Modulating the behaviour of pancreatic tumour cells

Vlecken, D.H.W.

Chapter 1: Introduction

Danielle Vlecken

Pancreatic cancer

The pancreas is a glandular organ in the abdomen, partly insulated by the peritoneum, that comprises an exocrine part (for excretion of digestive juices into the duodenum) and an endocrine part (secretion of hormones into the bloodstream), both have distinct functions, however, both can be affected by malignant transformation. The most common cancer of the pancreas is the pancreatic ductal adenocarcinoma (PDAC), which is characterised by moderately- to poorly- differentiated glandular exocrine structures (Thiruvengadam M. et al., 2013). These neoplasms are often referred to as PDAC's, whereas the terms PET's and PNET's indicate pancreatic endocrine tumours and pancreatic neuro-endocrine tumours which are generally rare (Benson AB. et al., 2013; Yao JC. et al., 2007).

Amongst risk factors that might facilitate hypertrophy and resulting malignant transformation of pancreatic tissue are: excessive consumption of animal fats (and resulting obesity), lack of exercise, and consumption of alcohol and tobacco (Thiruvengadam M. et al., 2013). Epidemiological studies showed that consumption of whole grains, fruits and vegetables is inversely proportional to the risk of developing pancreatic cancer (Hendrix MJC., 2007; Li L. and Leung PS., 2014). Hypertrophy is caused by the hyperactivity the pancreas has to show in order to produce enough enzymes to digest the lipids from the food in the duodenum (Pericleous M. et al., 2014). Signalling from adipose tissue and feedback loops with insulin and insulin-like growth factor are common events in obese people and were suggested to play major roles in the onset and progression of pancreatic cancer (Aleman JO. et al., 2014). Several genes, for example BRCA2 (breast cancer 2) (Klein A., 2013), were identified that were responsible for familial pancreatic cancer susceptibility, which also plays a major role in malignancies of the mammary glands.

Clinical presentation of patients with pancreatic cancer occurs relatively late in the course of the disease, since physical complaints arise in a late stage of the disease. In most cases, cells have already disseminated throughout the human body where they can form micro-metastases. If a patient presents himself before the onset of metastatic disease with an exocrine neoplasm, surgery can be applied in some cases (approximately 20 % of the patients are diagnosed with a localized tumour) although the complex positioning of the pancreas often prevents tumour resection (Corbo V. et al., 2012). In 2012, pancreatic cancer caused 330,000 deaths globally, and it is the seventh most common cause of deaths due to cancer ("Pancreatic Cancer Treatment (PDQ®) Patient Version". National Cancer Institute. 17 April 2014. Retrieved 8 June 2014).

Pancreatitis was suggested to be one of the risk factors for pancreatic cancer too. This can be explained by the fact that infiltration of immune cells and recruitment of vasculature takes place,

thereby creating a suitable environment for malignancies to develop. Both the innate and adaptive immune system are activated in cancer. However, cancer cells induce immune dysfuction by secreting immune-suppressors such as transforming growth factor (TGF), or vascular endothelial growth factor (VEGF). Additionally, cancer cells have the capability to interfere with MHC class I so that transformed cells are no longer recognized by the immune system (Sideras K. et al., 2014). Furthermore, pancreatic adenocarcinomas express and secrete tumour antigens which can recruit the immune system in some cases so that it will eliminate the tumour cells (Bazhin AV., 2014). Release of molecules (such as VEGF) by macrophages in the stroma or colony stimulating factor 1 (CSF 1) by the tumour itself) can result in a better micro environment for the tumour; these small (signalling) molecules can aid in attraction of the vasculature which can supply the tumour with more oxygen. This event can be triggered by hypoxia which occurs when the tumour is too large to depend on diffusion for its nutrient and gas exchange in the tumour (Wang W., et al., 2007; Bertout J. et al., 2008). Additionally, pancreatic tumour cells were observed to have acquired the ability to thrive on lactate metabolism, which enables them to survive in hypoxic conditions. These new properties were suggested to be regulated by oncogene products through a feed-back loop (Guillaumond F. et al., 2014).

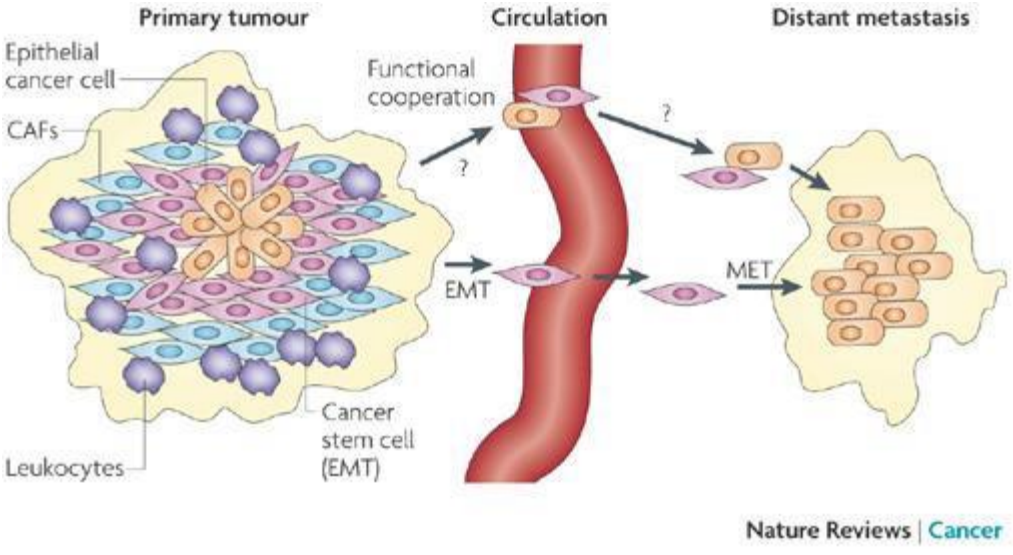

Figure 1: Transitions between epithelial and mesenchymal states during carcinoma progression. The cell's morphology changes from cubic to elongated and the cell loses its polarity whereas the capability of migration is gained. (This figure was adapted from Nature Reviews Cancer 9, 265-273 (April 2009)).

Epithelial Mesenchymal Transition (EMT) is a process that occurs when cancer stem cells are formed from epithelial cells. Epithelia contain a high level of integrity when healthy, however, when epithelial cells transform into mesenchymal cells (Figure 1) they lose their highly specialized properties and become able to invade surrounding tissues (World Cancer Report 2014. World Health Organization. 2014. pp. Chapter 5.7. ISBN 9283204298). Mesenchymal cells are motile and able to break loose from the rigid epithelial monolayers. Cell-cell and cell-matrix contacts are broken leading to a loss of adhesion and cell polarity are lost and contact inhibition as well as loss of contact with the extracellular matrix occurs when an EMT occurs. These metastatic properties were postulated to occur very early during tumour progression and were implicated to develop from signals from the tumour micro-environment. These signals, if they last long enough, will make the cell adapt (e.g. acquire mutations) to an unhealthy environment (Wang W., et al., 2007; Hanahan D. and Weinberg RA., 2000).

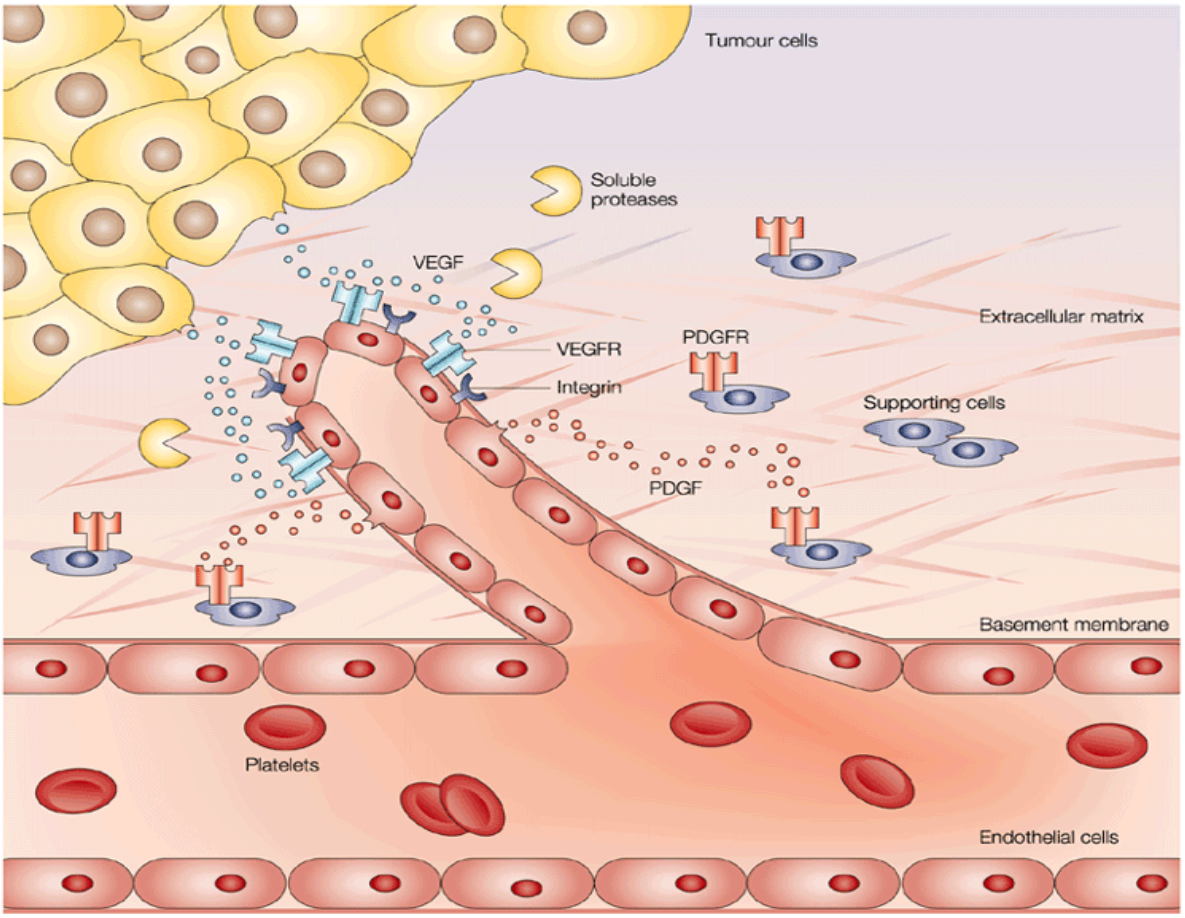

Figure 2: Tumour cell-induced angiogenesis. In this process new blood vessels are formed out of existing nearby vessels to supply the tumour with nutrients and oxygen. This occurs by sprouting and branching in response to platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and other molecules. (This figure was adapted from Nature Reviews Drug Discovery 1, 415-426 (June 2002)).

Tumour cell-induced angiogenesis (Figure 2) is a malignant process in which nearby vasculature is attracted to the tumour in order to meet the oxygen demand of the tumour. This vascular network, together with the lymphatic network which is also hijacked by the tumour is at the same time a transport medium for detached tumour cells which are capable of spreading to other sites in the human body to form a secondary mass (Wang W., et al., 2007). Invasion can take place after EMT has started. Since the transformed malignant cells have acquired the ability to move, they will extend their population into neighbouring tissues, travel in the bloodstream and settle at a distant site where they can form new colony, also after reversing EMT (MET, mesenchymal epithelial transition).

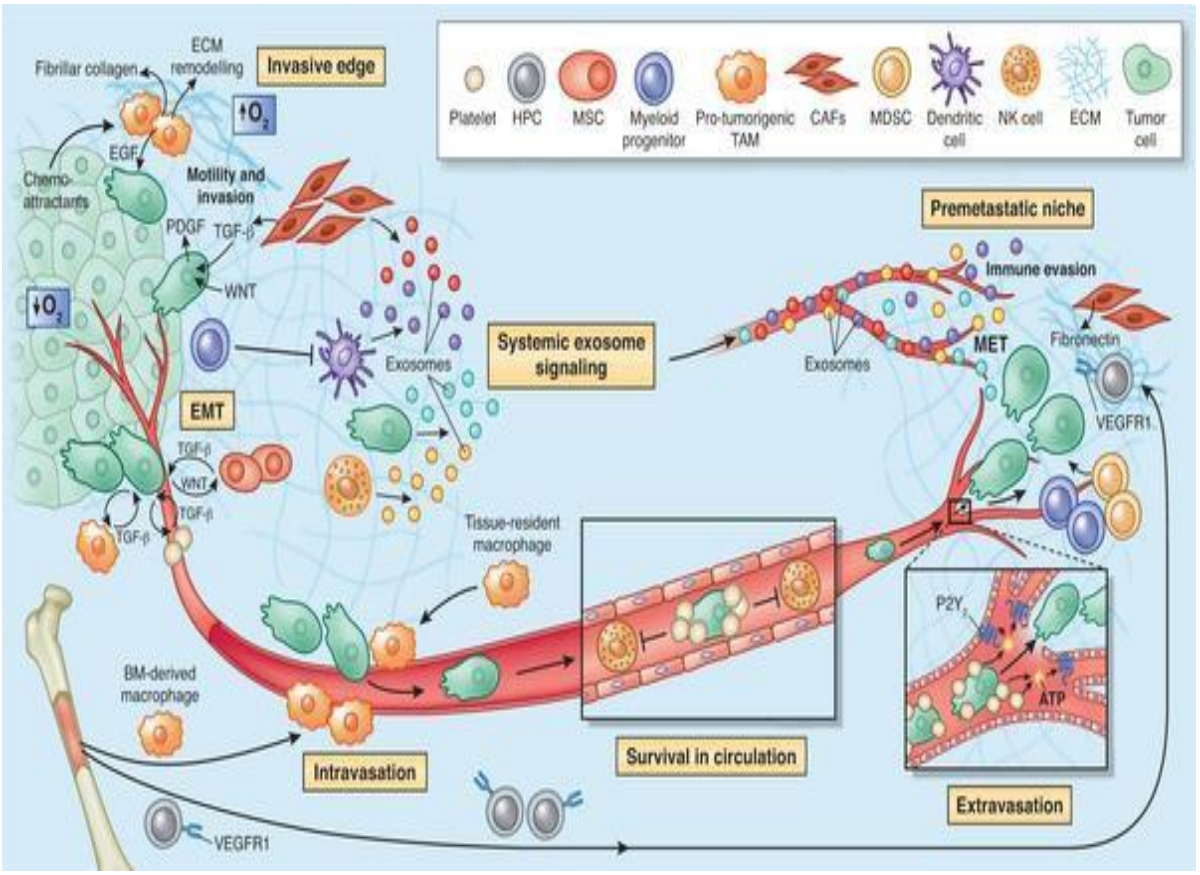

Figure 3: Metastatic events during tumour progression. By multiple signaling pathways and acquired mutations, malignant cells adapt to extra-vasate and survive in distant sites to form new micro-metastic cell masses. (This figure was adapted from Nature Medicine 19, 1423–1437 (2013)).

When searching for an option to reverse or neutralize the events caused by genetic mutations, identification of the genes involved is essential. Many gene products are currently identified as factors that can affect tumour-progression. Genes involved in the development and patterning of an embryo were implicated too in the context of malignancies. HOX (homeobox) genes which are involved in cell destination, patterning and migration (Durston AJ., 2012), could play a role in metastasis and angiogenesis since the growth of a tumour could be seen as a developmental process. Another group of genes are important for invasion and metastasis: genes that regulate cell motility by remodelling the actin cytoskeleton, which is subsequently suitable for movements (Wang W., et al., 2007). Cell motility can be controled by down regulation of adhesion proteins such as matrix

metalloproteins and integrins, or by up-regulation of proteins that control remodelling of the actin cytoskeleton, such as the Limkinases or members of the small GTP-ases like Rho and Rac (Croft DR. and Olson MF., 2006).

Also, the extracellular matrix is broken down, because a transformed cell has altered lysosomes that are capable of digesting it. In some cancer cells lysosomes are bigger and more centred in the cell, additionally, the integrity of their membranes has decreased, so that they can spill their contents into the cell and subsequently into the extracellular matrix (ECM). Conversely, when the autophagocytic pathway is blocked in a cell, the cell will undergo apoptosis as a self-protective mechanism (Kroemer G. and Jaatela M., 2005).

Table 1

Cell lines studied in this work

CELL LINE	TISSUE OF ORIGIN	MALIGNANT PROPERTIES (METASTASIS AND ANGIOGENESIS	DOUBLING TIME	MORPHOLOGY	CULTURE CONDITIONS
PATU 8988T	pancreas, metastatic site: liver	all	26 hours	mesenchymal	DMEM (Dulbecco's minimal essential medium), 10%FCS (fetal calf serum) 28^0 C
PATU 8988S	pancreas; adenocarcinoma metastatic site: liver	not metastatic	34 hours	epithelial-like	DMEM, 10%FCS 37^0 C
ASPC-1	pancreas; derived from metastatic site: ascites	all	28 hours	epithelial-like	RPMI (Roswell Park Memorial Institute 1640 Medium) with 10%FCS 37^0 C
PANC-1	Pancreas; ductal carcinoma	all	30 hours	epithelial-like, hypertriploid	DMEM 10%FCS, 37^0 C
ZF-4	*Danio rerio*, zebrafish embryo	n.a.	36 hours	fibroblast, adherent	L15 (Leibowitz-15 medium) 15%FCS, 28^0 C

Animal model and platforms used in this thesis

The zebrafish emerged as an *in vivo* cancer model to study both tumour-induced neovascularization and the formation of micro-metastases of xeno-transplanted cancer cells and tumour fragments. In the transparent zebrafish embryos, invasion and migration of tumour cells, their circulation in the vascular system, as well as the formation of micro-metastasis and the tumour-induced neovascularization can all be followed with high resolution in real-time. Importantly, these zebrafish models allow to quantitate both metastatic behaviour of transplanted tumour cells and tumour-cell induced neovascularization, as was described in detail before (Marques I., et al, 2009; Nicoli S. et al 2008; Nicoli S. and Presta M., 2007). Tg(fli1:eGFP) zebrafish embryos were used for the studies in this thesis because they express EGFP in their vascular endothelial cells.

The platforms that were used to test malignant properties comprised of two pancreatic adenocarcinoma cell lines and a set of two cell lines derived from two separate secondary sites, which originated from the same primary tumour (Table 1). PaTu 8988t cells were observed to be very aggressive and these cells disseminated readily upon transplantation ectopically of the zebrafish embryo as well as in *in vitro* migration assays, whereas it counterpart, PaTu 8988s cells did not. However, all cell lines tested showed the ability to induce angiogenesis. As can be anticipated, there are many other pathways and mechanisms that contribute to or inhibit malignant processes.

Aims

This work forms an assessment and a comparison of factors that influence or can influence malignant processes, such as metastasis (Figure 3) and angiogenesis, in the background of pancreatic cancer. Two approaches were employed, in order to obtain a broad dataset of information regarding the behaviour of pancreatic tumour cells under different conditions. Genetic input, both inhibition and facilitation of malignant behaviour, was determined for the 39 HOX genes and the two Lim kinases. Also, potential therapeutic drug candidates were tested for their properties to eradicate pancreatic tumour cells.

Scope

This work was performed using the animal and human cell line platforms and the in vivo zebrafish model as is indicated in Table 1. A special feature of the chosen cell lines is that PaTu 8988t and PaTu

8988s cells were derived from a metastatic foci in the liver belonging to the same primary pancreatic tumour. PaTu 8988t showed the ability to migrate in an aggressive manner both *in vitro* and in vivo, while PaTu 8988s cells do not.

HOX genes are essential to axial patterning, they have many essential functions during embryonic development including cell migration and are highly conserved amongst species and in time. Whether individual HOX genes play a role in metastasis or angiogenesis of pancreatic tumour cells was studied and described in **chapter 2**. All 39 HOX genes were investigated by means of *in vitro* and *in vivo* assays using zebra fish embryos. Not all HOX genes were observed to be involved in metastasis or angiogenesis. Loss of HOX genes from paralogue groups 10-13 were found to facilitate malignant processes whereas loss of HOX genes from the lower paralogue groups mostly seemed to inhibit metastasis or angiogenesis.

Chapter 3 describes the influence of LIM kinase 1 and – 2 on pancreatic tumour cells using cell migration, angiogenesis and metastasis assays. These assays were performed by silencing LIMK1 and -2 mRNA with addition of si-RNA (small interference RNA) against LIMK1 and -2 to the cells two days before assessment. The results showed that both kinases play a facilitating role in metastasis and angiogenesis of pancreatic cancer cells. These molecules are involved with the formation of actin fibers and function in motility. One of the results was that knocking down both kinases, completely abolished malignant processes in pancreatic tumour cells.

Chapter 4 is based on a drug-screen for candidate chemotherapeutic compounds and their effect on angiogenesis and neovascularization, induced in pancreatic cancer cells and in zebrafish embryos, respectively. In this study, the compound Iridium and its effects were characterized. As is shown in Chapter 4, Iridium complexes are able to inhibit carcinogenic processes if applied in the suitable therapeutic window. Also, if tested on zebrafish embryos, it is clear that the embryonic development of its own vasculature is inhibited. If the administered dose is too high, severe adverse effects can occur. Also, the vehicle in which the drug is suspended has pathological effects when administered in a high concentration. The results show that this compound is a serious candidate for an anti-metastasis drug; however, administration routes should be subjected to further investigation as well as the extent of the therapeutic window in humans.

Chapter 5 provides more insight in the effects of whole snake venoms and candidate therapeutic applications of these. From the experiments that are described in the fifth chapter it becomes clear that some snake venoms have inhibitory effects on metastasis and angiogenesis of pancreatic cancer cells in an *in vivo* system. In this study, a different method was used in the *in vivo* model than the ones described in the other chapters. First, the cells were treated a day before transplantation,

however no effects were observed. Then, venoms were added to the water which the fish resided in (as is done in vascularization assays). In this setting anti-malignant effects became evident for venoms derived from cobras (*Naja* species).

Finally, in **chapter 6**, the results are placed in context to the real situation, which underlines the importance of a healthy lifestyle and absence of damaging habits.

Relevance

With a rising incidence of pancreatic cancer during the last decades, public demand for therapeutics continues to be strong. Public awareness and knowledge regarding malignancies of the pancreas are low. Additionally, scientific understanding also does improve significantly, and the expected resulting reduction of the incidence and prevalence of this disease remain cumbersome, in contrast with the strong progress made in the treatment of other cancer types such as mammary cancer.

This is especially striking since the overall survival time of pancreatic cancer is very short for patients. For these reasons, it is of major importance to identify essential properties and elucidate mechanisms of pancreatic cancer progression which are responsible for the poor prognosis for patients with this disease.

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