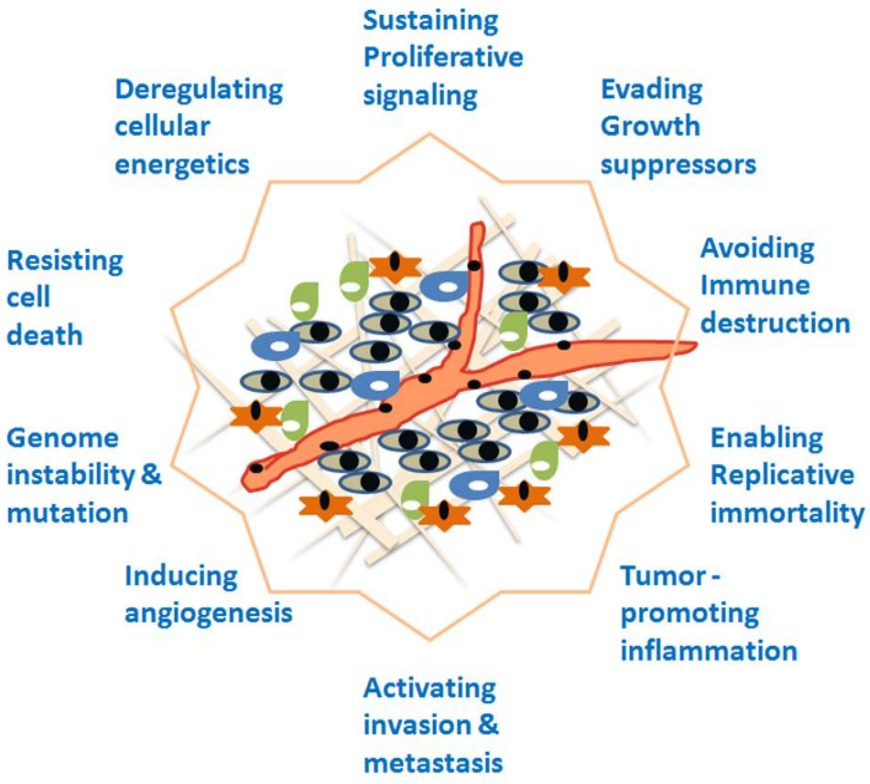

Figure 2. Acquired capabilities of the tumor cells (Hanahan & Weinberg, 2011).

(I) Sustaining Proliferative Signaling

Normal tissue cell controls their mitotic cycle by carefully orchestrating the dynamic level of multiple growth signals that are involved in the cell growth and cell division, thereby maintaining the homeostasis of the cell and the whole body. These growth signals function by binding to

different classes of signaling molecules: diffusible growth factors, extracellular matrix components and cell-to-cell adhesion molecules. Cancer cell, however, re-manipulates these signals and acquire the capability to sustain proliferative signaling in alternative ways.

The malignant cell can generate growth factor ligands itself and bind to cognate receptors, resulting in autocrine signals which facilitate the proliferative stimulation of the malignant cell independently (Hanahan & Weinberg, 2011). Alternatively, the malignant cell can interact with the normal cell in the tumor stroma to gain additional growth factors (Mao, Keller, Garfield, Shen, & Wang, 2013). Besides, the cancer cell is able to disrupt the normal negative feedback loops which operate to maintain the homeostatic status of various types of signaling. The mutation of oncogenic RAS gene, for example, results in a high level of cellular Ras-GTP and the abolishment of the negative feedback by Ras-GDP. The high level of Ras-GTP leads to sustained activation of proliferative signal pathways (Eser, Schnieke, Schneider, & Saur, 2014). However, the mechanisms that control the release of these mitogenic signals remain largely unknown.

(II) Evading Growth Suppressors

In normal cells, a variety of growth factors collaborates with dozens of growth suppressors to ensuring a homeostasis balance. Two typical tumor suppressors, RB (retinoblastoma-associated) and TP53 proteins, cooperate to serve as the central cellular regulatory circuits that master the decisions of proliferation, senescence and the apoptotic process of cells (Sherr & McCormick, 2002). The RB protein, in response to diverse extracellular signals, functions as a critical gatekeeper by deciding whether or not a cell should enter into its division cycle. Cancers with RB signal mutations, however, miss its control of cell cycle in cancer cells, thereby permitting sustaining proliferation of cancer cell. Whereas TP53 is a tumor suppressor mainly govern internal abnormality like damage of genome or abnormal metabolism status. TP53 protein is able to halt further cell-cycle progression until these abnormal conditions have been normalized, otherwise

induceing apoptosis if the damage of the cell is irreparable. In addition to tumor suppressor proteins, evasion from contact inhibition also matters in a variety of cancer types. In ensuing years, NF2 gene and LKB1 epithelial polarity protein are discovered to be key regulators in cell-cell contact inhibition (Faubert et al., 2014; Martin-Belmonte & Perez-Moreno, 2012). These two proteins could both strengthen the adhesion of cell-cell attachment and suppress further cell proliferation when yielding confluent cell monolayer *in vitro*. In numbers of cancers, NF2 and LKB1 has represented the most frequently lost tumor suppressor proteins involved in cell contact inhibition.

(III) Avoiding immune destruction

Immune surveillance denotes that the whole body is monitored by the sensitive immune system which is in charge of recognizing and obliterating the most of the incipient cancer cells. However, a fraction of cancer cells can escape the detection and eradication by the immune system. In recent years, the immune system has been indicated to operate as a significant barrier to tumor formation and progression. Interestingly, cancer cells that originally generated in immune deficient mice are often inefficient at initiating secondary tumors in syngeneic immune competent hosts, while cancer cells from tumors arising in immune competent mice are able to initiate transplanted tumors in both types of hosts (Teng, Swann, Koebel, Schreiber, & Smyth, 2008). This could be explained by ‘‘immune editing’’, indicating that the surviving cancer cells in immune competent host gained a capability to colonize into a new tumor mass when transplanted to a new host, while tumors originate from immune deficient mice lack of the select process of the host immune system (Smyth, Dunn, & Schreiber, 2006).

(IV) Enabling replicative immortality

Another important ability of cancer cells is their unlimited replicative potential, compared with normal cells only going through a limited number of growth-division cycles. The senescence

and crisis have been two distinct barriers that are responsible for this limitation of normal cells. Senescence indicates a non-proliferative but viable state while crisis denotes cell death. A vast majority types of tumor cells cultured *in vitro* appear to be immortalized, suggesting that this limitless replicative ability was gained *in vivo* during tumorigenesis. Most evidence indicates that telomeres in cancer cells protect the ends of chromosomes, thereby enabling the capability of proliferation.

Telomeres locate in the end of chromosomes. They are composed of several thousand repeats of a short 6 bp sequence element. DNA polymerases are unable to completely replicate the 3'ends of chromosomal DNA, lead to every chromosome loses 50-100bp telomeric DNA from the ends during each S phase. The telomeres shorten progressively after cell cycle in normal cells, thus failing to protect the ends of chromosomal DNA from end-to-end fusions (Shay & Keith, 2008). Cancer cells, however, enjoy functional levels of telomerase enzyme which guarantees stable chromosomal DNA by replenishing the lost telomere repeat segments (Shay & Keith, 2008). In this way, the cancer cells acquire the ability of resistance to induction of both cell senescence and cell crisis/apoptosis.

(V) Induced and Sustained Angiogenesis

Angiogenesis, the tumor-associated neo-vasculature which is important for the acquirement of nutrients, oxygen as well as the evacuation of metabolic waste, is carefully regulated by pro-angiogenic and anti-angiogenic factors. Cancer, when the growth of which is beyond 1–2 mm diameter, can interact with the hypoxic microenvironment to guide the sprouting of blood vessels from surrounding tissues into the tumor (Nishida, Yano, Nishida, Kamura, & Kojiro, 2006). A variety of cells within the cancer stroma, such as tumor-associated fibroblasts, macrophages, mast cells and CD11b+Gr1+ myeloid cells, participate in the complex angiogenesis signal networks in cancer. One of the well-known angiogenesis inducers and inhibitors are vascular endothelial

growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1), respectively. VEGF ligand triggers the angiogenesis signaling via its binding to receptor tyrosine kinases (VEGFR-1–3) which are regulated by both hypoxia and oncogene signaling (Fong, 2008). However, TSP-1 serves as an anti-angiogenic factor which binds to trans-membrane receptors in endothelial cells and therefore counterbalances pro-angiogenic effect. Generally, tumor angiogenesis is characterized by precocious capillary sprouting, excessive vessel branching, distorted and enlarged vessels, erratic blood flow, microbleeds, leakiness and aberrant levels of endothelial cell proliferation and apoptosis.

(VI) Invasion and Metastasis

During the progression of cancer, primary cancer cells grow gradually beyond adjacent tissue and spread to distant sites to establish new tumor colonies. So far, cancer related death are of more than 90% caused by such metastasis. This kind of invasion-metastasis cascade is organized by a sequence of independent steps: (1) Local invasion of cancer cells through surrounding extracellular matrix (ECM) and stromal cell layers, (2) Intravasation of cancer cells into nearby blood and lymphatic vessels, (3) Survival and transition of cancer cells through the vasculature, (4) Arrest of cancer cells at distant organ sites, (5) Extravasation of cancer cells from vessels into the parenchyma of distant tissues, (6) Survival and colonization of cancer cells in metastasis site. These complex events are orchestrated by not only molecular pathways within cancer cells but also multi-level interactions between cancer cells and stroma cells.

In order to invade the stroma, cancer cells must first detach from epithelial sheets with acquired highly motility and invasiveness. To overcome the E-cadherin-mediated intercellular junctions that knit together epithelial cell sheets and other barriers, carcinoma cells undergo a cell-biological program known as the epithelial-mesenchymal-transition (EMT) (Seton-Rogers, 2016). During the EMT process, cancer cells break down cell-cell adhesion and tight junctions, followed

by the change of cell polarity which exhibits mesenchymal morphology and unregulated invasiveness. Specifically, the cancer cells undergo EMT begin to produce fibronectin, an extracellular matrix protein that is normally produced by mesenchymal cells such as the fibroblast. The expression changes of fibronectin and N-cadherin thus affects the organization of cells' intermediate filament cytoskeleton and mortality. A set of important transcriptional factors, including Snail, Slug, Twist, and ZEB1/2, coordinates to manage the EMT of cancer cells. These transcription factors directly repress expressions of E-cadherin which maintain epithelial states. Besides, certain miRNAs belonging to miR-200 family could promote epithelial phenotypes by repressing ZEB1 and ZEB2 EMT-inducing transcription factors (Park, Gaur, Lengyel, & Peter, 2008). Moreover, cancer cells can secrete matrix metalloproteinase (MMPs) to degrade ECM elements like IV collagen, laminin and vitronectin. Stroma cells like fibroblasts and myofibroblasts, endothelial cells, adipocytes, mesenchymal stem cells, as well as macrophages and other immune cells also determine the invasion and metastasis ability of cancer cells. These stromal cells are capable of improving aggressive behaviors of carcinoma cells via various signaling. For example, stromal CD4+ T-lymphocytes stimulate tumor-associated macrophages (TAMs) to trigger epidermal growth factor receptor (EGFR) signaling in the carcinoma cells and thus promoting mammary carcinoma invasion (Mao et al., 2013). In fact, only a small minority of disseminated cancer cells can finally succeed in terms of metastatic colonization and clinically detectable metastases.

(VII) Tumor-Promoting Inflammation

Pathologists noticed that virtually every neoplastic lesion was infiltrated with immune cells at densities ranging from subtle infiltrations to gross inflammations. This was initially viewed as a systematic immune response of the host to eliminate tumor cells. However, recent evidence revealed that immune cell infiltrations could unanticipated enhance tumorigenesis and tumor progression in the way of providing various bioactive molecules (DeNardo, Andreu, & Coussens,

2010). Those factors include growth factors, survival factors, pro-angiogenic factors, extracellular matrix-modifying enzymes and EMT inductive signals. In addition, inflammatory cells can release reactive oxygen species which accelerate the evolution speed of tumor cells towards more malignancy status (Chaffer, San Juan, Lim, & Weinberg, 2016).

1.1.4 Genetics of cancer

The genome of cancer is based on a constant succession of alterations at multiple levels ranging from subtle mutation to chromosomal lesions. Certain mutant genotypes ensure survival advantage of sub-clones of cancer cells, facilitating their growth dominance in a local tissue environment. Normal cells have DNA-maintenance system that keeps genomic integrity and induces genetically damaged cells to either senescence or apoptosis. In order to acquire mutant genes, cancer cells increase their rates of gene mutation which enable them to escape from the DNA-maintenance system. Inductions of genetic alterations of several critical genes determine different fate of the cells. Cell that harbors hundreds of these susceptible cancer gene experiences spontaneous mutation at a very low speed all the time. These critical genes can determine the fate of cancer cells and they are divided into two categories: tumor suppressor gene and oncogene (You & Jones, 2012).

1.1.5 Oncogene

Oncogene is the gene that can stimulate growth, division and survival of cells. The abnormal activation of oncogene will lead to eventual immortalization or transformation of cells. Oncogenes with non-activated state existing in all normal cells are called proto-oncogenes. Namely, products of proto-oncogenes can induce cell mutations under abnormal activation. The activations of proto-

oncogenes include retroviral transduction or retroviral integration, point mutation, insertion mutation, gene amplification, chromosomal translocation and protein-protein interaction. So far more than hundred oncogenes, like KRAS and Myc, are reported (Croce, 2008).

1.1.6 Tumor suppressor gene

Tumor suppressor gene functions as ‘‘caretakers’’ of the cell damage. The functions of this tumor suppressor gene comprise: (1) Detecting and repairing DNA damage, (2) Inactivating or preventing mutagenic molecules before they have damaged the DNA, (3) Preventing unrestrained cellular growth, (4) Promoting cell cycle checkpoint activation (E. Y. Lee & Muller, 2010). Among the most important tumor suppressor genes are TP53, BRCA1/2, APC and RB1 gene. Up to date, more than 200 tumor suppressor genes are identified in human and other species (E. Y. Lee & Muller, 2010).

1.1.7 Tumor microenvironment

In ensuing years, it is emphasized that tumor is more than simple aggregations of proliferating tumor cells. Instead, cancer is comprised of multiple different cell types as well as distinct stromal elements that participate in complex interactions with other elements. This viewpoint reveals that the biology of the tumor should be no longer understood as the simple accumulation of the traits of the cancer cells, but instead should encompass the contributions of the ‘‘tumor microenvironment’’ (Visvader & Lindeman, 2008). The tumor microenvironment represents the surviving environment of cancer which is constructed during the course of multistep tumorigenesis. As mentioned above, cancer cells with clonal heterogeneity comprise of cells with various degrees of differentiation, proliferation, vascularity, inflammation and invasiveness. Accumulated evidence

in recent years revealed a new subclass of neoplastic cells within tumors, termed as cancer stem cells (CSCs) (Visvader & Lindeman, 2008). More and more human tumors are found to contain such subpopulation with the properties of CSCs, which exert their efficient tumor-initiating capabilities *in vivo* experiment. In recent years, researchers have also interrelated the acquisition of CSC features with the EMT progression. The EMT of some cancer cells in certain models could induce defining features of CSC, such as self-renewal ability. Moreover, cells with properties of CSC are shown, in a variety of cancers, to be more resistant to commonly used chemotherapeutic and radiotherapeutic treatments.

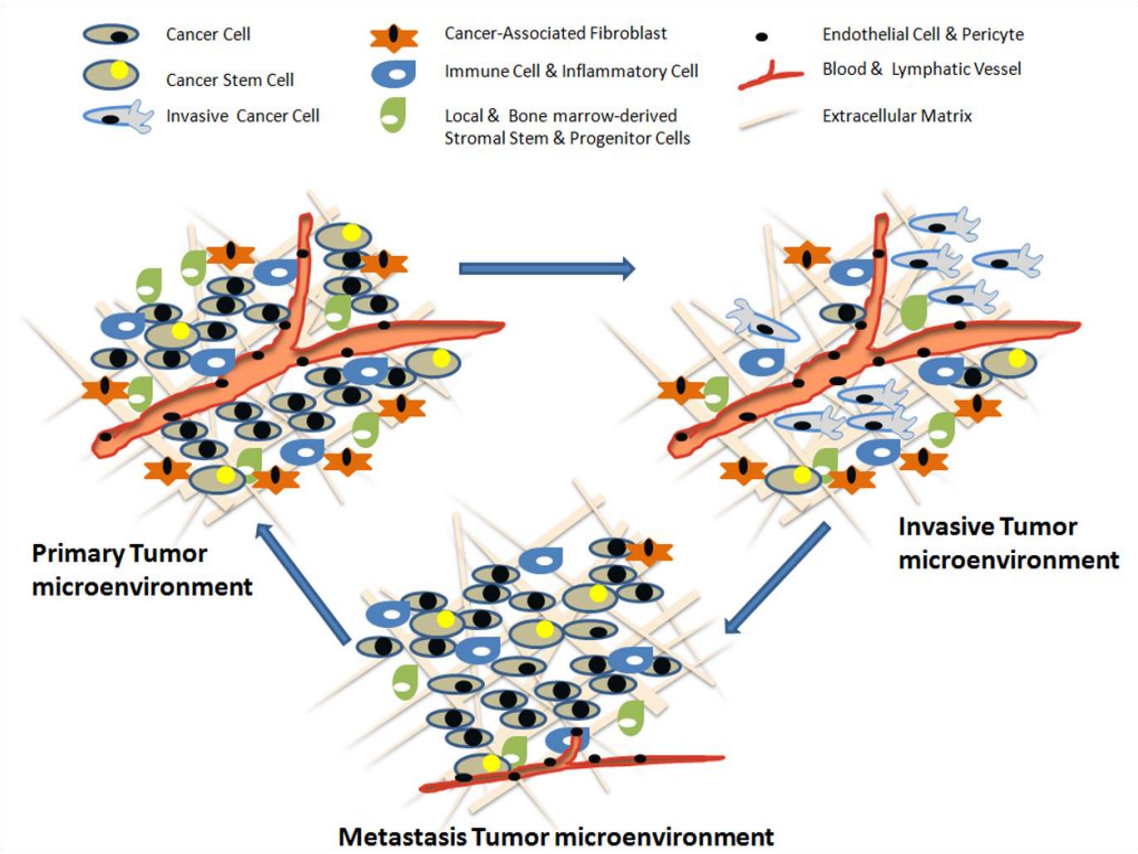

Figure 3. Cells in tumor microenvironment. Adjusted from (Hanahan & Weinberg, 2011)

Besides, CSCs display their ability of cell dormancy, in which way they keep quiescent and survive from diverse therapy. In this case, dormant CSC turned to proliferate again in one time after the clinical termination of radiotherapy or chemotherapy. What's more, several other cell types within stromal, such as endothelial cells, cancer-associated macrophage, immune inflammatory cells, also participate in the complex network to regulate the proliferation, migration and invasion of cancer cells (Mao et al., 2013).

1.2 The pancreas

The pancreas is comprised of the exocrine pancreas and the endocrine pancreas. The exocrine pancreas mainly regulates digestion while endocrine pancreas is responsible for glucose metabolism. Acinar cells locate in the exocrine pancreas and constitute the most part of the pancreatic tissue. Acinar cells are organized as acini structure which consists of several hundreds of acinar cells. Adjacent acinar cells are linked by numerous gap junctions that allow intercellular communication. Acini is the smallest termini of the branching duct system. It secretes a variety of enzyme mixture, together with mucous and bicarbonate produced by duct cells, into the main pancreas duct and then empties into the duodenum.

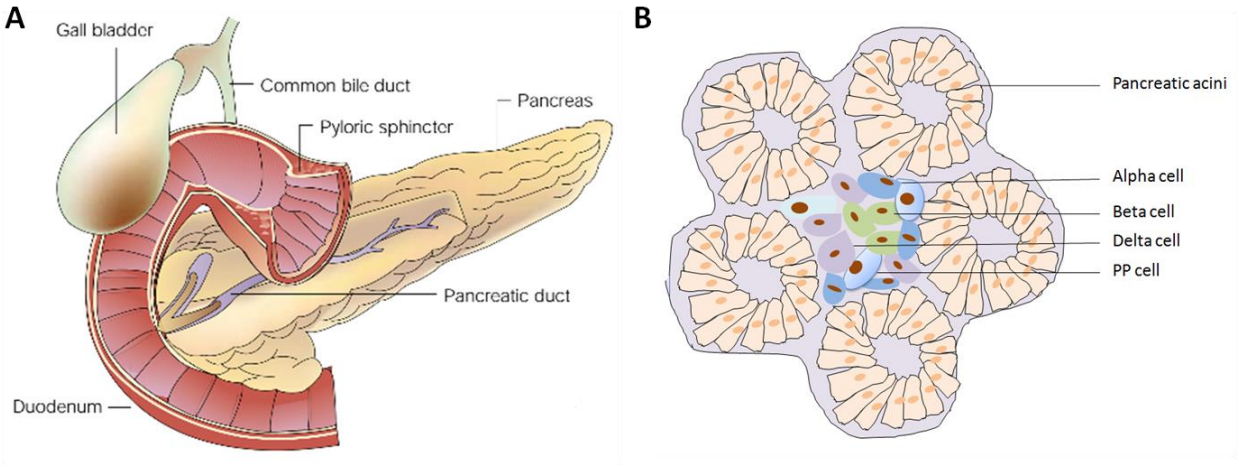

Figure 4. Anatomy of pancreas (Bardeesy & DePinho, 2002). (a) Gross anatomy of the pancreas. (b) The exocrine pancreas. Adjusted from (Bardeesy & DePinho, 2002)

Another function of the pancreas is the regulation of blood glucose level, which is regulated by endocrine pancreas. There are totally four cell types (A-, B-, PP and δ-cells) which are arranged within the islets in the endocrine pancreas. Specialized hormones generated by these cells are secreted into the bloodstream. For example, A- and B-cells regulate the glucose level by the production of glucagon and insulin, respectively. While PP cell and δ-cells can produce pancreatic polypeptide and somatostatin to modulate the secretory properties of the other pancreatic cell types (Bardeesy & DePinho, 2002). Normal function of the pancreas is crucial for the body to maintain the homeostasis of metabolism.

1.3 Pancreatic Duct Adenocarcinoma (PDAC)

PDAC is the fourth most common cause of cancer-related death in western countries, with a quite low 5 years survival rate. PDAC frequently eludes detection at its early stage due to diagnostic

limitations and the lack of specific symptoms in the early stage of the disease. Up to date, surgery still serves as the main treatment for PDAC patients who are under potentially curative resection. Despite, adjuvant chemotherapy and radiotherapy are emerging as potential options that could improve the survival in PDAC patients (Kamisawa, Wood, Itoi, & Takaori). Gemcitabine monotherapy or the systemic chemotherapy are recommended as the first option for these patients with locally advanced, unresectable PDAC. Chemotherapy strategies like “FOLFIRINOX” or “gemcitabine plus albumin-bound paclitaxel” represent the main strategy for the treatment of metastatic PDAC (M. A. Tempero et al., 2010). However, no remarkable progress, despite the toxicity of therapy, are achieved in terms of long-term survivals due to the intensive resistance of PDAC to a variety of therapy strategy.

1.3.1 Epidemiology and Risk Factors of PDAC

PDAC is diagnosed mainly in the elderly population with the median age of 71 years. In western countries, PDAC is the fourth most common cause of death from cancer both in men (after lung, prostate and colorectal cancer) and women (after lung, breast and colorectal cancer) (M. Tempero, Malafa, & Hawary, 2016). In 2015, nearly 367,000 new cases of pancreatic cancer were diagnosed worldwide. In the United States, black individuals are found to suffer from the disease more often (12–15 cases per 100,000 men and 8–10 cases per 100,000 women) than their white counterparts. A higher incidence rate was recorded in high-income countries, so is that of urban populations higher than rural populations. The lowest rates were recorded in India, Northern and Central Africa (<2 cases per 100,000 men and 1 case per 100,000), which is probably due to the quality of diagnosis despite the difference of involved risk factors). Recent statistics and prediction model show the incidence will continue to climb in the United Kingdom and in North America (M. Tempero et al., 2016).

The survival rate correlates closely with the tumor stages at the time of diagnosis. Specifically, the 5-year survival rate of stage IA pancreatic cancer is about 14% and stage IB cancer is about 12%. While 5-year survival rate of stage IIB pancreatic cancer reaches to only 5%, compared with the rate of stage IIA cancer 7%. Stage III and stage IV PDAC patients has a 5-year survival rate of about 1%-3%. ("Pancreatic Cancer Survival Rates, by Stage," 2016.3.14)

Variable	**Approximate Risk**
Risk factor	
Smoking	2–3
Long-standing diabetes mellitus4	2
Nonhereditary and chronic pancreatitis	2–6
Obesity, inactivity, or both	2
Non–O blood group	1–2
Genetic syndrome and associated gene or genes - %	
Hereditary pancreatitis (*PRSS1*, *SPINK1*)	50
Familial atypical multiple mole and melanoma syndrome (CDKN2A/*p16*)	10–20

	Hereditary breast and ovarian cancer syndromes (*BRCA1, BRCA2, PALB2*)	1–2
	Peutz–Jeghers syndrome (*STK11*/*LKB1*)	30–40
	Hereditary nonpolyposis colon cancer (Lynch syndrome) (*MLH1, MSH2, MSH6*)	4
	Ataxia–telangiectasia; Familial pancreatic cancer (monoallelic) (*ATM*)	Unknown
	Li–Fraumeni syndrome (P53)	Unknown

Table 1. Risk Factors and Inherited Syndromes Associated with Pancreatic Cancer (Kamisawa et al.).

There is a variety of risk factors that associate with PDAC incidence (Table 1). Although the definitely genetic basis of the disease heredity is unknown, almost 5% to 10% of PDAC origin from the family with a pancreatic cancer history. The patients' relative risks of developing pancreatic cancer increase by factor 2, 6 and 30 with one, two and three family members surfed from pancreatic cancer compared with general population, respectively. Besides, general oncogenic factors like smoke, obesity, diabetes and chronic pancreatitis also increase the risk of PDAC (Kamisawa et al.).

1.3.2 Genetic mutations in PDAC

PDAC develops from pancreatic intraepithelial neoplasias (PanINs) and subsequent progressive stages of neoplastic growth. The most frequently altered genes in PDAC include KRAS, CDKN2A, TP53 and SMAD4.

Mutation of KRAS gene is usually the first genetic change that occurs in the mouse model of PDAC, detected in about 30% of early lesions. In PDAC patients, KRAS was found mutated in more than 90% cases (M. A. Tempero et al., 2010). Activated mutations of RAS-family oncogenes result in remarkable changes of cellular effects, including the induction of abnormal proliferation, survival and invasion through the stimulation of several effectors pathways such as epidermal growth factor (EGF) family (Ardito et al., 2012).

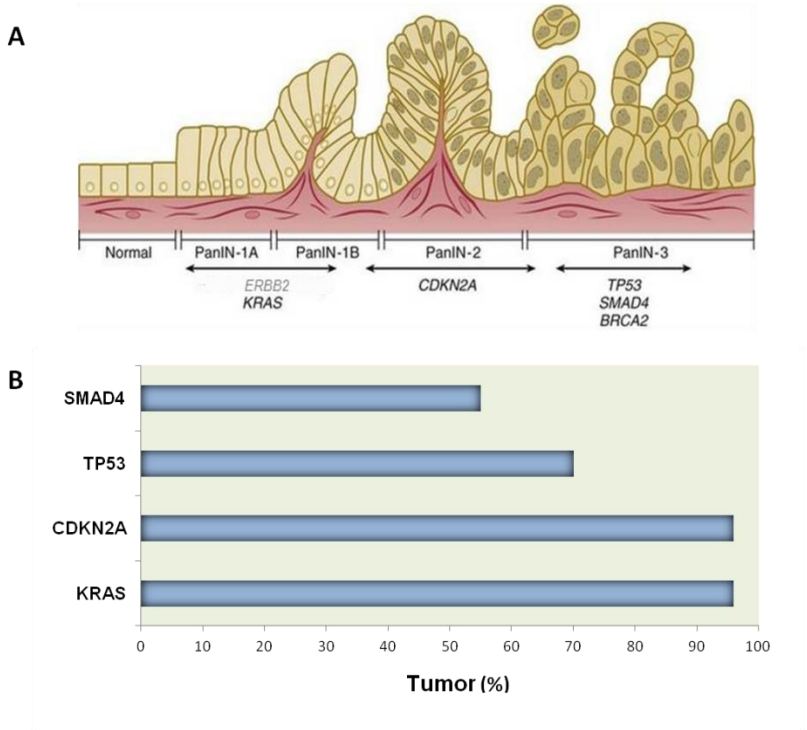

Figure 5. Genetic alterations of PanIN progression model and frequencies of mutations in DPAC patients (Kamisawa et al.).

The mutation of CDKN2A, an important tumor suppressor gene, is detected in 80–95% PDAC patients. Loss of CDKN2A gene occurs frequently in moderately advanced lesions with features of dysplasia. The mutation of CDKN2A also exists in patients diagnosed with the familial atypical mole-malignant melanoma (FAMMM) syndrome, who have a 13-fold increased risk to develop pancreatic cancer (Ardito et al., 2012).

The TP53 tumor-suppressor gene is also frequently mutated in more than 65% of PDAC, usually in the way of missense alterations of the DNA binding domain. TP53 mutations arise in later-stage PanINs which has already acquired significant features of dysplasia with mutations of KRAS and CDKN2A. This mutation facilitates the genetic instability which leads to consistent genomic re-arrangements which are characterized by frequent aneuploidy, cytogenetic re-arrangements and intratumoral heterogeneity.

DPC4/SMAD4 mutation serves as another key contributor for the development of PDAC. SMAD4 gene is inactivated in nearly 55% of PDAC, usually in the way of homozygous deletion and intragenic mutation. This gene encodes a protein that plays crucial roles in the transforming growth factor-beta (TGF-B)–mediated growth inhibitory pathway (Makohon-Moore & Iacobuzio-Donahue, 2016).

1.3.3 The microenvironment of PDAC

The microenvironment of PDAC is characterized by an abundant and compact collagenous stroma, resulting in an elevated interstitial fluid pressure and considerable hypoxic environment for cancer cells. PDAC stroma is mainly composed of extracellular matrix (ECM) proteins — collagens, fibronectin, laminin and non-collagenous proteins such as glycoproteins, proteoglycans

and glycosaminoglycans (Gore & Korc, 2014). The over-expression and deposition of these proteins in the interstitial results in high-level internal pressures which can reach 100 mmHg by the creation of a large gel-fluid phase. This unique microenvironment results in the robust fibro-inflammatory response, widespread vascular collapse and hypoperfusion, which lead to the primary resistance of chemotherapy.

The ECM in PDAC is mainly produced by activated pancreatic stellate cells (PSCs), which have also been termed as cancer-associated fibroblasts (CAFs). PSCs are crucial for the remodeling of PDAC microenvironment. They can interact with cancer cells, as well as other stromal cells like immune cell and endothelial cells, facilitating the progression of PDAC. PSCs have been initially identified to inhibit cancer cell apoptosis and promote cell survival and resistance to chemotherapy by facilitating the niche formation of CSCs. Other cellular components in the stroma comprise of infiltrating immune cells, endothelial cells and neuronal cells. The fact that CD4+ regulatory T cells are infiltrated in the stroma suggests that the PDAC microenvironment is immune suppressive. This feature has generated the therapeutic attempt to target factors that mediate the immune suppression, such as PD-L1 (Hamanishi et al., 2016).

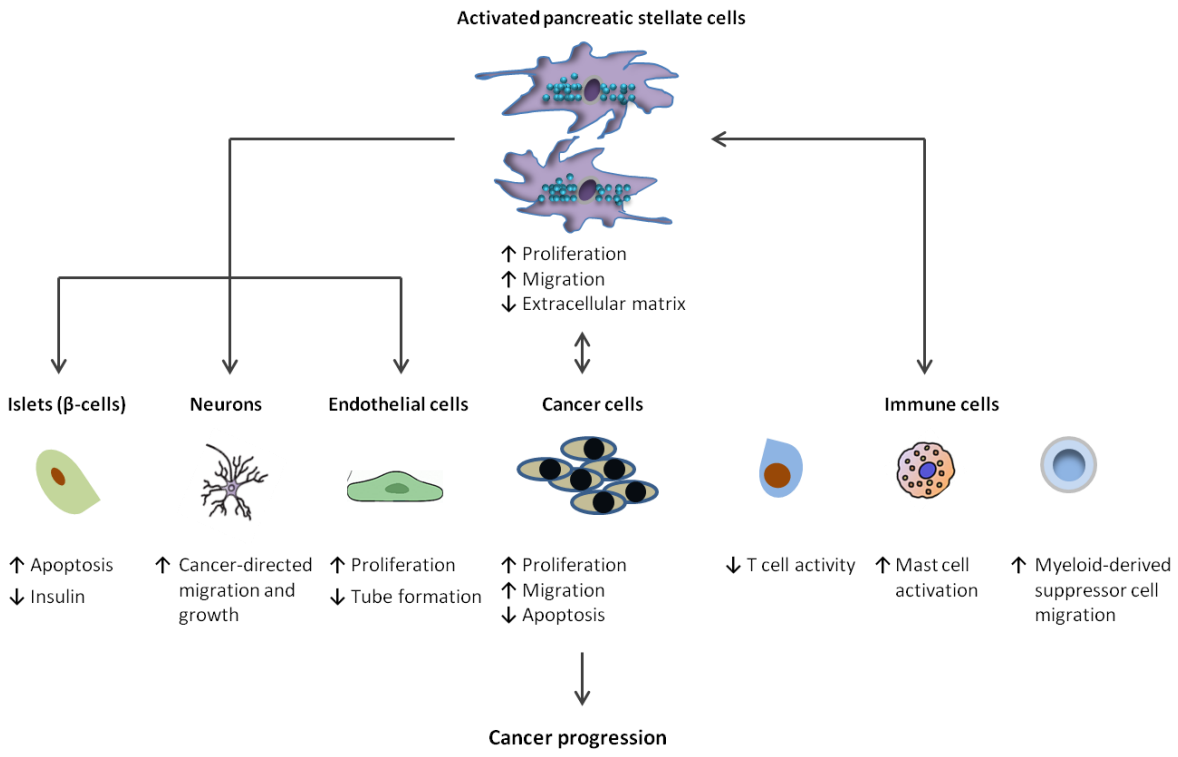

Figure 6. Activated pancreatic stele cells in PDAC. Adjusted from (Jorg Kleeff, 2016)

Up to date, it is still controversial whether and how the stroma participates in cancer progression. Contradictory results from *in vitro* experiment and in vivo trail make it more complicated (Gore & Korc, 2014).

1.3.4 Aberrant signal pathways in PDAC

In PDAC, a variety of alterations in signaling pathways is reported in the past decades. In 2008, Jones *et al* and his colleges have systemically investigated the genome and whole transcripts in 28 PDAC patients. They found that each of these cancers contains an average of 63 genetic alterations, which can be further grouped and categorized into 7 most important cellular signaling pathways and processes. These pathways are involved in modulating the hallmarks of cancer cells

as well as remodeling the stroma (Table 2) (Jones et al., 2008). For example, over-expression of multiple ligands in the tumor context can bind to EGFR, HGFR, HER2 or HER3 and result in elevated activation of RAS protein (Eser et al., 2014). Moreover, excessive activation of oncogenic RAS protein leads to sustained activation of downstream signaling pathways, such as mitogen-activated protein kinase (MAPK) signaling, signal transducer and activator of transcription 3 (STAT3), phosphatidylinositol 3-kinase (PI3K) and AKT signaling (Eser et al., 2014). Activated MAPK serves as a nuclear transcriptional factor that can induce, among others, the hypoxia-inducible transcription factor 1A (HIF1A), which is regularly induced in respond to hypoxia in pancreatic cancer. HIF1A can, in turn, induce the expression of vascular endothelial growth factor A (VEGFA) which can promote angiogenesis and glucose metabolism. Similarly, TGFB binds with II TGFB receptor (TGFBRII) homodimer to activate type I TGFB receptor (TGFBRI) homodimer. This leads to the subsequent phosphorylation of nuclear transcriptional factors SMAD2, SMAD3 and SMAD4 (Ikushima & Miyazono, 2010). Besides, mutation of CDKN2A results in enhanced mitogenic activities by increasing cyclin D1, CDK4 and CDK6 activity. Moreover, loss of RB1 protein communicates with TGFB to activate non-canonical signaling pathways including MAPK, PI3K and WNT7B signaling (Ikushima & Miyazono, 2010).

Regulatory pathway genetically altered	Number of genetically altered genes detected	Fraction of tumors with genetic alteration of at least one of the genes	Representative altered genes
Hedgehog signaling	19	100%	TBX5, SOX3, LRP2, GLI1, GLI3, BOC, BMPR2, CREBBP
Integrin signaling	24	67%	ITGA4, ITGA9, ITGA11,

			LAMA1, LAMA4, LAMA5, FN1, ILK
C-Jun N-terminal kinase signaling	9	96%	MAP4K3, TNF, ATF2, NFATC3
KRAS signaling	5	100%	KRAS, MAP2K4, RASGRP3
Small GTPase–dependent signaling (other than KRAS)	33	79%	AGHGEF7, ARHGEF9, CDC42BPA, DEPDC2, PLCB3, PLCB4, RP1, PLXNB1, PRKCG
TGF-b signaling	37	100%	TGFBR2, BMPR2, SMAD4, SMAD3
Wnt/Notch signaling	29	100%	MYC, PPP2R3A, WNT9A, MAP2, TSC2, GATA6, TCF4

Table.2 Core signaling pathways and processes genetically altered in the majority of pancreatic cancers. (Jones et al., 2008)

1.3.5 Subtypes of PDAC defined by gene mutation and global gene profiles

Emerging genomic technologies have been applied to analyze whole gene profiles in either PDAC tissues or cells. Genomic investigation of global gene mutations and expression provides new insight to understand the cancer genetics and biology. Genomic analysis of PDAC could also

serve as the basis of precision medicine in the future.

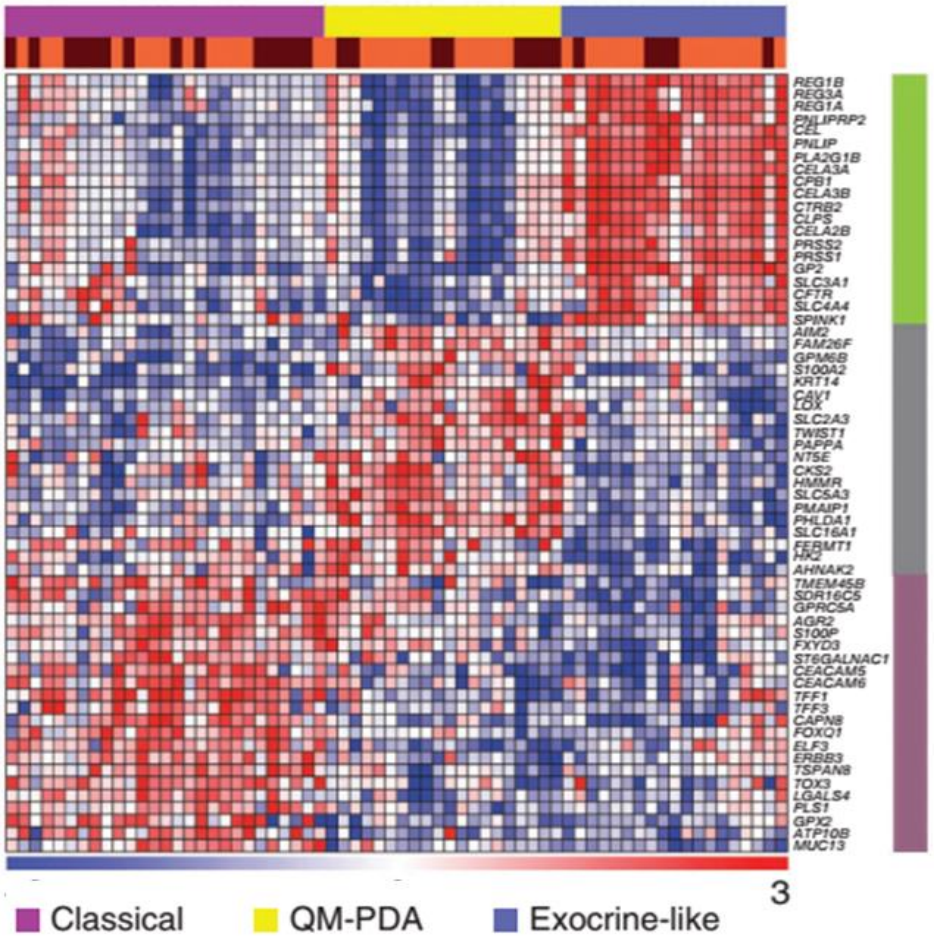

Figure 7. Three PDAC subtypes generated by microarray data sets using the PDAssigner gene set (Collisson et al., 2011).

In 2011, Collison *et al* have integrated the analysis of transcriptional profiles of primary PDAC specimens along with human and mouse PDAC cell lines. They defined three PDAC molecular subtypes with gene signatures: classical, quasi-mesenchymal, exocrine-like, with the therapeutic response and clinical outcome varying quite different (See Figure 7) (Collisson et al.,

2011).

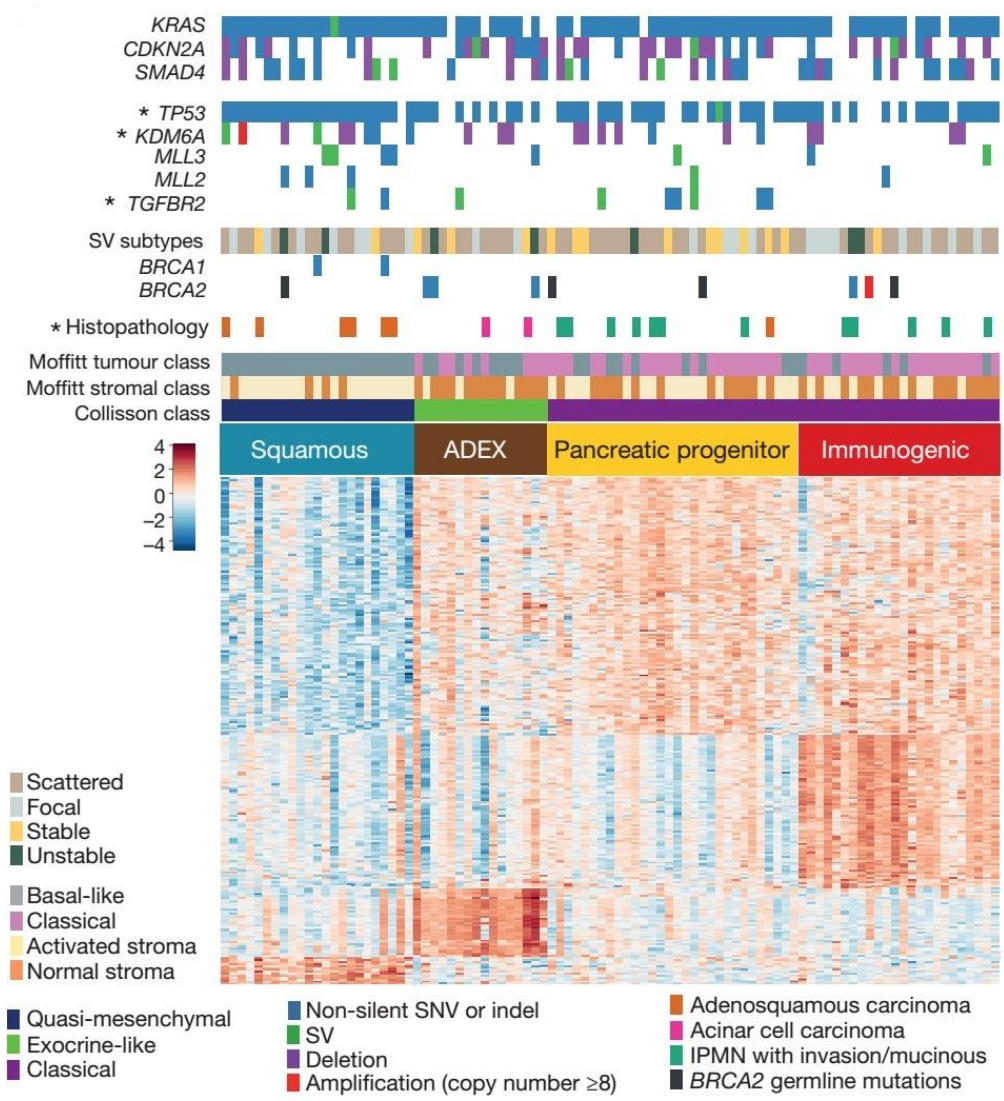

Figure 8. Molecular subtypes of pancreatic cancer defined by Grimmond *et al* (Bailey et al., 2016). RNA-seq of pancreatic cancer identified 4 PDAC classes: squamous (blue); ADEX (abnormally differentiated endocrine-exocrine; brown); pancreatic progenitor (yellow); and immunogenic (red).

Another group has also perform integrated genomic analysis of PDAC tissues from 456 PDAC patients (Bailey et al., 2016). They categorized 32 frequently mutated genes into 10 tumorous pathways: KRAS, TGF-B, WNT, NOTCH, G1/S transition, chromatin modification, SWI-SNF, ROBO/SLIT signaling, DNA repair and RNA processing. Based on these aberration signal pathways, four subtypes were subsequently identified: (1) Squamous; (2) Pancreatic progenitor; (3) Immunogenic; and (4) Aberrantly differentiated endocrine exocrine (ADEX) (Figure 8) (Bailey et al., 2016). Each subtype of PDAC patients was suggested with different treatment strategies. Moreover, the PDAC with different molecular subtypes could response differently to therapy.

1.4 Retinoic acid and retinoid receptors

1.4.1 The biology of retinoic acid and retinoid receptors

Retinoic acid, the metabolite of vitamin A, is important for growth and differentiation of both healthy and cancerous cells. All-trans retinoic acid (ATRA), the major physiologically active form of vitamin A, regulate the expression of many genes. Recently, retinoic acid and its derivatives are reported that they are able to inhibit proliferation and migration of various cancer types including PDAC (Garattini, Bolis, Fratelli, Paroni, & Terao, 2016; Guan et al., 2014; Kalitin & Karamysheva, 2016; Y. Zhang et al., 2013). Disturbances of vitamin A metabolism are prevalent in various cancer types. Previous studies showed that concentration of ATRA and ATROL are reduced in PDAC cancer tissues compared to normal pancreatic tissue (Bleul et al., 2015).

Physiological effects of retinoid are mainly mediated by two families of nuclear receptors: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). RAR and RXR are bothe ligand-dependent transcription factors. They belong to the nuclear hormone receptor superfamily. RAR and RXR family comprise several isotypes: RARA, RARB, RARγ and RXRA RXRB, RXRγ.

RARA, RXRA and RXRB are found ubiquitously expressed in human, while the others have tissue-specific expressions. The A-, B- and γ-isotype of RAR can be activated by all-trans RA and 9-cis RA, while RXR-A,-B and -γ are mainly activated by 9-cis RA. Activated nuclear receptors are able to form the hetero- or homodimer (RAR/RAR and RAR/RXR). RXR can also dimerize with other receptors like the vitamin D3-receptor (VDR), thyroid hormone receptor (TR), peroxisome proliferator-activator receptor (PPAR) and orphan receptors. However, retinoid receptors regulate gene transcription mainly, under physiological conditions, by the formation of RAR/RXR transcriptional complexes (Amann, Eichmuller, Schmidt, & Bazhin, 2011; Evans & Mangelsdorf, 2014; Urvalek, Laursen, & Gudas, 2014).

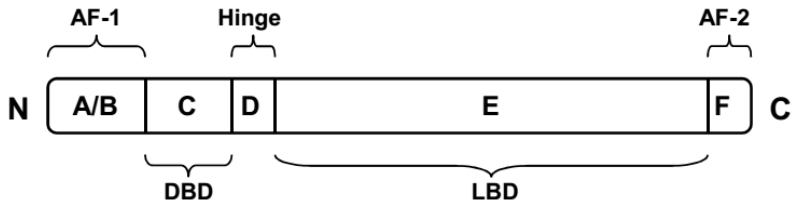

Figure 9. The schematic functional domain of the retinoid receptor (Amann et al., 2011). Retinoic acid receptors consist of six domains, termed A-F, with independent functions. AF-1 contains several phosphorylation consensus sites for cyclin-dependent- and MAP kinases. The C domain is highly conserved and functions as the DNA-binding domain. The D domain promotes the steric conformation of the DNA-binding domain and interacts with transcriptional co-regulator. E domain is the ligand binding domain.

1.4.2 Retinoic acid response elements

Retinoic acid response element (RARE) is the specific sequence within many promoters. Both RAR-RXR heterodimers and individual retinoid receptor can recognize and bind to the RARE sequence. In addition, direct repeats (DR) of RARE with a space of two to five nucleotides (DR2-

DR5) are also involved in the binding with homodimers of RXR. The dimerization partners of RXR can be RXR itself (for DR1), RAR (for DR1, DR2 and DR5), VDR (for DR3), PPAR (for DR1) and other receptors. The number of the combinatorial interaction of these receptors can be enlarged by changing their polarity and bind alternatively to the upstream or downstream of the DR. Such polarity reversion is important for switching the activity of the receptor heterodimer from an activator to a repressor of the retinoid-responsive gene (le Maire et al., 2012).

1.4.3 RA signaling

Transcriptional effects of activated retinoid receptors are achieved by following steps: (i) Ligand binding, (ii) Receptors dimerization, (iii) DNA binding, (iv) Recruitments of co-activators and (v) The transcriptional modulation of the gene (Balmer & Blomhoff, 2002).

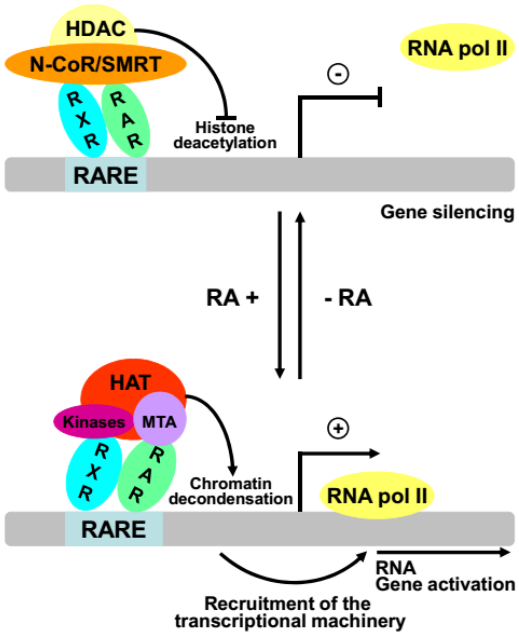

Figure 10. RA signaling. RA binds to RAR–RXR heterodimer to form complex which further bound to RAREs in the target gene, resulting in the control of gene transcription (Amann et al., 2011).

In the absence of RA, the receptor complex assembled by retinoid receptors and co-repressors N-CoR and SMRT can bound to the RARE sequence of target genes and repress the gene expression. However, the complex undergoes conformational changes upon RA binding, leading to the release of co-repressors and meanwhile the recruitment of co-activators to form an activator complex with histone acetyltransferase, methyltransferase (MTA) and kinases (Grosshans & Chatterjee). Co-activators possess a leucine-rich LXXLL motif while co-repressors share an L/IXXI/VI motif. Importantly, some co-activators share an interactive domain with each other, indicating their cooperativeness of gene regulation. Chromatin is decondensed after the co-activator complex binding with the RARE region, thereafter p160 coactivators dissociate and the retinoid receptors complex start to recruit the transcriptional enzyme, including RNAPol II and general transcription factors which in turn increase chromatin remodeling at the promoter regions (Amann et al., 2011).

Retinoid receptors are nuclear receptors functioning as a part of transcription factors which play important role in a variety of cellular events. The diverse complex formed by these receptors, such as heterodimeric complex RAR-RXR, homodimerized RXR-RXR or RXR dimerized with another receptor such as PPAR, functions as a key transcriptional factor in retinoic acid signaling and other related signaling pathways.

Number	Directly Regulation	Indrectly Regulation	Total
Up Regulated	63	248	311
Down Reglated	40	69	109
Variable	30	82	112
Total	133	399	532

Table 3. Up- or down-regulated genes regulated by retinoic acid and retinoid receptor directly or indirectly (Balmer & Blomhoff, 2002).

It is estimated that more than 532 downstream genes can be regulated by retinoic acid and retinoid receptor, either in the direct or the indirect way (see Table 3) (Balmer & Blomhoff, 2002). For example, 133 genes were strongly identified as being regulated directly, of which 63 were up-regulated and 30 was variably regulated. The total number unregulated genes by retinoid and RA was 311. Interestingly, other 221 genes were either down-regulated or variably regulated (Balmer & Blomhoff, 2002). Besides, RA also acts on non-tumorous cells to affect the PDAC cells. RA was reported to induce quiescence and reduces motility of PSCs, and thus inhibiting proliferation and promoting apoptosis of surrounding PDAC cells (Balmer & Blomhoff, 2002).

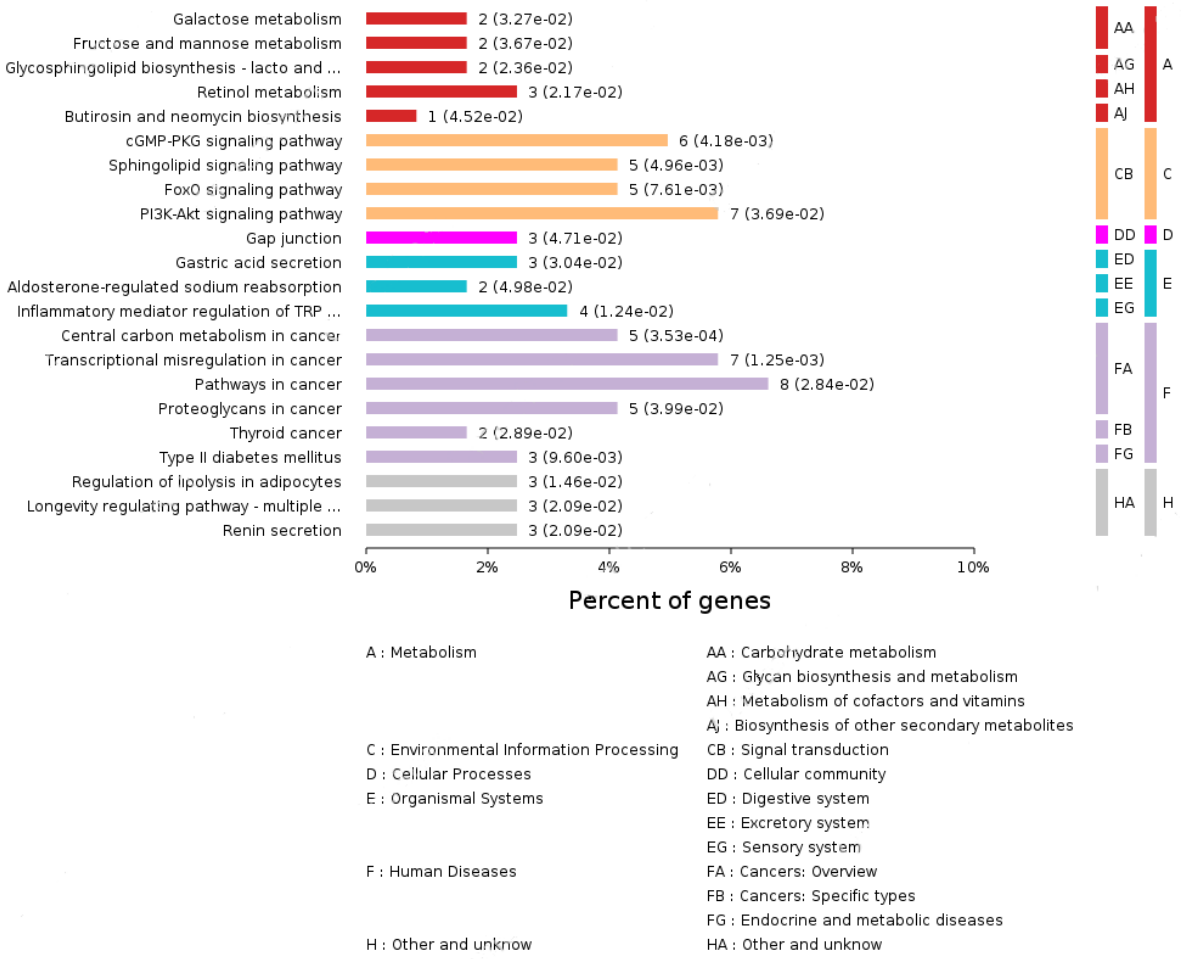

Figure.11 Classification of genes regulated by retinoic acid and retinoid receptors. Differentially genes (>1.5 fold change) regulated by RR and RA were categorized based on known or predicted functions.

Pathway enrichment analysis of genes regulated by retinoid receptors and retinoic acid shows that these genes are widely involved in a variety of cellular events. Responsive genes encoded transcriptional factors involved in biological processes, such as metabolic processes, cell-cell junctions and organismal systems development. In addition, retinoid signaling can affect lots of other signaling pathways like PI3K-Akt signaling pathway, p53 pathway and Wnt pathway (Figure

11) (GSE69850, GSE69844 and GSE71856).

1.4.4 Retinoid receptor and the PDAC

Disturbances of retinoic acid metabolism and dysregulation of retinoid receptors are very common in some types of cancer. We previously investigated the retinoids level in human and murine PDAC tissues using mass spectrometry and real-time RT-PCR analysis (Bleul et al., 2015). Moreover, the survival of PDAC patients was estimated using Kaplan–Meier method and Cox proportional hazards analysis. The analysis showed that the concentration of ATRA and all-trans retinol is significantly reduced in PDAC tissue compared to their normal counterparts in both human and mouse. The expressions of RARA, RARB, RXRA and RXRB are also significantly down-regulated in PDAC tissues. More importantly, the reduced expression of retinoid receptors correlates with the expression of markers of the EMT and stemness of cancer cell. Besides, the expression of RARA and RXRB is found to be associated with a better overall survival in PDAC patients. Accordingly, the reduced level of retinoids and their receptors is an important feature of PDAC (Bleul et al., 2015). However, the mechanism of the down-regulation of retinoid receptors is not well defined.

1.5 microRNAs

1.5.1 The biology of microRNAs

Genes can be regulated on various levels, such as epigenetic regulation (e.g. histone modifications, DNA methylation), transcriptional control (e.g. promoter regulation by transcription factors) or translational control by non-coding RNAs. MiRNAs are small non-coding endogenous

RNAs that are involved in multiple biological processes. These regulatory elements are first transcribed and then processed by Dicer and Drosha complexes into 21–23nt mature miRNAs (van Kouwenhove, Kedde, & Agami, 2011).

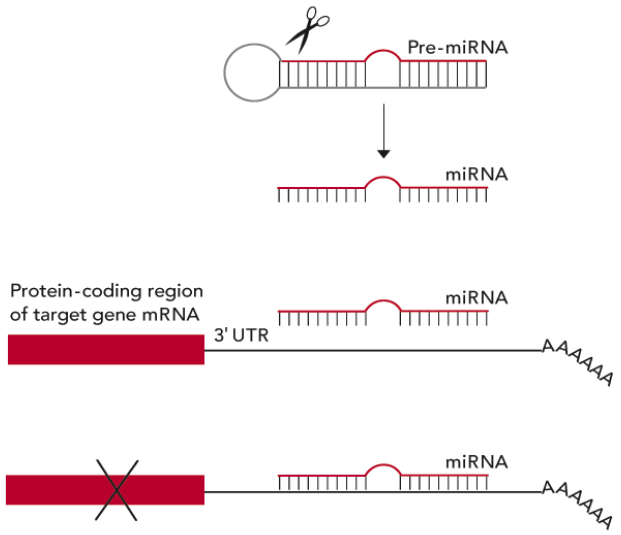

Figure 12. Mechanism of blocking gene expression by mature miRNA. A precursor hairpin-shaped pre-miRNA is sliced to form a mature miRNA, which binds to the 3' untranslated region (3' UTR) of a target gene's mRNA and blocks its translation (Lin He & Hannon, 2004).

The miRNA is incorporated into the RNA-induced silencing complex (RISC), regulating posttranscriptional mRNA expression typically by binding to the 3’ untranslated region (3’-UTR) of the complementary mRNA sequence. The binding of RISC with mRNA is able to induce the cleavage or repression of gene translation (van Kouwenhove et al., 2011). Due to the significance of miRNAs, it has provided a new horizon to better understand the regulation of important oncogene or tumor suppressor gene in cancer.

1.5.2 The complexity of gene regulations by miRNAs

Gene regulation by miRNAs is previously thought to be simple, by binding to the 3'UTR as the canonical pathway. However, non-canonical binding and targeting have also been reported (Helwak, Kudla, Dudnakova, & Tollervey, 2013). For instance, recent CLASH (cross-linking, ligation, and sequencing of hybrids) analysis has revealed that although the majority miRNAs bind to 3'UTR, they can also bound to 5'UTRs, gene bodies, long non-coding RNAs (lncRNAs) as well as introns (Helwak et al., 2013). Moreover, the binding of miRNAs with promoters of genes could result in either gene repression or gene activation. The activation of genes is usually caused by the active histone marks (H3K4 trimethylation), compared with gene repression which is induced by H3K9 dimethylation (Helwak et al., 2013).

Depending on the orientation, two strands of the microRNA generated from pre-miRNAs are termed as 5p and 3p. Studies have shown that both strands can function as mature miRNAs in gene regulation. For example, both 5p and 3p of miR-142 and miR-17 can be incorporated into the RISC complex to regulate the gene expression (Kasashima, Nakamura, & Kozu, 2004; X. Yang et al., 2013). Interestingly, both miR-17-5p and miR-17-3p are able to target the same gene TIMP3 which induce prostate cancer cell growth (X. Yang et al., 2013). However, there are also cases that 5p and 3p produced by the same pre-miRNAs target two different genes. In the colorectal cancer cell, for instance, overexpression of miR-28-5p and miR-28-3p lead to different effects (Almeida et al., 2012). Specifically, miR-28-5p altered the expression of CCND1 and HOXB3, whereas miR-28-3p regulate the expression of NM23-H1. Another interesting point about the complexity of miRNA is that one single miRNA can, based on experimental and computational analysis, target hundreds or potentially thousands of mRNA molecules. So far, it is still hard to predict the regulation effect of miRNAs due to the fact that expression of different targets could be repressed to different degrees by a given miRNA. Hence, it seems impossible to predict the effects of miRNA on gene

expression at the global level. Lastly, due to the fact that cells, organs and human body function as a uniform organism, miRNAs can only target transcripts that were induced in a particular pathological context. Hence, specific genes that could be regulated by miRNAs in one cell type is largely dependent on the whole transcription of that particular cell under given conditions. That is to say, functional target relationship in one cell type does not necessarily prove to be the same effective in a different cell type. For example, Blodin *et al* showed that the function of miR-146a was almost exclusively restricted to the hematopoietic system (Bustin et al., 2013; Zhao et al., 2011). This is vital for the prediction of the target and effect of miRNA manipulation across different cell types and tissue types. Hence, miRNA and mRNA interaction studies should be interpreted according to the specific cell type and the origin of tissue.

1.6 Potential miRNAs target of retinoid receptors

Using online database, our preliminary analysis of the miRNA expression signature of PDAC has revealed some novel miRNA – candidates. We found that these miRNAs are potentially upregulated in PDAC tissue, suggesting that these miRNAs are candidate onco-miRNAs. Using in silico analysis, we identified several dysregulated miRNAs that could potentially target retinoid acid signal pathway. These miRNAs, namely miR-138, miR-206, miR-613, miR-9, miR-27a/b, miR-10a/b, are predicted to target at least one of these retinoid receptors (RARA, RARB, RXRA and RXRB).

2. Specific Aims of the Thesis

The present study aims to identify novel oncogenic-suppressive miRNAs as the regulator of retinoid receptors in PDAC. Towards this end, the study will be conducted with the following specific objectives:

(1) To investigate the expression of retinoid receptors and miRNAs (miR-138, miR-206, miR-613, miR-9, miR-27a/b, miR-10a/b) in PDAC cells and HPDE cell *in vitro*.

(2) To investigate the expression of expression of retinoid receptors after the manipulation of the expression of these miRNAs and retinoid receptors.

(3) To identify the relevance of target miRNAs in migration and invasion of PDAC cell if any of these retinoid receptors are targeted by selected miRNAs.

3. Materials and Methods

3.1 Materials

3.1.1 Instruments

Instrument	Company
Centrifuge	Eppendorf
CO_2 incubator (37℃)	Thermo Fisher
Water bath	Grant
Light microscope	Olympus
StepOne Real-Time PCR Systems	Applied Biosystems
Pipetboy	Gilson
Pipettes	Eppendorf
Sterile work bench	Hera Safe Thermo Electron
Trans Blot Semi-Dry Electrophoretic transfer Cell	Bio-Rad laboratories
Vortexer	VWR analog Vortex mixer
Pipette holder	Accu-jet® Pro
Odyssey system	LI-COR

3.1.2 Disposables

Name	Company
24- well plate	Corning
96- well plate	Corning
6-well plate	Corning
Barrier sterile pipette tips	Avant Guard
Extra thick Blotting paper	Bio-Rad
Falcon tubes (15ml, 50ml)	BD
Cell culture dishes (10cm2)	CellStar ® Grenier Bio-One
Plastic pipettes (2, 5, 10, 25ml)	CellStar ® Grenier Bio-One
PVDF membrane (0.45um)	Millipore
Pipette tips	TipOne (STARLAB)
Cell culture flask	BD
MicroAmp Optical 96-Well Reaction Plate	Thermo Fisher

3.1.3 Chemicals and Reagents

Name	Company
Agarose	Gibco BRL
Ampicillin	Sigma
Chloroform	Sigma
Isopropyl alcohol	Sigma
Ethanol	Sigma
0.4% Trypan blue solution	Sigma
Dimethyl Sulfoxide (DMSO)	Sigma
Protein Ladder (1Kb)	BioRad
Ethylenediaminetetraacetic acid (EDTA)	Sigma
TEMED	Sigma
Hydrogen chloride (HCl)	VWR BDH Prolabo
Isopropanol	Merck
Methanol	Sigma Aldrich
Protease inhibitor tablets	Roche
Odyssey® Blocking Buffer	LI-COR
Tween 20	Roth

Sodium dodecyl sulfate (SDS)	SERVA
Ammonium Persulfate	Sigma

3.1.4 Kits

Kits	Company
HiperFect transfection kit	Qiagen
RNeasy Mini Kit	Qiagen
miScript II RT Kit	Qiagen
miScript SYBR Green PCR Kit	Qiagen
QuantiTect Reverse Transcription Kit	Qiagen
QuantiTect SYBR Green PCR Kits	Qiagen
QuantiTect Primer Assays	Qiagen
miScript Primer Assays	Qiagen
QIAzol Lysis Reagent	Qiagen
BCA Protein Assay Reagents	Peirce

3.1.5 Cell lines

Cell lines used in the study are listed in the following table:

Cell line	Origin	Source	Culturing medium
MiaPaCa-2	Pancreatic adenocarcinoma	ATCC	PRMI+10%FBS
DanG	Pancreatic adenocarcinoma	ATCC	PRMI+10%FBS
Panc-1	Pancreatic adenocarcinoma	ATCC	PRMI+10%FBS
BxPc-3	Pancreatic adenocarcinoma	ATCC	PRMI+10%FBS
HPDE	Normal human pancreatic duct epithelial	Gift, Dr. Kong Bo (TUM, Germany)	Keratinocyte Basal Medium + supplied supplements

3.1.6 Antibodies

Antibody	Company
Anti - MET antibody	Sigma
Anti-RARA antibody	Santcruz
Anti-RARB antibody	Santcruz
Anti-RXRA antibody	Santcruz
Anti-RXRB antibody	Santcruz
Anti-RARA antibody	CST

Anti-RARB antibody	CST
Anti-RXRA antibody	CST
Anti-RXRB antibody	CST
IRDye® 680LT Goat anti-Mouse	LI-COR
IRDye® 800CW Donkey anti-Rabbit	LI-COR
IRDye® 680CW Goat anti-Rabbit	LI-COR
IRDye® 800CW Donkey anti-Mouse	LI-COR
Protein ladder	Bio-Rad
Anti-c-met antibody	CST

3.1.7 Medium and solutions

DMEM, high glucose	Gibco BRL
Keratinocyte culture Medium	Gibco
Fetal Bovine Serum (FBS)	Gibco BRL
L-Glutamine	Sigma
Phosphate Buffered Saline (PBS)	Gibco
PRMI 1640 Medium	Gibco BRL

Trypsin-EDTA solution 0.25%	Sigma
RIPA buffer	Sigma

3.1.8 Buffers

Sample Buffer	58mM Tris, pH 6.8 1.67% SDS 5% Glycerol 0.00025% Bromophenol Blue
Separating Gel buffer	30% acrylamide mix 1.5 M Tris-HCl, pH 8.8 10% SDS 10% APS
Stacking gel buffer	30% acrylamide mix 0.5 M Tris-HCl, pH 6.8 10% SDS 10% APS
SDS-PAGE running buffer	25mM Tris 250mM glycine, pH 8.3

	0.1% SDS
Transfer buffer	25mM Tris base 192mM glycine 0.01% SDS 20% Methanol
Tris-acetate-EDTA (TAE) buffer	40mM Tris 20mM acetic acid 1mM EDTA
Washing buffer (PBS-T)	1000ml PBS 0.1% Tween 20
Blocking buffer	Alternative 1. Odyssey Blocking Buffer 2. Washing buffer + 5% milk powder

3.1.9 miRNAs and Primers

All miRNAs products are purchased from Qiagen

miRNA mimic or inhibitors	***Sequence***
Syn-hsa-miR-9-5p miScript miRNA Mimic	5'UCUUUGGUUAUCUAGCUGUAUGA
Syn-hsa-miR-27a-3p miScript miRNA Mimic	5'UUCACAGUGGCUAAGUUCCGC

Syn-hsa-miR-27a-5p miScript miRNA Mimic	5'AGGGCUUAGCUGCUUGUGAGCA
Syn-hsa-miR-27b-3p miScript miRNA Mimic	5'UUCACAGUGGCUAAGUUCUGC
Syn-hsa-miR-27b-5p miScript miRNA Mimic	5'AGAGCUUAGCUGAUUGGUGAAC
Anti-hsa-miR-9-5p miScript miRNA Inhibitor	5'UCUUUGGUUAUCUAGCUGUAUGA
Anti-hsa-miR-27a-3p miScript miRNA Inhibitor	5'UUCACAGUGGCUAAGUUCCGC
Anti-hsa-miR-27a-5p miScript miRNA Inhibitor	5'AGGGCUUAGCUGCUUGUGAGCA
Anti-hsa-miR-27b-3p miScript miRNA Inhibitor	5'UUCACAGUGGCUAAGUUCUGC
Anti-hsa-miR-27b-5p miScript miRNA Inhibitor	5'AGAGCUUAGCUGAUUGGUGAAC
miRNA negative control Oligos	
Hs_miR-9_1 miScript Primer Assay	
Hs_miR-27a_1 miScript Primer Assay	
Hs_miR-27b_2 miScript Primer Assay	
Hs_miR-138_1 miScript Primer Assay	
Hs_miR-206_1 miScript Primer Assay	
Hs_miR-613_1 miScript Primer Assay	
Hs_RARA_1_SG QuantiTect Primer Assay	

(QT00095865)

Hs_RARB_1_SG QuantiTect Primer Assay
(QT00062741)

Hs_RARG_1_SG QuantiTect Primer Assay
(QT00000987)

Hs_RXRA_1_SG QuantiTect Primer Assay
(QT00005726)

Hs_RXRB_1_SG QuantiTect Primer Assay
(QT00061117)

Hs_RXRG_1_SG QuantiTect Primer Assay
(QT00007238)

3.2 Methods

3.2.1 PDAC cell culture

PDAC cell lines were maintained as a monolayer. Prior to cell culture, trypsin and media were warmed up in a 37℃ water bath. Cells were cultured in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cell was incubated at 37℃ in a humidified atmosphere of 5% CO_2/95% air. Cells were passaged as the growth reach the confluence of 80% - approximately 1 or 2 times per week. Specifically, the medium was removed first. Cells were then washed with PBS and treated with trypsin. The same amount of complete medium was added to stop the digestion. Cells suspension were pelleted with a 200 × g centrifugation for 5–10 minutes and the supernatant was discarded. Then additional

complete growth medium was added to the flask and 10% of the suspension was transferred into each new flask. The flask was then supplemented with appropriate medium to make a 10-15ml total volume.

3.2.2 HPDE cell culture

Immortalized Human Pancreatic Duct Epithelial cells (HPDE) was a kind gift from Dr. Bo Kong in Technical University of Munich (who get the cell from Prof. M.S. Tsao in Ontario Cancer Institute (Toronto, Canada)). HPDE cell line was maintained in Keratinocyte Basal Medium supplied with additional supplements (Lonza, Clonetics KBM, Cat#CC-3111). The flask was incubated at 37℃ in a humidified atmosphere of 5% CO_2/95% air. Culture medium was replaced every two to three days following the general procedure of cell culture. Cells were passaged as the growth reach the confluence of 80%, following the same procedure applied in PDAC cell culture.

3.2.3 Freezing and thawing cells

For the freezing procedure, cells were first trypsinized and centrifuged at 200 × g for 5 minutes and the growth medium was then discarded. The cells were then resuspended in freezing medium (90% FBS and 10% DMSO) and distributed into sterile vials for cell freezing. Vials containing the cells were immediately placed on ice, then allowed to freeze at -20℃ for 3 hours and then at -80℃ overnight. Vials were then transferred to liquid nitrogen for long-term storage.

For the thawing procedure, working steps should be done quickly to ensure a high proportion of the survival rate of the cells from the frozen state. Specifically, the frozen vial was immediately placed into a 37°C water bath. Cells inside the vials were then quickly thawed (< 1 minute) by gently swirling the vial in the 37°C water bath. Then the vial was transferred to a 15ml falcon tube

in a laminar flow hood. Then 10 ml pre-warmed complete growth medium was transferred into the falcon tube dropwise. After a centrifugation of the cell suspension at approximately 200 × g for 5–10 minutes, the supernatant was carefully removed without disturbing the cell pellet. Cells were then gently resuspended in an approximate volume of complete growth medium and transferred into the cell culture flask. Cells were cultured in the incubator under normal conditions as mentioned before.

3.2.4 Cell counting

Cells were counted using a hemocytometer. Briefly, equal volumes of single cell suspension and 0.4% trypan blue solution (with the volume ratio 1:1) were mixed together in a 1.5 ml tube. Trypan blue is a dye which stains only dead cells, thus live cells were unstained and visualized. The chamber of the hemocytometer was filled with the stained cell suspension by capillary action.

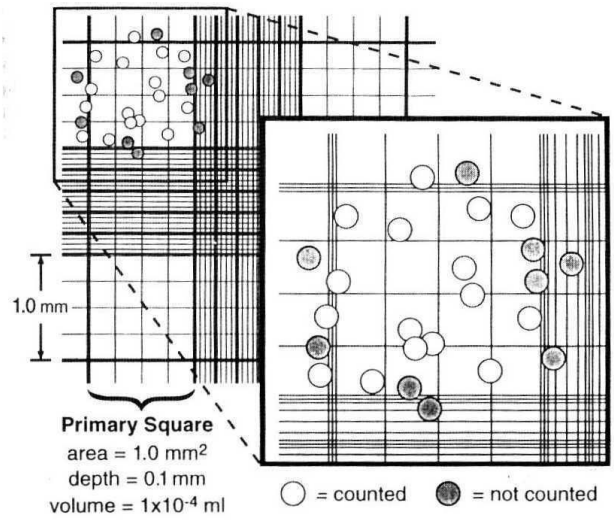

Figure 13. Cells counting with hemocytometer (Neubauer). Cells stained with trypan were loaded onto the hemocytometer and the total number of cells in the four big squares (shown with blue numbers) was counted. The number of cells/ml of cell suspension is then calculated as described.

The total number of cells in the four marked squares (Figure 13) was counted using a microscope. Each chamber of the hemocytometer is divided into nine 1.0mm squares. The chamber was then covered with a cover glass. So that the total volume over each square is 0.0001ml (0.1cm × 0.1cm × 0.1cm). Since 1cm^3 is equivalent to 1mL, the cell concentration per ml will be the average count per square × 2 (dilution factor) × 10^4.

3.2.5 The isolation of microRNAs from cultured cells

Total miRNA from cells was isolated using QIAzol (Qiagen) following the manufacturer's instruction (miRNeasy Mini kit). In brief, cells were trypsinized under 37℃ and collected and centrifuged at 300 × g for 5 minutes. The cell pellet was then washed with cold 1× PBS 3 times. Cells were then pelleted again in a 1.5ml Eppendorf tube. By adding 700 µl QIAzol lysis reagent, cells were lysed after 5 minutes' incubation at room temperature. Then 140µl chloroform was added and the tube was shaken vigorously for 15 seconds. The tube was centrifuged at 8000 × g at 4°C 15 min after 3 minutes' incubation. The upper aqueous phase which contains miRNA was transferred into a new collection tube. Then 1.5 volumes 100% ethanol was added and well mixed, which was later on put into an RNeasy Mini spin column in a 2 ml collection tube for centrifugation at 8000 × g (RT, 1min). The RNeasy Mini spin column which contains miRNAs was then washed with 700 µl RWT Buffer (included in the Qiagen kit) once and 500 µl RPE Buffer (included in the Qiagen kit) twice. 30-50µl RNase-free water in directly onto the RNeasy Mini spin column membrane and miRNAs were then obtained by centrifugation at 8000 × g for 2min. RNA was quantified using a spectrophotometer. The quality of isolated miRNA was evaluated by the A260/A280 value.

3.2.6 The isolation of RNA from cultured cells

Total RNA from cells was isolated using RNeasy Mini Kit following the manufacturer's instruction. Briefly, cells were trypsinized under 37℃ and then centrifuged at 300 × g for 5 minutes. After 3 times' wash with 1 × PBS, cells were transferred into a 1.5ml Eppendorf tube and the cell pellet was lysed by adding 600 µl RLT Buffer (included in the Qiagen kit). To homogenize the cell lysis, it was transferred into the QIAshredder spin column placed in a 2 ml collection and centrifuged at 8000 × g for 1min. Thereafter, an equal volume of 70% ethanol was added into the 2ml tube and mixed well. Then 700µl mixture was transferred into the RNeasy spin column placed in a 2 ml collection tube. The RNeasy spin column was centrifuged again at 8000 × g. Total RNA that stayed in the spin column was washed with 700µl RW1 Buffer (included in the Qiagen kit) once and 500µl RPE Buffer twice (included in the Qiagen kit) by centrifugation at 8000 × g for 1 min. The spin column was then put into a new 2 ml collection tube and 30-50µl RNase-free water was directly added to the spin column membrane. The total RNA was finally precipitated by a centrifugation at 8000x g for 1 minute. RNA was quantified using a spectrophotometer. The quality of isolated miRNA was evaluated by the A260/A280 value.

3.2.7 SYBR Green quantitative PCR for miRNAs

3.2.7.1 cDNA synthesis for miRNA RT-PCR

miScript II RT Kit (Qiagen) was used for the generation of cDNA from the miRNA. The reverse transcription reaction was set up by mixing the following setup:

Component	Volume/reaction

5x miScript HiSpec Buffer	4 µl
10 × Nucleics Mix	2 µl
RNase-free water	Variable
miScript Reverse Transcriptase Mix	2 µl
Template RNA	Variable (500ng)
Total volume	20 µl

An equal amount of miRNA (500ng) from the miRNA sample was added into each reaction at the last step. The reagent was then gently mixed and briefly centrifuged. The reverse transcription reaction was incubated in the machine (60 minutes' incubation at 37℃ and 5 minutes at 95℃). After the incubation, each tube was diluted with 200µl RNase-free water for later use in RT-PCR analysis. The cDNA can be stored in -20℃ for later use.

3.2.7.2 Real time PCR analysis of mature miRNAs

Expression of mature miRNAs was determined by miScript miRNA PCR Array (Qiagen), and normalized using the $2^{-\Delta\Delta CT}$ method relative to U6-snRNA. For this purpose, 1µl cDNA was added in 25 µl reaction in 96-well plate. The reaction consists of the following components:

Component	**Volume/reaction (96 well)**
2x QuantiTect SYBR Green PCR Master Mix	12.5 µl

10 × miScript Universal Primer	2.5 µl
10 × miScript Primer Assay	2.5 µl
RNase-free Water	RNase-free Water
Diluted cDNA	1 µl
Total volume	25 µl

The 96 well plate was briefly centrifuged for 1 minute to remove any bubbles. The reaction was then carried out with the following parameters for 40 cycles in StepOne™ Real-Time PCR System (Thermo Fisher):

Step	**Time**	**Temperature**
Initial activation step	15 min	95ºC
3-step cycling:		
Denaturation	15 s	94ºC
Annealing	30 s	55ºC
Extension	30 s	70ºC

3.2.8 SYBR Green quantitative PCR for retinoid receptor (RARA, RARB, RXRA, RXRB)

3.2.8.1 cDNA synthesis of retinoid receptor mRNA

The cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen). For this purpose, an equal amount of RNAs (500ng) from each RNA sample was used. The RNA was placed on ice and the reverse transcription reaction was initiated with genomic DNA elimination reaction as the following recipe:

Component	Volume/reaction
gDNA Wipeout Buffer, 7x	2 µl
Template RNA	Variable (500ng)
RNase-free water	Variable
Total volume	14µl

The reaction was put directly on the ice after incubation for 2 min at 42°C.

The reverse transcription master mix was performed on ice according to following:

Component	Volume/reaction
Reverse-transcription master mix	

Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT Buffer, 5x	4µl
RT Primer Mix	1 µl
Template RNA	
Entire genomic DNA elimination reaction	14 µl
Total volume	20 µl

The reaction was carried out with the following parameters in a PCR machine (Thermocycler):

Temperature	**Time**
42°C	15min
95°C	3min

Synthesized cDNA can be stored at -20°C or directly for real time PCR analysis.

3.2.8.2 Real time PCR of retinoid receptor

Expression of the retinoid receptor was determined by QuantiFast SYBR Green PCR kit (Qiagen) and normalized using the $2^{-\Delta\Delta CT}$ method relative to GAPDH mRNA. For this purpose, 1µl of cDNA synthesized before was added in 25 µl reaction in 96-well plate. The reaction consists of the following components:

Component	Volume/reaction (96 well plate)
2x QuantiFast SYBR Green PCR Master Mix	12.5 µl
10 x QuantiTect Primer Assays	2.5µl
RNAse-free water	9µl
Template cDNA	1µl
Total reaction volume	25µl

The 96 well plate was briefly centrifuged for 1 minute to remove any bubbles. The reaction was then carried out with the following parameters in StepOne™ Real-Time PCR System (Thermo Fisher):

Step	Time	Temperature
PCR initial activation step	5 min	95 ℃
Two-step cycling		
Denaturation	10 s	95ºC
Combined annealing/ extension	30 s	60ºC
for 40 cycles		

3.2.9 Transfection of miRNAs

Transfection is the process of introduction of new genetic material, DNA or RNA into a eukaryotic cell. Here, we use HiPerFect Transfection Reagent (Qiagen) to transfect miRNAs into pancreatic cancer cells. Transfection was done following manufacturers' manual. The initial step of transfection of miRNA was to determine optimal transfection conditions of different cells. Briefly, 1×10^5 cells were suspended in 0.5 ml growth medium and seeded into one well of a 24-well plate. Then appropriate amount miRNA and HiperFect Transfection Reagent was added into 100 µl culture medium without serum. The mixture was vortexed and incubated for 5-10min at room temperature to allow the formation of transfection complexes. Transfection was achieved by adding the mixture dropwise into each well. The plate was gently swirled and incubated under normal growth condition (5% CO2, 37℃, 90% humidity) for 48-72 h. The Pipetting scheme to optimize transfection of miRNA is shown in the table below:

Amount (conc.) of miRNA	75 ng (10 nM)	75 ng (10 nM)	75 ng (10 nM)
Volume of HiPerFect Reagent	1.5 µl	3 µl	4.5µl
Amount (conc.) of miRNA	37.5 ng (5 nM)	37.5 ng (5 nM)	37.5 ng (5 nM)

Volume of HiPerFect Reagent	1.5 µl	3 µl	4.5µl
Amount (conc.) of miRNA	7.5 ng (1 nM)	7.5 ng (1 nM)	7.5 ng (1 nM)
Volume of HiPerFect Reagent	1.5 µl	3 µl	4.5µl

After the incubation for 48-72 hours, cells were collected and miRNA were isolated with the protocol as described before. The level of target miRNA was determined by RT-PCR again compared with non-transfected cells.

3.2.10 Protein extraction

Cells growing in 24 well-plate were washed with the cold phosphate-buffered saline (PBS) buffer three times and lysed in extraction buffer (RIPA buffer) by incubation on ice for 15 min (RIPA buffer recipe: 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50mM Tris, pH 8.0). Cell lysis was centrifuged for 20 min (12000 × g, 4℃). The supernatant containing the total protein was used for western blot or stored at -80℃.

3.2.11 Protein quantification

The concentration of the isolated protein was investigated by utilizing the BCA Protein assay

(Pierce). This assay is based on the detection of Cu1+ ions by bicinchoninic acid (BCA). Peptide bonds in protein can transfer Cu2+ ions into Cu+. In the end, a purple-colored water soluble complex was generated which has an absorbance-max at 562nm. The total protein concentration is proportional correlated with the color change, which can be measured using colorimetric techniques. Briefly, 200µl of AB reagent (50 parts of BCA Reagent A with 1 part of BCA Reagent B) was mixed with 25µl of the diluted protein in 96 well plate and incubated at 37℃ for 1h. The purple color has then developed and the 96 well plate was allowed to cool down to room temperature before the measurement. The absorbance of each well at 562nm was measured on the plate reader (Spectra Max). The protein concentration of the unknown samples was determined by the standard curve of BSA standards.

3.2.12 Protein gel electrophoresis (SDS-PAGE)

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method widely used to achieve a semi-quantitative measurement of target proteins based on the molecular weight. The principle is that SDS can denature the protein by binding to their hydrophobic regions, thus making them negatively charged. The charged proteins are then loaded on a polyacrylamide gel and start to separate when given an appropriate electric field. In combination with Western blotting, this method was able to determine the size and relative expression of target proteins among different groups. Western blotting is a method to detect the target protein in a PVDF membrane by using the specific antibody directed against that target protein. In SDS-PAGE, a stacking gel at the top and a separating gel at the bottom was combined. The stacking gel has a large pore size and is used to stack proteins at the beginning. The components of each gel were indicated in the following table. The separating gel at the bottom was made first and then covered with isopropanol to get a horizontal gel surface. Polymerization was allowed for 30 minutes or even longer until a clear liquid-gel interface was visible. Then the isopropanol was removed completely and the 4% stacking

gel was cast on top of the separating gel. The comb was set into the stacking gel from the top side. When the stacking gel had polymerized for nearly another 40 minutes or longer, the complete gel complex was immersed in a chamber containing the running buffer. The comb was carefully removed, avoiding any scratches of the gel surface. Typically 25-30 µg protein was loaded into each well. 5 µg protein standard mix was loaded as the standard marker. The gel was then run at 120V until the required separation of the marker was obtained.

Recipes for separating gel and stacking gel:

Component	**Separating Gel**			**Stacking Gel**
	8%	10%	12%	4%
Distilled Water	9.3 ml	7.9 ml	6.6 ml	6.1 ml
Acrylamide mix	5.3 ml	6.7 ml	8 ml	1.3 ml
Tris	5 ml	5 ml	5 ml	2.5 ml
10% SDS	200 µl	200 µl	200 µl	100 µl
10% APS	200 µl	200 µl	200 µl	60 µl
Temed	20 µl	20 µl	20 µl	20 µl

3.2.13 Western blotting

3.2.13.1 Protein transfer

The proteins was separated by SDS-PAGE. The gel was then transferred onto a PVDF membrane by wet electrophoretic transfer. The membrane was first wet in 100% methanol for 5 seconds and then equilibrated in transfer buffer. The blot was assembled on a wet blotting apparatus in the following order, from the anode to cathode, 1 sponge, 2 Whatman filter papers soaked in transfer buffer, PVDF membrane, gel, 2 Whatman filter papers and finally 1 sponge. Transfer of proteins was done for 1.5-2 hour by applying stable 200 mA electricity on the apparatus.

TBS 10 × (concentrated TBS) recipe: 24.23 g Trizma HCl/ 80.06 g NaCl Mix in 800 ml ultra-pure water, adjust pH to 7.6 with 1mol/L HCl, top up to 1L.

TBST recipe: 100 ml of TBS 10 × + 900 ml ultra-pure water + 1 ml Tween 20

3.2.13.2 Protein detection

The membrane was then blocked in the blocking solution (TBS+5%BSA) for 1 hour at room temperature. The membrane was then incubated overnight with the primary antibody diluted properly in blocking solution. Following three 10-min washes with TBST buffer (0.1% Tween 20 in TBS), the blot was incubated for 1 hour at room temperature with the fluorescence-conjugated secondary antibody diluted in blocking solution in the dark. After the incubation, the membrane was washed 3 × 10minutes with TBST buffer. The protein band was visualized in the Odyssey Reader machine.

3.2.14 Bioinformatic analysis of the miRNA target

To explore the potential target sequence in the 3'UTR of retinoid receptors mRNA, different public database website were utilized:

Database	Website
miRNA.org:	http://www.microrna.org/microrna/getGeneForm.do
miRDB:	http://www.mirdb.org/miRDB/
TargetScanHuman:	http://www.targetscan.org/vert_71/

Any potential target were selected out if it is predicted by one of these databases. Specific complementary target sequence were recorded. Besides, another public database (miRTarBase) of experimentally validated miRNA targets was also utilized to combine with the information of already published data.

Database	Website
miRTarBase:	http://mirtarbase.mbc.nctu.edu.tw/

3.2.15 Statistics analysis

Statistical analysis was performed using either SPSS software or Graphpad software. Values

were presented as mean±SEM. Different statistical programs, such as one-way Anova, Pearson's correlation were utilized depending on specific data types, as indicated in the figures. Significance were considered when p-value<0.05.

4. Results

4.1 Prediction of retinoid receptors targeted by miRNAs

The down-regulation of retinoid receptors in PDAC and their correlations with patients' survival was presented in our previous study, indicating that they could function as tumor suppressors (Bleul et al., 2015). To investigate the mechanism of down-regulation of retinoid receptors, we focus our interests on the regulation function of miRNA family. More and more convincible evidence suggests that miRNAs collaborate with classical tumor suppressors and oncogenes to functionally regulate key pathways involved in the progress of cellular growth control (Lin He & Hannon, 2004). To investigate whether one retinoid receptor is targeted by a specific miRNA, the first step is to predict the specific binding sites in the 3'UTR of the retinoid receptor mRNA. Therefore, we combined several prediction databases (Targetscan, mirbase, Pictar and miRDB, see Materials and methods) to analysis the targeting sites. The analysis reveals that the miRNAs, namely miR-138, miR-206, miR-613, miR-9, miR-27a/b, can potentially target retinoid receptors. Retinoid receptors and predicted target miRNAs are represented in Table 4.

	miR-9a	miR-10	miR-27a/b	miR-138	miR-206	miR-613
RAR α			+	+		
RAR β					+	+
RXR α	+		+			
RXR β			+			

Table 4. Predicted retinoid receptors targeted by selected miRNAs.

4.2 Investigation of miRNAs target sites in the 3'UTR of retinoid receptors

To identify the specific targeting sites, each mRNA of the retinoid receptor was screened for complementarity to seed sequences of selected miRNAs. As a result, several 100%-match target sequences were found: (1) miR-138 targets at nt330-337 of the RARA-3'UTR; (2) miR-27a and miR-27b targets at nt740-747 of the RARA-3'UTR; (3) miR-206 targets at nt22-30 of the RARB-3'UTR; (4) miR-613 targets at nt21-29 of the RARB-3'UTR; (5) miR-9-5p targets at nt637-644 of the RXRA-3'UTR; (6) miR-27a/b-3p targets at nt2818-2826 of the RXRA-3'UTR. Besides, a non-100%-match target sequence was also identified: miR-27a targets at nt177-196 (see Figure 14).

```
      3' GCCGGACUAAGUGUUGUGGUCGA 5' has-miR-138
           || ||  ||  |||||||
314 : 5' UCACCACAUCUUCAUCACCAGCA 3' RARA

      3' CGCCUUGAAUCGGUGACACUU   5' has-miR-27a
           |||     |||||||
726 : 5' UUUUCUCUUUAAAACUGUGAA   3' RARA

      3' CGCCUUGAAUCGGUGACACUU   5' has-miR-27b
           |||     |||||||
726 : 5' UUUUCUCUUUAAAACUGUGAA   3' RARA

      3' GGUGUGUGAAGGAAUGUAAGGU  5' has-miR-206
           ||||  |  |||||||
  9 : 5' CUAGCUACUU--CAAACAUUCCC  3' RARB

      3' CCGUUUCUUCCUUGUAAGGA    5' has-miR-613
           ||||||||
 10 : 5' UAGCUACUUCAAACAUUCCC     3' RARB

      3' UAUGUCGAUCUAUUGGUUUCU   5' has-miR-9-5p
                 |||||||
623 : 5' GGUAGCCCCUUUUUCCAAAGA   3' RXRA

      3' CGCCUUGAAUCGGUGACACUU   5' has-miR-27a/b-3p
                |||||||
3804 : 5' CUUCCUGUGACUGACUGUGAA  3' RXRA

      3' CGCCUUGAAUCGGU--GACACUU 5' has-miR-27a
           |||  |  ||  |  ||||||
175 : 5' AGGGACCCCAGAUACCUGUGAG  3' RXRB
```

Figure 14. Target sequence of selected miRNAs within 3' UTR of retinoid receptors predicted by integrated database.

4.3 Investigation of the expression of selected miRNAs in PDAC and HPDE cell lines

To find out whether there are correlations of selected miRNAs with these retinoid receptors, we investigated the basal expression of selected miRNAs in HPDE cell line and 4 PDAC cell lines: BxPC-3, Panc-1, DanG, MiaPaCa-2. Expression of mature miRNAs was determined by miScript miRNA PCR Array (Qiagen) and normalized using the $2^{-\Delta\Delta CT}$ method relative to U6-snRNA (housekeeping gene for miRNA), as described in the Materials and Methods.

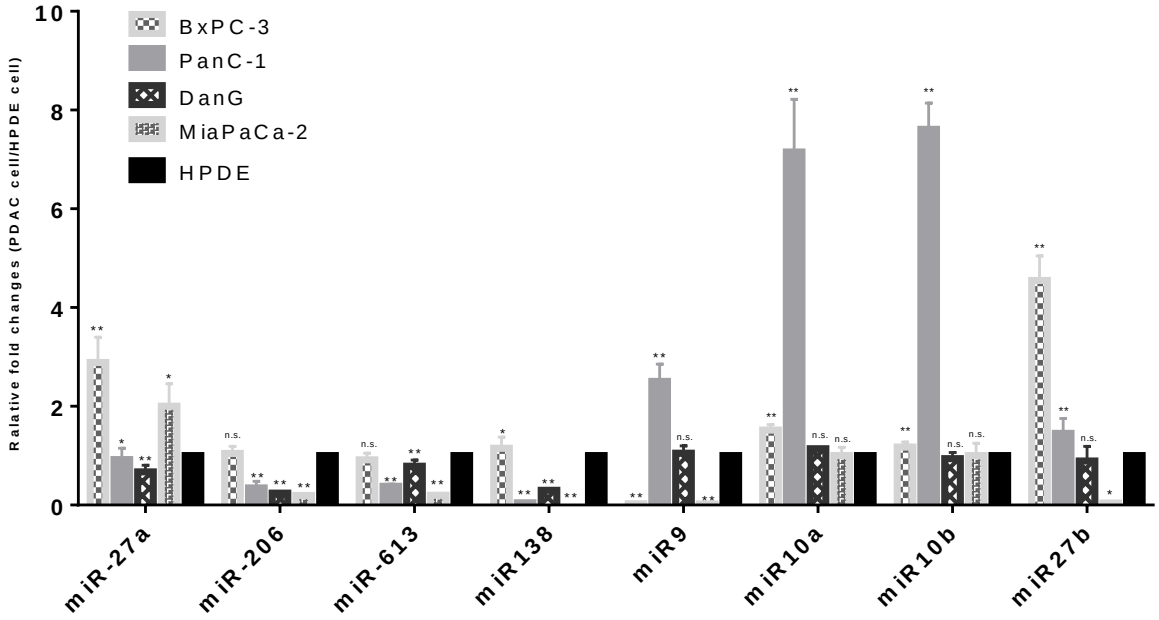

Figure.15 Overview of the miRNA expression in PDAC cell lines and HPDE cell line determined by qRT-PCR. 500 ng of total RNA extracted from cell line were used to determine miRNAs expression levels by qRT-PCR. All expression data were normalized by the U6 expression level and presented as the relative expression level. The relative expression

level of each miRNA in HPDE was arbitrarily set as 1.0. The fold changes of miRNA in PDAC cell line compared with HPDE cell show a P value of <0.05 (*) or <0.01 (**) in one-way ANOVA. n.s. indicate there is no significant difference between the PDAC group compared to HPDE group.

The miRNAs expression in PDAC cell lines (BxPC-3, Panc-1, DanG, MiaPaCa-2) and HPDE cell are shown in Figure 15. As shown in the figure, expression levels of these selected miRNAs varied considerably. Specifically, miR-27a increased by nearly 3 and 2 fold changes in BxPC-3 and MiaPaCa-2 respectively, compared to the expression level in HPDE cell. In contrast, the expression of miR-27a slightly decreases in PanC-1 and DanG (Figure 15).

Interestingly, the basal expression of miR-206, miR-613 and miR-138 are significantly decreased in PanC-1, DanG and MiaPaCa-2, but not significantly changed in BxPC-3 compared to the level in HPDE cell (Figure 15). Besides, miR-9, miR-10a and miR-10b are all high expressed in PanC-1 (Figure 15). For miR-27b, the expression levels increase significantly in BxPC-3 and PanC-1 with a fold change of 5 and 1.8, but decrease by 96% percentage in MiaPaCa-2 (Figure 15).

4.4 Analysis of retinoid receptor expression in PDAC cell lines

Next, we investigated the basal expression of the mRNAs of RARA, RARB, RXRA and RXRB in HPDE cell and four different PDAC cell lines: BxPC-3, Panc-1, DanG and MiaPaCa-2. Expression of retinoid receptors was determined by RT² Profiler PCR Arrays (Qiagen), and normalized using the $2^{-\Delta\Delta CT}$ method relative to GAPDH mRNA (housekeeping gene for retinoid receptors), as described in the Materials and Methods.

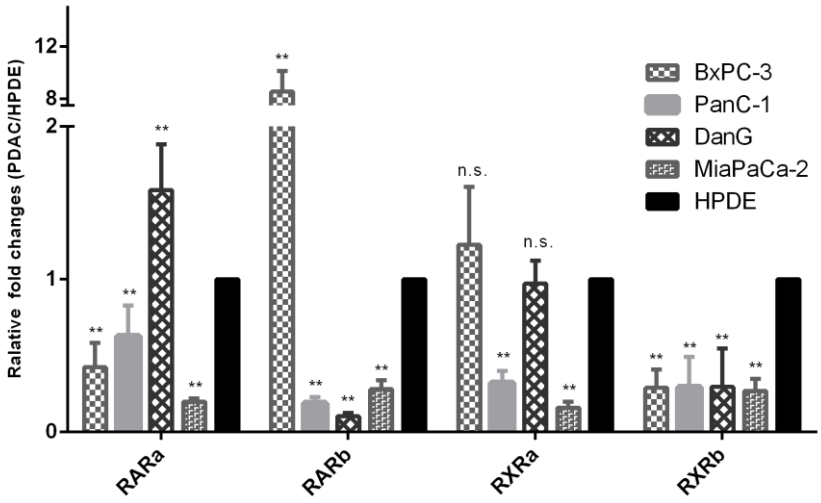

Figure 16. Overview of the retinoid receptors mRNA expression in PDAC cell lines and HPDE cell line determined by qRT-PCR. 500 ng of total RNA extracted from cell line were used to determine mRNAs expression levels by qRT-PCR. All expression data were normalized by the GAPDH expression level and presented as the relative expression level. The relative expression level of target mRNA in HPDE was arbitrarily set as 1.0. The fold changes of mRNAs in PDAC cell line compared with HPDE cell show a P value of <0.01 (**) in one-way ANOVA. n.s. indicate there is no significant difference between the PDAC group compared to HPDE group.

The retinoid receptors expression in PDAC cell (BxPC-3, Panc-1, DanG and MiaPaCa-2) and HPDE cell are shown in Figure 16. The results indicate that the expression of retinoid receptors varied in different cell lines. As showed in Figure 16, expression of RARA significantly decreased in BxPC-3, PanC-1 and MiaPaCa-2, while increased in DanG. Similarly, RARB is significantly decreased in PanC-1, DanG and MiapaCa-2, as compared to the level in HPDE cell. However, the expression of RARB increased with more than 8 times in BxPC-3 than in HPDE cell (Figure 16).

In PanC-1 and MiaPaCa-2, there is a significant down regulation of RXRA expression compared to in HPDE cell (Figure 16). While RXRB expressions are decreased in all four PDAC

cell lines compared to HPDE cell (Figure 16). n.s. indicate there is no significant difference between the PDAC cell line and HPDE cell.

4.5 Correlation analysis between selected miRNAs and retinoid receptors

Based on the basal expression of selected miRNAs and retinoid receptors in both PDAC cell lines and HPDE cell, we next want to find out whether the expression of selected miRNAs correlates with the gene expression level of retinoid receptors.

	RARA	**RARB**	**RXRA**	**RXRB**
miRNA27a	-0.8[a] 0.104[b]	+0.900* 0.037	+0.300 0.624	-0.600 0.285
miRNA206	+0.100 0.873	+0.700 0.188	+0.900* 0.037	+0.300 0.624
miRNA613	+0.500 0.391	+0.500 0.391	+0.900* 0.037	+0.700 0.188
miRNA138	+0.300 0.624	+0.600 0.285	+1.000* 0.000	+0.400 0.505
miRNA9	+0.700 0.188	-0.600 0.285	0.000 1.000	+0.600 0.285

miRNA10a	+0.051 0.935	-0.154 0.805	+0.154 0.805	-0.103 0.870
miRNA10b	-0.462 0.434	+0.359 0.553	+0.051 0.935	-0.308 0.614
miRNA27b	+0.000 1.000	+0.500 0.391	+0.700 0.188	+0.100 0.873

Table 5. Correlations between expression levels of selected miRNAs and retinoid receptors in HPDE and PDAC cell lines.

[a] Spearman's correlation coefficient

[b] *P* Spearman's rank correlation test

* Spearman's correlation coefficient > +0.50 or < −0.50 and $P < 0.05$ is considered significant

For this purpose, Spearman's rank correlation test was performed and the result was presented in Table 4. In HPDE cell and PDAC cell lines, expression levels of RARB were positively correlated with the level of miRNA27a (Spearman's correlation coefficient: +0.900 ($P = 0.037$)). Besides, expression levels of RXRA were also shown to be positively correlated with miRNA206, miRNA613 and miRNA138 ((Spearman's correlation coefficient: +0.900 ($P = 0.037$), +0.900 ($P = 0.037$), and +0.990 ($P = 0.001$), respectively)). However, no other significant correlations between these selected miRNAs and retinoid receptors were observed.

As is indicated in Table 4, there are significant correlations between specific miRNA and retinoid receptors such as the correlation between miR-613 and RXRA, but the correlation coefficient reveals that they are positively correlated rather than negatively correlated. From this

aspect, we cannot draw the conclusion that there are significant correlations which are meaningful for our experiments. Although there are no significant correlations from the result of Spearman's correlation analysis, it does not mean that there are definitely no correlations between selected miRNAs and retinoid receptors. One possibility is that although the expression level of miRNAs in PDAC cell line is relatively higher than that in HPDE cell, there can be no changes of the expression of the target gene in both PDAC cell and HPDE cell. The explanation can be that the absolute expression level of miRNAs in the cell is still not enough to cause significant changes in the expression of the target gene (See Figure 17).

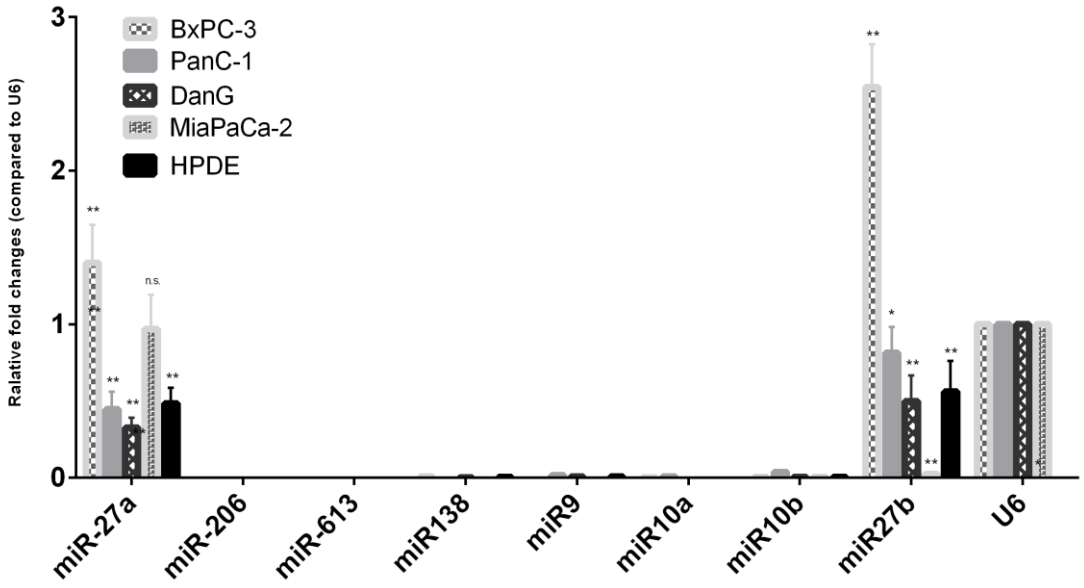

Figure 17. The relative expression of selected miRNAs in PDAC and HPDE cell lines. A-H, relative expression of miR-27a, miR-138, miR-206, miR-613, miR-9, miR-10a, miR-10b and miR-27b in PDAC cell lines and HPDE cell. All expression data were normalized by the U6 expression level. The expression level of U6 in each cell was arbitrarily set as 1.0. The relative level of miRNAs compared to U6 in PDAC cell and HPDE cell is shown. P <0.01 (**) indicate there is a significant difference between the miRNAs and U6 expression level.

Despite, we next want to investigate the effect of miR-27a, miR27b and miR9 on the gene expression of retinoid receptors due to the following fact: (1) the expression level of miR-27a, miR27b, miR9, miR10a and miR10b in PDAC cell lines are significantly higher than that in HPDE cell (Figure 15); (2) The absolute expression of miR-27a, miR27b, miR9, miR10a and miR10b are not very low or even higher compared to the U6 expression in PDAC cell lines and HPDE cell (Figure 17); (3) There are predicted retinoid receptor targets only for miR-27a, miR27b and miR9 but not miR10a and miR10b in the prediction database (Table 4).

4.6 Investigation of retinoid receptors expression after the manipulation of miRNAs expression

4.6.1 Investigation of the optimal transfection conditions

To investigate the retinoid receptors expression after manipulation of miRNAs, we first performed transfection with combinations of different concentration of miRNA mimics with transfect reagent in PanC-1 cell and DanG cell, as described in the Material and Methods. Transfected cells were then collected for total RNA isolation. 500ng total RNA was then used for Real-Time PCR analysis. The relative expression levels of miRNA after transfection were calculated compared to the miRNA expression in the cell transfected with negative control miRNAs. The results show that for the Panc-1 cell, the combination of 0.15µl miRNA mimics with 3µl HesPect buffer result in the maximum expression level of the miRNA mimics (Figure 18). For DanG cell, the combination of 0.15µl miRNA mimics with 1.5µl HesPect buffer result in the maximum expression level of the miRNA mimics (Figure 19).

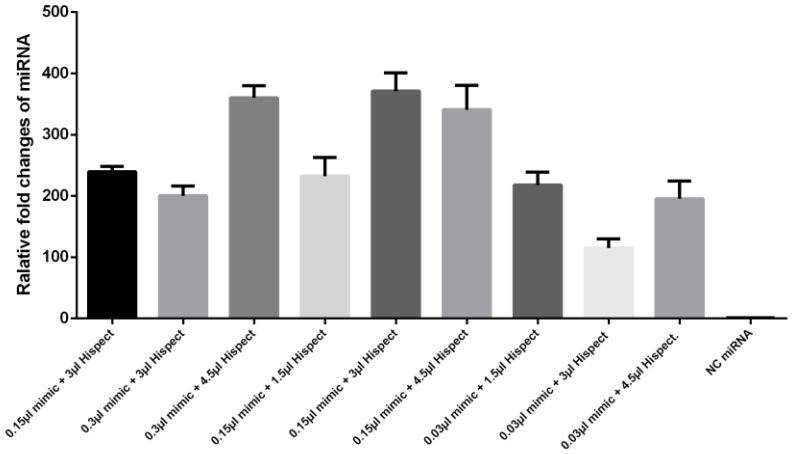

Figure 18. miRNA-9 level detected by RT-PCR in PanC-1 cell after transfection of miRNA mimic. Cells were transfected with miRNA mimics. Total RNA were isolated from the cell 48 hours after transfection. 500ng total RNA was used for Real-Time PCR analysis. The relative expression levels of miRNA after transfection were normalized with the expression of U6 and compared to the negative control (NC) group. The miRNA expression in the NC cell was set as 1.

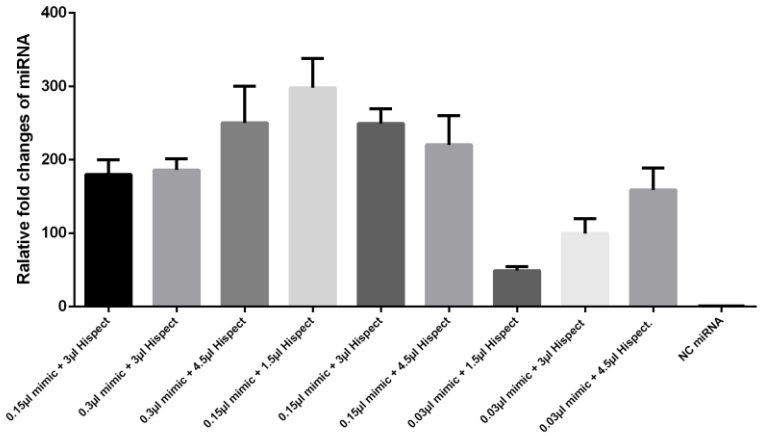

Figure 19. miRNA-9 level detected by RT-PCR in DanG cell after transfection of miRNA mimic. Cells were transfected with miRNA mimics. Total RNA were isolated from the cell 48 hours after transfection. 500ng total RNA was used for Real-Time PCR analysis. The relative expression levels of miRNA after transfection were normalized with the

expression of U6 and compared to the negative control (NC) group. The miRNA expression in the NC cell was set as 1.

4.6.2 Analysis of protein expression of retinoid receptors after miRNA transfection

Next, we investigated protein expressions of retinoid receptors in PDAC cell after the manipulation of miRNAs level. For this purpose, PDAC cell were transfected with miRNA mimics and incubated for 48-72 h under normal conditions for cell culture. The whole protein was isolated and proceeded with Western Blot analysis, as described in the Material and Methods. Results of Western Blot are presented in Figure 20 - 23.

After the transfection of miR-9, miR-27a or miR-27b, there are no significant inhibition of the expression of retinoid receptors in both PanC-1 cell and DanG cell compared with negative control (Figure 20 - 23).

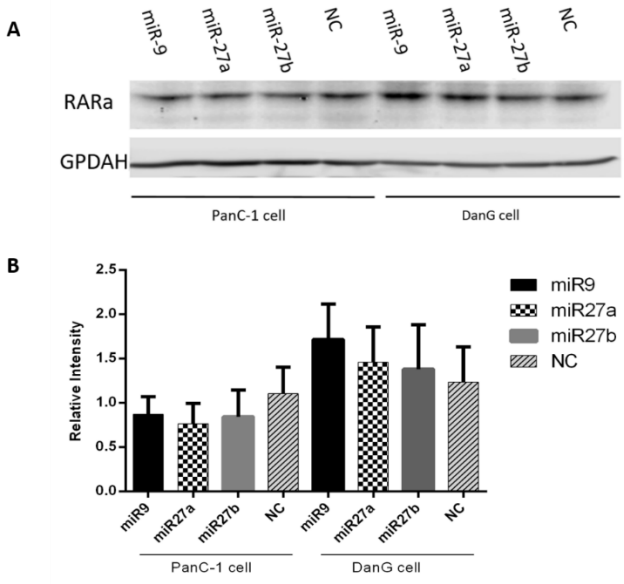

Figure 20. Expression of RARa in Panc-1 and DanG cells transfected with miR27a, miR27b or miR9. (A) 25 μg total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by western blotting. The membrane was incubated with rabbit polyclonal anti-RARa antibody as primary antibody and IRDye® 680CW Goat anti-Rabbit as secondary antibody. GAPDH was used as the loading control; (B) Densitometric values of the bands were calculated and normalized with background by using GelAnalyzer 2010 software. Relative intensity (i.e. density ratio of target protein/GAPDH) from triplicate experiments are represented as bar diagram, mean ± S.D. The data processing was confirmed by 2 person independently.

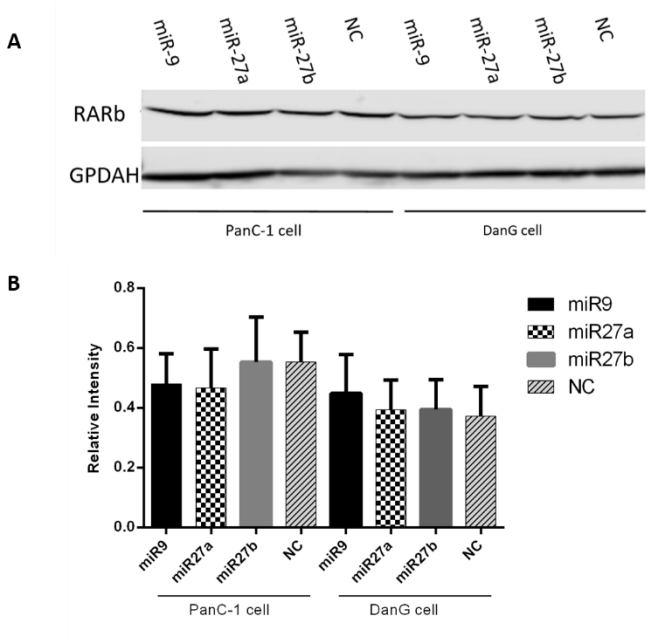

Figure 21. Expression of RARb in Panc-1 and DanG cells transfected with miR27a, miR27b or miR9. (A) 25 μg total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by western blotting. The membrane was incubated with rabbit polyclonal anti-RARb antibody as primary antibody and IRDye® 680CW Goat anti-Rabbit as secondary antibody. GAPDH was used as the loading control; (B) Densitometric values of the bands were calculated and normalized with background by using GelAnalyzer 2010 software. Relative intensity

(i.e. density ratio of target protein/GAPDH) from triplicate experiments are represented as bar diagram, mean ± S.D. The data processing was confirmed by 2 person independently.

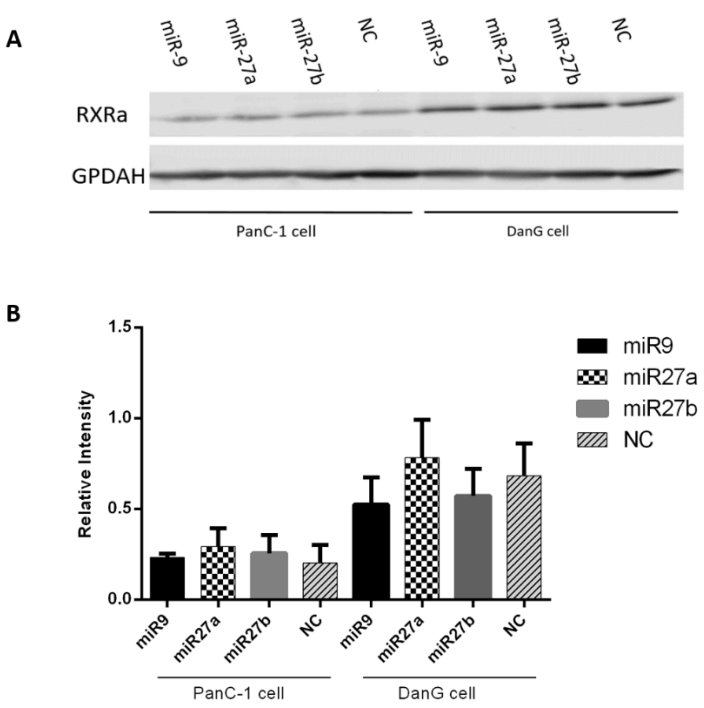

Figure 22. Expression of RXRa in Panc-1 and DanG cells transfected with miR27a, miR27b or miR9. (A) 25 μg total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by western blotting. The membrane was incubated with rabbit polyclonal anti-RXRa antibody as primary antibody and IRDye® 680CW Goat anti-Rabbit as secondary antibody. GAPDH was used as the loading control; (B) Densitometric values of the bands were calculated and normalized with background by using GelAnalyzer 2010 software. Relative intensity (i.e. density ratio of target protein/GAPDH) from triplicate experiments are represented as bar diagram, mean ± S.D. The data processing was confirmed by 2 person independently.

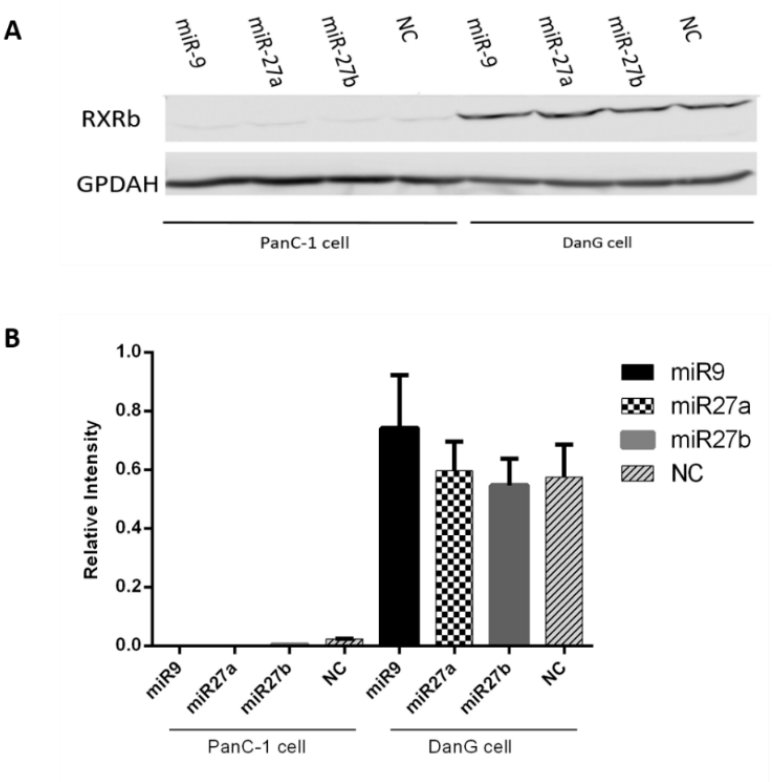

Figure 23 Expression of RXRb in Panc-1 and DanG cells transfected with miR27a, miR27b or miR9. (A) 25 μg total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by western blotting. The membrane was incubated with rabbit polyclonal anti-RXRb antibody as primary antibody and IRDye® 680CW Goat anti-Rabbit as secondary antibody. GAPDH was used as the loading control; (B) Densitometric values of the bands were calculated and normalized with background by using GelAnalyzer 2010 software. Relative intensity (i.e. density ratio of target protein/GAPDH) from triplicate experiments are represented as bar diagram, mean ± S.D. The data processing was confirmed by 2 person independently.

Next, we investigated protein expressions of retinoid receptors in PDAC cell after the transfection of miRNAs inhibitors. For this purpose, PDAC cell were transfected with miRNA inhibitor and incubated for 48-72 h under normal conditions for cell culture. The whole protein was isolated and proceeded with Western Blot analysis, as described in the Material and Methods.

Results of Western Blot and the densitometric analysis are presented in Figure 24-25. It shows there is no significant changes in the expression of retinoid receptors in both PanC-1 cell and DanG cell compared with negative control (Figure 25-26).

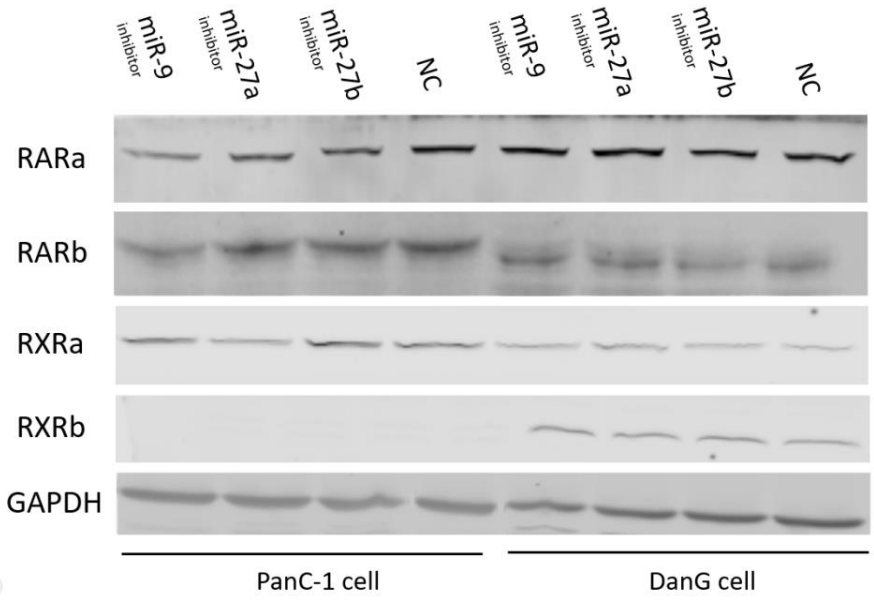

Figure.24 Expression of retinoid receptors in Panc-1 and DanG cells transfected with anti-miR27a, anti-miR27b or anti-miR9. 20 μg total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by western blotting. The membrane was incubated with rabbit polyclonal antibody (anti-RARa, anti-RARb, anti-RXRa or anti-RXRb) as primary antibody and IRDye® 680CW Goat anti-Rabbit as secondary antibody. GAPDH was used as the loading control.

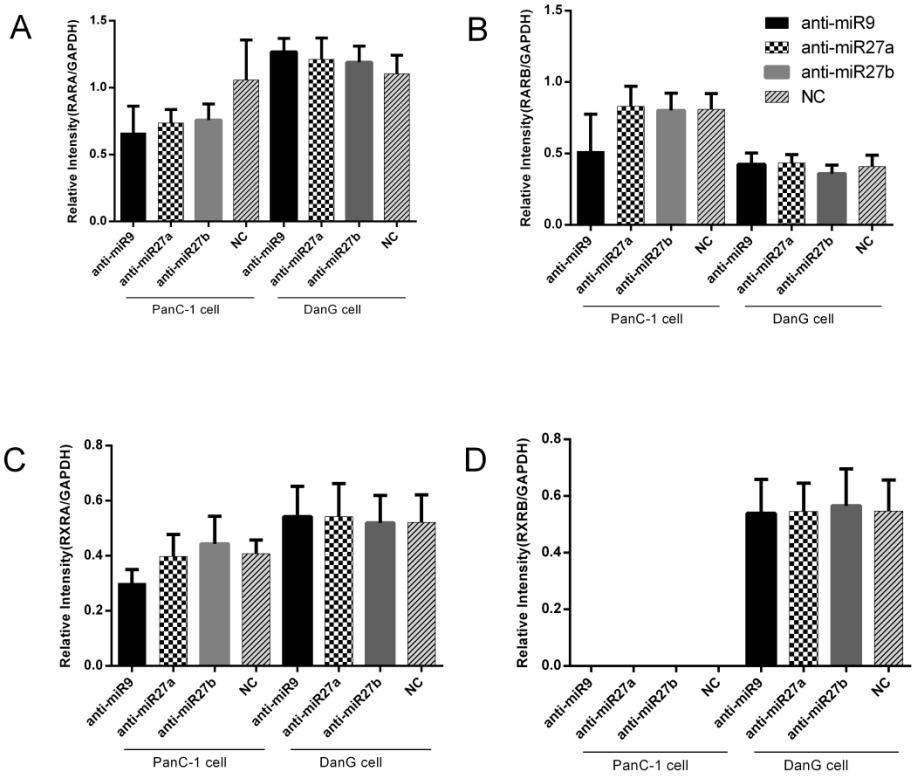

Figure 25. Relative intensity of retinoid receptors expression after the transfection of miRNA inhibitors. Densitometric values of the bands were calculated and normalized with background by using GelAnalyzer 2010 software. Relative intensity (i.e. density ratio of target protein/GAPDH) from triplicate experiments are represented as bar diagram, mean ± S.D. The data processing was confirmed by 2 person independently.

To find out whether these miRNAs are indeed transfected into the cells, MET, an experimentally validated target of miR27a, was selected as a positive control (Acunzo et al., 2013). The same amount of miRNA27a was transfected PanC-1 and DanG cell with the same procedure, as mentioned above. After incubated for 48-72 h under normal conditions, the whole protein was isolated and proceeded with Western Blot analysis, as described in the Material and Methods. Results of Western Blot are presented in Figure 26.

It showed that transfection of miR27a mimics into PanC-1 and DanG cell lead to a significant decrease of MET protein level by Western Blot analysis (Figure 26).

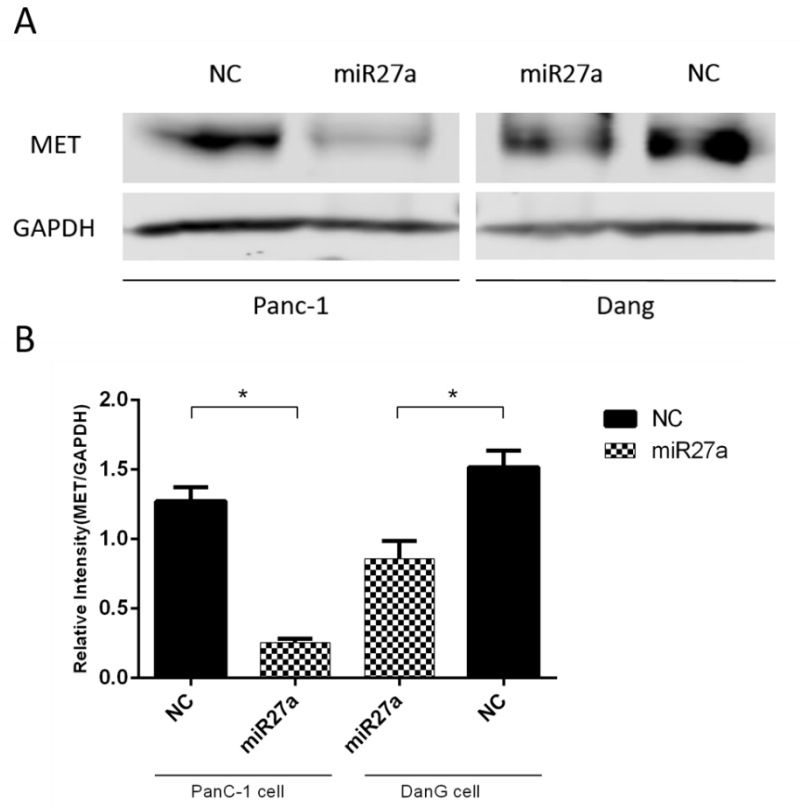

Figure.26 Expression of MET in Panc-1 and DanG cells transfected with miR27a (A) 25 μg total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by western blotting. The membrane was incubated with rabbit polyclonal anti-MET antibody, mouse polyclonal anti-GAPDH as primary antibody and IRDye® 680CW Goat anti-Rabbit and IRDye® 680CW Goat anti-Mouse as secondary antibody. GAPDH was used as the loading control; (B) Densitometric values of the bands were calculated and normalized with background by using GelAnalyzer 2010 software. Relative intensity (i.e. density ratio of target protein/GAPDH) from triplicate experiments are represented as bar diagram, mean ± S.D. The data processing was confirmed by 2 person

independently.

5. Discussion

PDAC is one of the most deadly cancers, with the death ranking fourth among cancer-related deaths. Despite emerging progression of the understanding of cancer in past decades, PDAC remains the major burden of human health. The extremely poor prognosis results largely from the diagnosis of cancer at advanced stages. Frequently perineural and vascular local growth and invasion of PDAC cell usually lead to the distance metastasis at an early stage. Most conventional treatment strategies, like chemotherapy, radiotherapy and molecular immune therapy, experienced remarkable resistance when applied in treating PDAC. PDAC is characterized by multiple genetic and epigenetic aberrations and the unique dense tumor microenvironments, which result in the heterogeneity of PDAC and variation of treatment outcomes (Jorg Kleeff, 2016).

5.1 Aberrant level of retinoid receptors in cancer

As discussed in the Introduction, all-trans-RA derives from the metabolism of vitamin A and functions through binding to the retinoid receptors. A successful case of cancer treatment using all-trans-RA is well known in acute promyelocytic leukemia (APL). In APL patients, the RARA gene is fused mainly to promyelocytic leukemia (PML) gene which generates abnormal PML-RARA fusion protein. The fused protein complex increases HDACs level and related DNA methyltransferase complexes, leading to the epigenetic silencing of retinoid-regulated genes, including those involved in the regulation of myeloid differentiation and proliferation (Di Croce, 2005; Freemantle, Spinella, & Dmitrovsky, 2003).

In other cancer types, the retinoid signaling pathway is frequently attenuated in tumorous tissue versus the surrounding normal epithelial tissue. Besides, RARA, RARB and RARγ are reported to have tumor-suppressor effects in various cancer (Farias et al., 2002; Chen, Goyette, &

Lohnes, 2004; Treuting et al., 2002). For instance, in squamous cell carcinoma cells, RARB function as a key mediator of cell growth inhibition (Le, Dawson, Soprano, & Soprano, 2000). Restoration of RARB expression in breast cancer cells can restore the ability of RA to induce both growth arrest and apoptosis (Seewaldt, Johnson, Parker, Collins, & Swisshelm, 1995). Similarly, thyroid cancer cells expressing both RARB and RXRγ show significant growth suppression when treated with retinoids (Haugen et al., 2004).

In fact, different retinoid receptor subtypes were dsyregulated in a range of cancer types, resulting in a decreased efficacy or the failure of comprehensive therapy with retinoid (Pawlyn et al., 2016; Suh et al., 2002; Y. Wang et al., 2016; Xue & Lito, 2016). RARA was also demonstrated to be expressed with higher level in differentiated, estrogen receptor (ER)-positive (ER+) mammary carcinoma cells compared with undifferentiated, estrogen-independent, ER-negative (ER−) cells, which were responsible for the response to retinoid therapy (Schneider, Offterdinger, Huber, & Grunt, 2000).

In PDAC, global gene expression has also been investigated in PDAC tissues compared to normal pancreas. Totally 62 genes have been reported to be significantly down-regulated with more than 2 fold changes in PDAC tissues, compared to that in normal pancreas. Moreover, 140 significantly up-regulated genes were also identified in PDAC tissues (Schneider et al., 2000; Suh et al., 2002) (GSE28735, GSE85589). These abnormal expression levels of genes could form complex networks regulating the tumorigenesis and tumor metastasis.

5.2 Mechanism of aberrant level of retinoid receptors in PDAC

In consistent with our previous study, we also showed in this study that retinoid receptors in PDAC cell were markedly down-regulated compared to the normal pancreatic cell. The mechanism of the down-regulation of the retinoid receptor in cancer could be the following possibilities: (1) the mutation or deletion of retinoid receptor gene; (2) the transcriptional repression; (3) the post-

transcriptional repression. The fact that the retinoid receptor genes are not mutated in the majority of human cancer specimens indicates that epigenetic events could be involved. Studies have reported that RARB can be repressed by the hypermethylation of the promoter of RARB gene and histone deacetylation in some types of cancer, resulting in the associated resistance to the growth inhibitory effects of RA in some cancer types (Cristiano et al., 2016; Kim, Kim, Park, & Kim, 2016; M. F. Lee, Hsieh, Huang, & Li, 2016; Long et al., 2016; Schneider, Offterdinger, Huber, & Grunt, 2000; Sirchia et al., 2000; Sirchia et al., 2002; S. Yang et al., 2016). Interestingly, Song et al reported another possibility that the carcinogen existing in tobacco smoke and environmental pollution, Benzo Pyrene Diol Epoxide (BPDE), can inhibit expression of the specific retinoid receptors, such as RARB2 gene. In their study, BPDE was able to inhibit RARB2 by methylation in a time-dependent manner in esophageal cancer cells (Song et al., 2005). Besides, the RARB2 was found to be a polycomb group target, providing another manner of epigenetic silencing of this tumor-suppressor gene (Bracken, Dietrich, Pasini, Hansen, & Helin, 2006). However, whether the loss of retinoid receptors expression in cancer cell drives the initiation of tumor formation or the aberrant level of these receptors is the outcome of cancer progression is not well determined (Liu et al., 1996; Seewaldt, Johnson, Parker, Collins, & Swisshelm, 1995).

Aberrant retinoic acid signaling in stromal cell has also been shown to act on the PDAC cell. RA was shown to be able to induce quiescence and reduce motility of PSCs, leading to reduced proliferation and increased apoptosis of surrounding PDAC cells (Froeling et al., 2011).

5.3 Aberrant expression level of miRNA in PDAC

miRNA is produced from the transcript pre-miRNA (longer primary transcripts), which is generated mainly by RNA polymerase II (Cai, Hagedorn, & Cullen, 2004; Y. Lee et al., 2004). miRNAs genes usually locate in introns of protein-coding genes. Usually, several miRNAs genes that are arranged in clusters within the genome can be transcribed into one pri-miNRA, which will

be further processed into several miRNAs functioning as a regulator group (Rodriguez, Griffiths-Jones, Ashurst, & Bradley, 2004).

The biogenesis of miRNA comprises four main processes: (1) Microprocessor complex (Drosha/DGCR8) liberates a hairpin-sturcture miRNA precursor (pre-miRNA). (2) Export receptor transport pre-miRNAs to the cytoplasm. (3) Dicer cleaves the pre-miRNA molecule to become a short-lived double stranded intermediate. (4) The double strand was diced into two strands and one strand becomes the mature miRNA. miRNA expression can be regulated transcriptionally and post-transcriptionally at the level of RNA processing (van Kouwenhove et al., 2011). In addition, the degradation of miRNAs can be regulated both by proteins and by cell divisions, during which the pool of miRNAs was diluted if the rate of miRNAs production is less than the sum of dilution and degradation (Grosshans & Chatterjee, 2010). For example, the tumor suppressor p53 can induces the transcription of miR-34 family members, which in turn inhibit factors like Bcl2 and Cdk4/6, being important for cell proliferation and survival (Chang et al., 2007; L. He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007).

Besides, miRNAs expression pattern can be affected at different levels. Some miNRAs can be strongly induced by stimuli like LPS (Androulidaki et al., 2009; O'Connell, Chaudhuri, Rao, & Baltimore, 2009). Interestingly, one study has shown that despite that specific miRNAs can be up-regulated in cancer, global miRNAs are usually down-regulated in human malignancies through several mechanisms. For instance, Sun et al report that Erk activation can decrease expression of miRNAs through phosphorylating exportin-5 (Sun et al., 2016). Similarly, Martello et al identify that in breast cancer, miR-103/107 attenuates miRNA biosynthesis by targeting Dicer (Martello et al., 2010), a key factor in miRNA production.

Thus, disease-related changes of miRNA expression should include the understanding of both global expression and temporal expression pattern, particularly when the phenotype could be induced by multiple biological process and various stimuli.

5.4 miRNA profiles by microarray in PDAC

In general, the whole genome miRNA profile provides high-through data demonstrating that dysregulated miRNA signatures are indeed found in many cancers (Calin & Croce, 2006). For example, several groups have applied microarray in PDAC specimens to investigate the expression alteration of miRNAs as well as global RNA transcriptome. They identified the change of miRNAs landscape in PDAC tissue compared to either adjacent normal tissue or normal pancreas. For example, a study involved in 7 PDAC patient specimens and 5 non-neoplastic pancreases have revealed that totally 91 significantly up-regulated miRNAs and 109 down-regulated miRNAs with at least 1.5 fold changes are identified in PDAC. Among them, hsa-miR-135b ranks at the top of the up-regulation list, with a 13 fold changes' increase. While has-miR-216a decreased with 36 fold changes in PDAC tissue compared with the non-neoplastic pancreas. The selected miRNAs in our work, namely miR-27a, miR-27b, miR-10 and miR-9, are up-regulated while miR-613 and miR138 experienced no significant up-regulation in PDAC tissue compared with normal pancreas.

Other research groups also investigated the changes of miRNAs levels in the short-overall-survival group compared with long overall survival group. They identified differentially expressed miRNAs between the two groups (Giovannetti et al., 2012). Despite the alteration of miRNAs expression, RNA transcriptome in PDAC are also widely focused.

Some of the dysregulated miRNAs are meaningless, while others can play important role in regulating tumorigenesis and metastasis. Hence, these functional miRNAs are defined as the tumor suppressor or onco-miRNA. For instance, miR-21 was found to be unregulated and serve as oncogenic miRNAs in various malignancies, such as glioblastoma, colorectal, breast and PDAC (Calin & Croce, 2006). In contrast, miR-146a is decreased as a result of gene deletion of chromosome 5 in the malignant hematopoietic progenitor cells in myelodysplastic syndrome

(Boldin et al., 2011; Calin & Croce, 2006; Zhao, Rao, O'Connell, Garcia-Flores, & Baltimore, 2013). However, not all dysregulated miRNAs are involved in the pathogenetic process or even to be functional. In general, cancer phenotypes induced by overexpression of miRNAs seem to be more common than those generated by loss-of-function. This feature may be explained by the fact that miRNAs can only function in a transcriptional program under given condition. Hence, miRNA gain of function in a cell type could repress target genes that are not properly repressed, while the loss of function would restore the functions in cells where target genes and corresponding miRNA of interest are concurrently expressed. This indicates that gain and loss-of-function of miRNA should be investigated in the appropriate cell types and the appropriate biological system.

5.5 The regulation of retinoid receptors by microRNAs in PDAC

Regulation of retinoid receptor genes in post-transcriptional level can also contribute to the deregulation of the retinoid receptor in PDAC cancer. Among these mechanisms, miRNA serves as one major pattern to regulate up to 30% of protein-coding gene in mammals. Regulation by miRNAs results in the destabilization of target mRNA, translational repression and even activation (Huntzinger & Izaurralde, 2011).

In this study, we aimed to figure out up-regulated miRNAs which could target retinoid receptor in PDAC cell lines. We showed the selected up-regulated miRNAs (miR27a, miR27b and miR9) were found not to be associated with the down-regulation of retinoid receptors. However, the high expression of miR27, miR-21and miR-23a in PDAC patients is associated with aggressive tumor behavior and poor survival after PDAC resection (Frampton et al., 2014). Specifically, miR-27a was found to target BTG2, ZBTB10, Spry2 which are associated with cancer survival, growth and angiogenic responses in PDAC. From their study, they concluded that the triple combination of miR-21/23a/27a could be a prognosis factor of PDAC (Frampton et al., 2014). Besides, the

combination of serum miR-27a and CA19-9 in peripheral blood mononuclear cells could help diagnose PDAC (Wang et al., 2013). Those evidence are inconsistent with our results that miR-27a is significantly up-regulated in PDAC cell lines. For the down-regulation of retinoid receptors in PDAC, Pettersson *et al* have shown that activation of RAR could cause apoptosis in PDAC cells (Pettersson et al, 2002). Similar result was also obtained by Tsujie et al that activation of RXRA involved pathway could inhibit the growth of PDAC cells (Tsujie et al, 2003). These results match our previous study about the down-regulation of retinoid receptor in PDAC. Interstingly, Tombolan and his colleges demonstrate that miR-27a is implicated in tumorgenesis by targeting the RARA and RXRA in 293T cell line (one kind of rhabdomyosarcomas cell) (Tombolan et al, 2015). This is possible because specific miRNAs could have different effect in specific cell types or pathological context. The specific genes that could be regulated by miRNAs in one cell type is largely dependent on the whole transcription of that particular cell under given conditions, as discussed in the Introduction.

RARA	RARB	RARg	RXRA	RXRB
hsa-miR-125a-5p	hsa-miR-16-2-3p	hsa-miR-182-5p	hsa-miR-423-3p	hsa-miR-3180
hsa-miR-218-5p	hsa-miR-30c-5p	hsa-miR-124-3p	hsa-miR-128-3p	hsa-miR-4467
hsa-miR-615-3p	hsa-miR-15a-5p	hsa-miR-335-5p	hsa-miR-574-3p	hsa-miR-6077
hsa-miR-138-5p	hsa-miR-16-5p	hsa-miR-320c	hsa-miR-486-3p	hsa-miR-8072
hsa-miR-6780a-5p	hsa-miR-33a-3p	hsa-miR-34c-5p	hsa-miR-7106-5p	hsa-miR-3960
hsa-miR-6779-5p	hsa-miR-15b-5p	hsa-miR-10b-5p	hsa-miR-4734	hsa-miR-6732-5p
hsa-miR-3689c	hsa-miR-195-5p		hsa-miR-4532	hsa-miR-6805-5p
hsa-miR-3689b-3p	hsa-miR-424-5p		hsa-miR-596	hsa-miR-6794-5p
hsa-miR-3689a-3p	hsa-miR-497-5p		hsa-miR-3940-3p	hsa-miR-4716-3p
hsa-miR-30b-3p	hsa-miR-545-5p		hsa-miR-3692-3p	hsa-miR-4524b-3p

hsa-miR-1273h-5p	hsa-miR-4307	hsa-miR-6817-5p	hsa-miR-320e
hsa-miR-519e-5p	hsa-miR-6792-5p	hsa-miR-4769-3p	mmu-miR-677-5p
hsa-miR-515-5p	hsa-miR-6838-5p	hsa-miR-1976	mmu-miR-466m-5p
hsa-miR-6778-5p	hsa-let-7c-5p	hsa-miR-6747-3p	mmu-miR-669m-5p
hsa-miR-1233-5p	hsa-miR-548c-3p	hsa-miR-4722-3p	mmu-miR-466h-5p
hsa-miR-6780b-5p	hsa-miR-548x-3p	hsa-miR-6727-3p	mmu-miR-466j
hsa-miR-4725-3p	hsa-miR-548j-3p	hsa-miR-660-3p	hsa-miR-6083
hsa-miR-4271	hsa-miR-548aq-3p	hsa-miR-4667-3p	hsa-miR-6754-3p
hsa-miR-6799-5p	hsa-miR-548am-3p	hsa-miR-4258	hsa-miR-412-3p
hsa-miR-5196-5p	hsa-miR-548aj-3p	hsa-miR-2681-5p	hsa-miR-6756-3p
hsa-miR-7106-5p	hsa-miR-548ah-3p	hsa-miR-3180-5p	hsa-miR-3127-3p
hsa-miR-4747-5p	hsa-miR-548ae-3p	hsa-miR-7702	hsa-miR-6769a-3p
hsa-miR-6797-5p	hsa-miR-578	hsa-miR-525-5p	hsa-miR-197-3p
hsa-miR-1249-5p		hsa-miR-520a-5p	hsa-miR-346
hsa-miR-6133		hsa-miR-4724-3p	hsa-miR-335-5p
hsa-miR-6130		hsa-miR-488-5p	hsa-miR-26b-5p
hsa-miR-6129		hsa-miR-6782-3p	hsa-miR-484
hsa-miR-6127			hsa-miR-92a-3p
hsa-miR-4510			hsa-let-7b-5p
hsa-miR-4419a			hsa-miR-573
hsa-miR-605-3p			hsa-miR-6851-5p
hsa-miR-3925-3p			hsa-miR-3616-5p
hsa-miR-766-3p			hsa-miR-3689d
hsa-miR-194-3p			hsa-miR-298
hsa-miR-6783-5p			hsa-miR-7854-3p

hsa-miR-4756-5p	hsa-miR-5002-3p
hsa-miR-4739	hsa-miR-4795-5p
hsa-miR-1321	hsa-miR-3158-5p
hsa-miR-940	hsa-miR-509-5p
hsa-miR-6893-5p	hsa-miR-509-3-5p
hsa-miR-3714	hsa-miR-4418
hsa-miR-6808-5p	hsa-miR-6891-5p
	hsa-miR-3173-3p
	hsa-miR-4278
	hsa-miR-6885-5p
	hsa-miR-328-5p
	hsa-miR-4697-5p
	hsa-miR-4488
	hsa-miR-1237-5p
	hsa-miR-3196
	hsa-miR-6816-5p
	hsa-miR-3180-3p

Table 6. Experimentally validated miRNAs that target retinoid receptors in all human cell types.

As PDAC is a disease involved with complex and dynamic molecular networks, global gene and miRNA research and integrated analysis combining genomic and bioinformatics could be applied to elucidate the complicated mechanism in tumor heterogeneity, tumorigenesis, metastasis and chemo-radiotherapy resistance.

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