# Modulating the behaviour of pancreatic tumour cells

Vlecken, D.H.W.

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**Daniëlle Vlecken**

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# Modulating the behaviour of pancreatic tumour cells

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# 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).

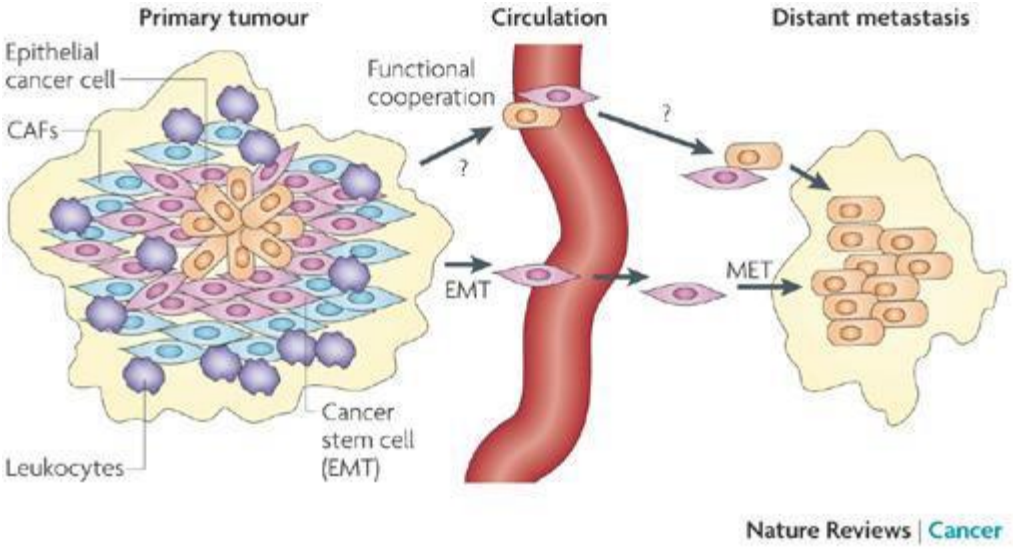


Figure 1: Transitions between epithelial and mesenchymal states during carcinoma progression. The cell's morphology changes from cubic to elongated and the cell loses its polarity whereas the capability of migration is gained. (This figure was adapted from Nature Reviews Cancer 9, 265-273 (April 2009)).

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).

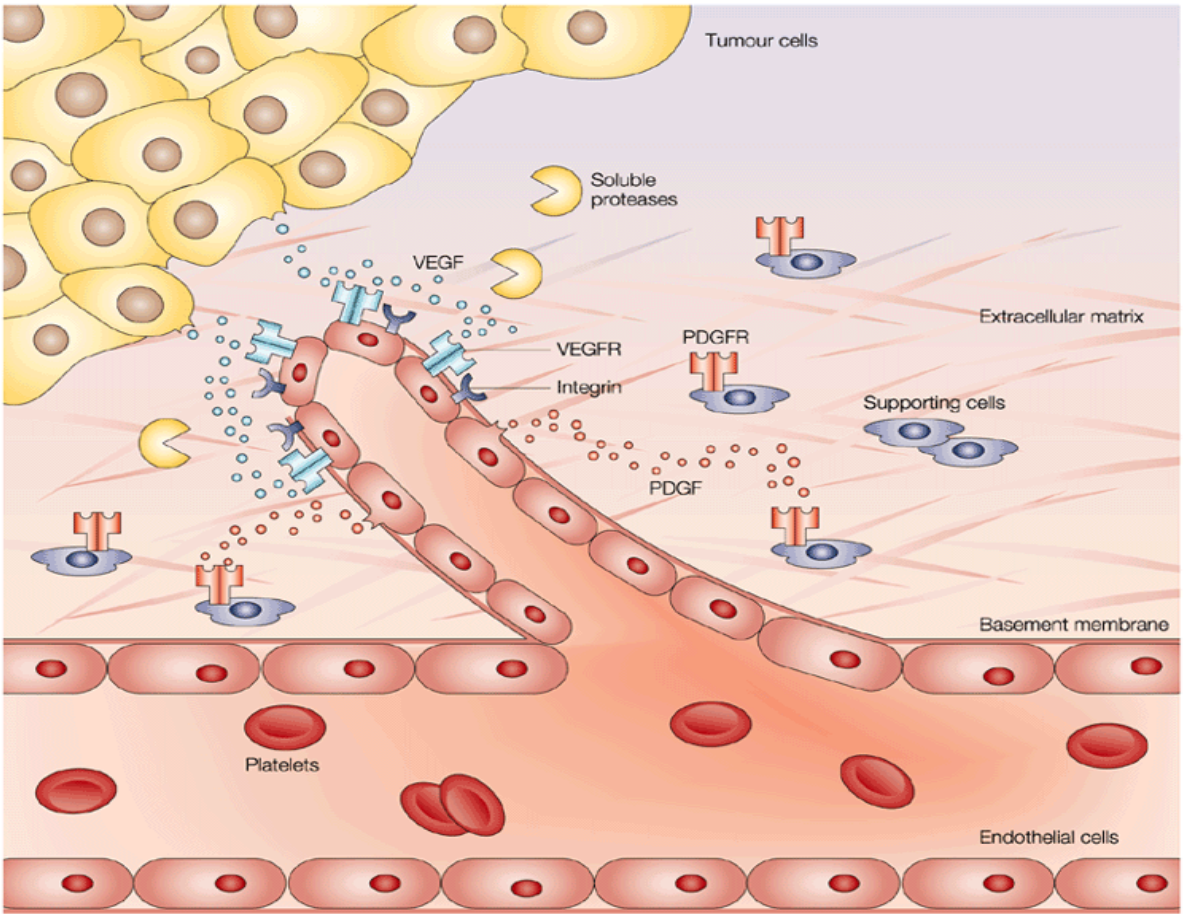


Figure 2: Tumour cell-induced angiogenesis. In this process new blood vessels are formed out of existing nearby vessels to supply the tumour with nutrients and oxygen. This occurs by sprouting and branching in response to platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) 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).

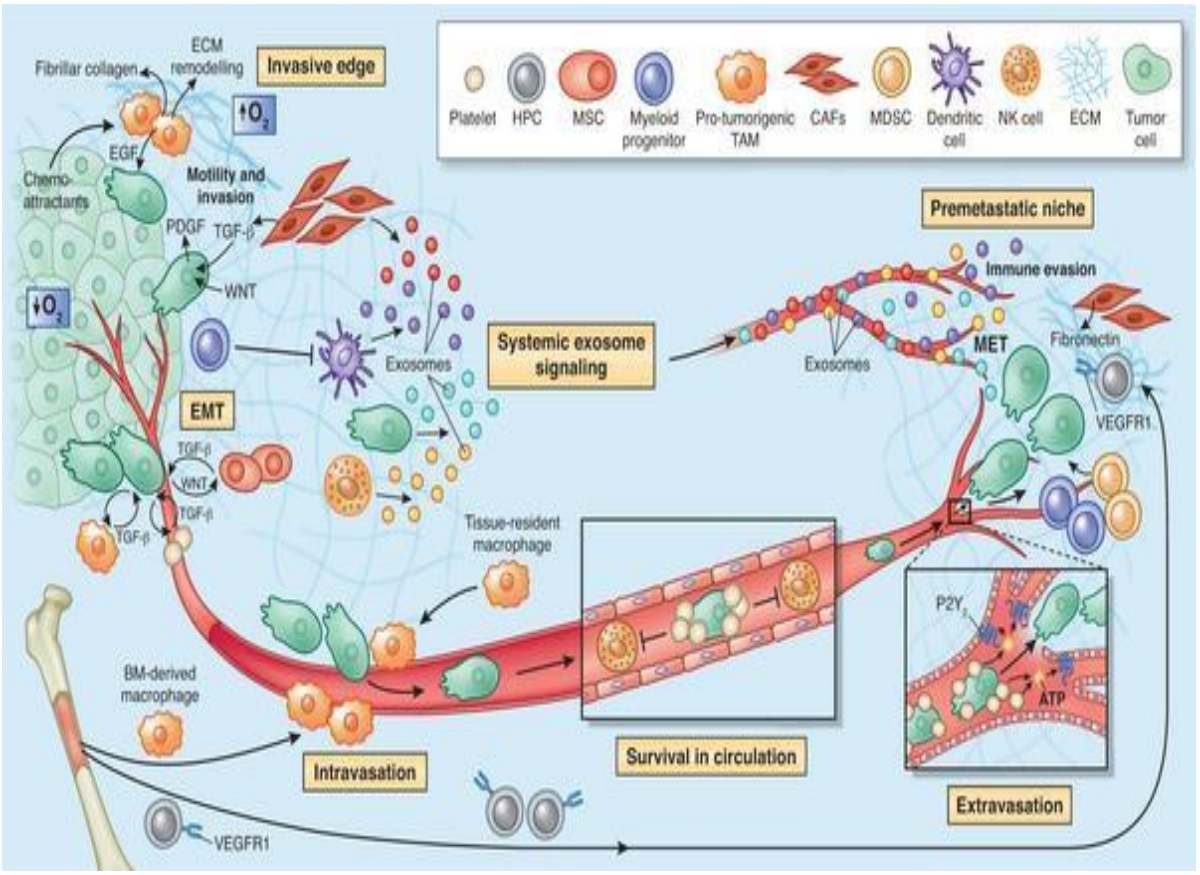


Figure 3: Metastatic events during tumour progression. By multiple signaling pathways and acquired mutations, malignant cells adapt to extra-vasate and survive in distant sites to form new micro-metastic cell masses. (This figure was adapted from Nature Medicine 19, 1423–1437 (2013)).

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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## Chapter 2: The oncogenic potential of HOX genes in pancreatic cancer cell dissemination and tumour-induced angiogenesis

**Danielle Vlecken, Prisca Leferink, Ulrich Weiss, Christoph Bagowski**

**Abstract**

HOX genes belong to the conserved super family of homeodomain-containing transcription factors and play fundamental roles in morphogenesis, organogenesis and differentiation. In vertebrates, increasing evidence suggests that HOX genes play a role in cancer progression. The oncogenic potential of HOX genes in certain human cancers has been described, but their role in most cancer types is still elusive. In this study, the contribution of the human HOX genes to metastatic behaviour and tumour-cell induced angiogenesis of four human pancreatic cancer cell lines (PaTu 8988t, PaTu 8988s, AsPC-1 and Panc-1) was assessed.

Initially, expression of the 39 human HOX genes in the studied cell lines was confirmed. Subsequently, their roles in the four pancreatic cancer cell lines were compared using using siRNAs for targeted gene silencing. The gene-specific siRNAs were added to the cells for specific genetic silencing and subsequent knock down effects were screened for altered migration *in vitro* and metastatic behaviour and tumour cell-induced angiogenesis *in vivo*. We show here that HOX genes of the paralogue groups 9 and 10 have a significant effect on pancreatic cancer cell behaviour. Furthermore HOXA2, HOXA5, HOXA7 and HOXB8 are shown here to have profound effects on the malignant behaviour of the pancreatic cancer cell lines studied.

**Introduction**

Tumourigenesis and tumour-progression involve interactions of cancer cells with each other, neighbouring cells and their microenvironments (Wang W. et al, 2007; Hannahan D. and Weinberg RA., 2000; Murdoch C. et al., 2008). Cancer cells often induce surrounding endothelial cells to build new vessels as a response to VEGF signalling by the vasculature in order to balance the blood supply with the demands of a growing tumour (Goel HL. and Mercurio AM., 2013). The vascular network is further employed by metastasizing cancer cells for their long and short distance traveling (Cao Y., 2005). The complex mechanisms that regulate these "nomadic" cancer cells include intravasation and extravasation. To successfully form a micro-metastasis, cancer cells need to use the vasculature for their transport (Fiedler IJ., 2003). The importance to understand and prevent metastasis formation is underlined by the fact that over 90% of all cancer related deaths are due to secondary tumours arising from metastasizing cancer cells (Jemal A., 2007). This is especially true for pancreatic cancer types which have a poor prognosis in 85-90% of the patients due to metastatic complications (Ganeh P. et al, 2007). The 5-year survival is estimated at 5% (WHO, 2014) and pancreatic cancer is responsible for 6% of cancer deaths each year (Jemal A., 2007).

Links between HOX gene expression and malignant transformation have been investigated based on the rationale that Hox expression in cancer cells is considered to reflect re-emergence of embryonic cell behaviour (Shah N., and Sukumar S., 2010). It is widely accepted that the processes of normal embryogenesis and neoplasia share many of the same pathways, and that tumour development is an aberrant form of organogenesis (Htuchin ME. et al., 2005; Hendrix MJC. et al., 2007).

The oncogenic potential of HOX genes has clearly been implicated in several human cancer types (Shah N. and Sukumar S., 2010). For example, HOX genes were shown to play a role in regulating lineage determination and maturation in hematopoietic tissues (Mahdipour E. and Mace KA., 2011; Magli MC., 1997) and a linkage to increased HOX gene expression was shown in acute leukaemia's (Kongsuwan K. et al., 1988; Alharbi RA., 2013).

The zebrafish has become an *in vivo* cancer model that allows us to study both tumour-induced angiogenesis (Nicoli S. and Presta M., 2007; Nicoli S. and Ribatti D., 2007) and the formation of micro-metastases of xeno-transplanted cancer cells (Haldi M., 2006) and tumour fragments (Marques I, 2009). In the transparent zebrafish embryos, invasion and migration of tumour cells, their circulation in the vascular system, as well as the formation of micro-metastases and the tumour-induced angiogenesis can be followed with high resolution in real-time (Marques I, 2009). Importantly, these zebrafish models allow to quantitate both metastatic behaviour of transplanted tumour cells and tumour-cell induced angiogenesis (Nicoli S. and Presta M, protocols 2007; Marques I., 2009). In this

study, two *in vivo* zebrafish models were used to characterize and compare the involvement of the human HOX genes in tumourigenesis of human pancreatic cancer cells. HOX genes are members of the Homeobox gene family. These genes code for transcription factors controlling morphogenesis, organogenesis, differentiation and regulate specification of positional identity in vertebrates. HOX genes are homeobox-containing genes. They code for transcription factors that help regulate morphogenesis, organogenesis, differentiation and positional specification in metazoans (Krumlauf R. and McGinnis W., 1992; Pearson JC. et al., 2005).

In humans, HOX genes are located in four clusters (A-D) which consist of a total of 39 genes, assigned to 13 paralogue groups based on their sequence similarity. Clusters A-D are located on different chromosomes, 7p15, 17p21, 12q13, and 2q31, respectively (Bayley WJ., 1997). Temporal and spatial control of HOX gene expression is essential for correct developmental patterning in many animals (Durston AJ. et al., 2012; Duboule D. 1994). Regulation of embryonic segmentation and the formation of the antero-posterior axis involve cell motility, morphogenesis, division, differentiation and apoptosis. These processes have prominent roles in tumourigenesis and –progression (Hannahan D. and Weinberg R.A., 2000).

Because of the redundancy of genes that belong to the same paralogue groups, all 39 genes of the four clusters were screened for metastatic behaviour and tumour induced angiogenesis. Expression of HOX RNA was assessed using PCR in the four cell lines. Then, HOX genes were knocked down using siRNA and these cells were subsequently used in an *in vitro* migration assay, an *in vivo* metastasis assay and in an *in vivo* angiogenesis assay. Using the results obtained, an estimation can be made about the set of HOX genes that might be involved in pancreatic cancer.

**Materials and Methods**

*Animal care and handling*

Tg(fli1:eGFP) zebrafish were kept at 28° C in tanks with day/night light cycles of 10 hours dark alternated with 14 hours light. The zebrafish were handled in compliance with Dutch animal care regulations and standard operating procedures. Zebrafish eggs were harvested at two hours post fertilization (hpf), followed by incubation at 28° C in egg water (sea salt, 60µg/ml in tap water). The developing embryos were kept in incubators at constant temperatures dependent on experimental requirements.

*Cell culture*

All cell lines (PaTu 8988t, PaTu 8988s, PANC-1 and AsPC-1) were cultured at a temperature of $37^0$ C and a $CO_2$ pressure of 5%.The PaTu 8988t cell line and the PaTu 8988s cell line were isolated from liver metastases over a decade ago (Elsässer HP., 1992). Their behaviour in an *in vitro* wound healing assay and in an *in vivo* zebrafish xenotransplantation assay were described in detail before (Marques I., 2009). PaTu 8988t and PaTu 8988s cells were cultured in DMEM (Gibco) high glucose supplemented with 10% FCS (Gibco). AsPC-1 cells (ATCC® (American Tissue and Culture Collection) CRL-1682™), first cultured 30 years ago, derived from the metastatic site (ascites) of a pancreatic adenocarcinoma. (Chen WH. et al., 1988) were cultured in RPMI (Gibco) with 10% FCS. PANC-1 (ATCC® CRL-1469™), first described in 1975, originated from an epithelioid carcinoma of the pancreatic duct. (Lieber MJ., 1975) were cultured in RPMI with 15% FCS.

*PCR and gene silencing using siRNA*

SiRNA polynucleotides for silencing the HOX genes and the corresponding primer pairs, were purchased from Santa Cruz Biotechnology (HOXA1 siRNA (h) sc-35583, HOXA2 siRNA (h) sc-38673, HOXA3 siRNA (h) sc-38675, HOXA4 siRNA (h) sc-75277, HOXA5 siRNA (h) sc-38678, HOXA6 siRNA (h) sc-89444, HOXA7 siRNA (h) sc-38680, HOXA9 siRNA (h) sc-38682, HOXA10 siRNA (h) sc-38684, HOXA11 siRNA (h) sc-60802, HOXA13 siRNA (h) sc-45666, HOXB1 siRNA (h) sc-38686, HOXB2 siRNA (h) sc-38688, HOXB3 siRNA (h) sc-38690, HOXB4 siRNA (h) sc-38692, HOXB5 siRNA (h) sc-75279, HOXB6 siRNA (h) sc-38694, HOXB7 siRNA (h) sc-45835, HOXB8 siRNA (h) sc-75281, HOXB9 siRNA (h) sc-45669, HOXB13 siRNA (h) sc-43851, HOXC4 siRNA (h) sc-60804, HOXC5 siRNA (h) sc-75287, HOXC6 siRNA (h) sc-45673, HOXC8 siRNA (h) sc-60806, HOXC9 siRNA (h) sc-75289, HOXC10 siRNA (h) sc-44810, HOXC11 siRNA (h) sc-75283, HOXC12 siRNA (h) sc-95877, HOXC12 siRNA (m) sc-146071, D1 siRNA (h) sc-38696, HOXD3 siRNA (h) sc-38698, HOXD4 siRNA (h) sc-75295 HOXD8 siRNA (h) sc-94725, HOXD9 siRNA (h) sc-35585, HOXD10 siRNA (h) sc-44814, HOXD11 siRNA (h) sc75291, HOXD12

siRNA (h) sc-75293, HOXD13 siRNA (h) sc-45656)). SiRNA and control siRNA were used in a final concentration of 50 nM and subsequently delivered into the pancreatic cancer cell lines using EndoPorter™ (Gene Tools, LLC). The control siRNA contains a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. The efficiencies of the HOX siRNAs and HOX expression in the used cells were tested by RT-PCR, which was described previously including total RNA preparation (van der Meer DLM., 2006). The annealing temperature was set to 55° C and the extension temperature to 72° C.

In vitro *migration assay*

The *in vitro* migration assay was carried out as was previously described (Liang CC., 2007). Cells were grown to confluence in 6-well plates (Greiner). Then, the monolayer was scraped along a straight line with a 200 µl pipette tip. Pictures of the scratch were taken under a Leica stereomicroscope at 0h, 24h and 48h and the gap was measured and gap closure was calculated as a percentage of the initial gap (scratch).

*Cell staining*

Cells were plated in 24-well plates (Greiner) at a seeding density of 4,0 $*10^4$ cells and incubated for 48 hours at $37^0$ C and 5% $CO_2$. SiRNA and EndoPorter® (Gene Tools, LLC) were added according to manufacturers' protocol, and the cells were incubated again at $37^0$ C and 5% $CO_2$ for 24 hours. After detaching the cells with trypsin (Gibco), two washing steps were done with DPBS (Dulbecco's PBS, Gibco), after which the cells were transferred to 1.5 ml Eppendorf tubes and centrifuged 5 min, at 1500 rpm. Then, the cells were stained according to manufacturer's protocol after which the cells were suspended in 25 µl DPBS for injection into the embryos.

*Cell transplantation into zebrafish embryos*

After dechorionation, 2 dpf zebrafish embryos were anesthetised with Tricaine methanesulfonate (TMS, MS-222, Sigma). Using a manual injector (Eppendorf, Celltram), the cell suspension was loaded into an injection needle (15 µm internal- and 18 µm external-diameter (Oxford Apparatus) and cells were injected in 48 hpf Tg(fli1:eGFP) zebrafish embryos. After injection, the embryos were incubated at $35^o$ C and checked for cell presence at 2 hours post transplantation (hpt). Embryos with fluorescent cells outside the implantation area at 24 hpt were excluded from further analysis. All other embryos were incubated at 35° C for analysis during the following days. Two independent experiments are combined for each treatment and cell type (Table 2). Every experiment consisted of 50 injected and approved embryos. For statistical analysis, a student t-test was performed which showed that all changes observed were significant (with p-values <0.01).

*Tumour cell-induced angiogenesis*

The tumour-cell induced angiogenesis assay was in general performed as previously described (Nicoli S., Presta M., 2007; Nicoli S., Ribatti D., 2007).The 48 hpf zebrafish embryos were dechorionated and anesthetised using 0.042 mg/ mL Tricaine (Sigma). Then, the embryos were positioned on a 1. 8% agarose dish for injections. Cells were stained as described above, then suspended in 20 µl 12. 0 mg / ml Matrigel® solution (Cultrex R Basement Membrane Extract, R&D systems, Minneapolis, USA) and kept on ice for a short period until injection. The CellTram Oil injector (Eppendorf, Germany) was loaded with an injection capillary (Harvard apparatus) which was filled with 5 µl of the cell suspension in Matrigel®, and embryos were injected as described in more detail in previous studies (Nicoli S., Protocols 2007; Nicoli S., Cancer Res 2007; Vlecken DH. and Bagowski CP., 2009). Every experiment consisted of 50 injected and approved embryos. For statistical analysis, a student t-test was performed which showed that all changes observed were significant (with p-values <0.01).

*Imaging*

After injection, embryos were incubated in egg-water at 35°C for 24 hours before screening. Embryos were photographed using a Bio-rad confocal microscope equipped with a 1024ES lens (Zeiss) and a Krypton/Argon laser. For light-microscopy images of pancreatic cells, an MZ16FA stereomicroscope (Leica) and a DFC 420C camera (Leica) were used. The data obtained were processed using ImageJ software and Photoshop (Adobe).

**Results**

*Human HOX genes are expressed in pancreatic cancer cells*

The four pancreatic cancer cell lines were subjected to PCR to obtain information regarding HOX gene expression (Table 1 and Supplemental Table 1). From this data set it is clear that most HOX genes are expressed in the pancreatic cancer cell lines; HOXD4, HOXD8, HOXD9 and HOXD13 were not detected in any of the cell lines. As an internal assay control, GAPDH expression was analysed by PCR of the same RNA samples.

Table 1

Overview of HOX gene silencing scores in all experiments

| Not expressed in any cell line | Expressed but no effects | Effect in PaTu 8988t: migration | Effect patut 8988t: dissemination | Effect patu 8988s: dissemination | Effect: AsPC-1: dissemination | Effect Panc-1: dissemination | Effect in PaTu 8988t: angiogenesis |
|---|---|---|---|---|---|---|---|
| | A6, A13 | A1, A2, | A1, A2, A7, *A9* (induced) | [A4] | A2, A5, A7, A9 | A2, A5, A7, A9 | A1, *A2*, (induction), A3 and A5 |
| | B6, B13 | B2, B8, B9 | B2, B8, B9 | [B1, B3] | B9, B5 (induced) | B1, B3 (induced), B2, B8, B9 | B1,B3, B7, B9, B8, |
| | C6, C8, C11, C12 | C9, C10 | C9, *C10* (induced) | [C5, C13] | *C10, C13* (both induction) | | *C4, C10* (both induced) |
| D4,D8, D11, D13 | | D10 | D10 | [D9, D11] | | | D10 |

HOX genes shown in italic were observed to induce a malignant feature. The genes that reduced malignant processes are shown in normal text.

*HOX genes affect pancreatic cancer cell migration* in vitro

Despite their well-documented role in regulation of morphogenesis and development in vertebrate and non-vertebrate species limited information is available on the function of HOX genes in cell migration and invasion.

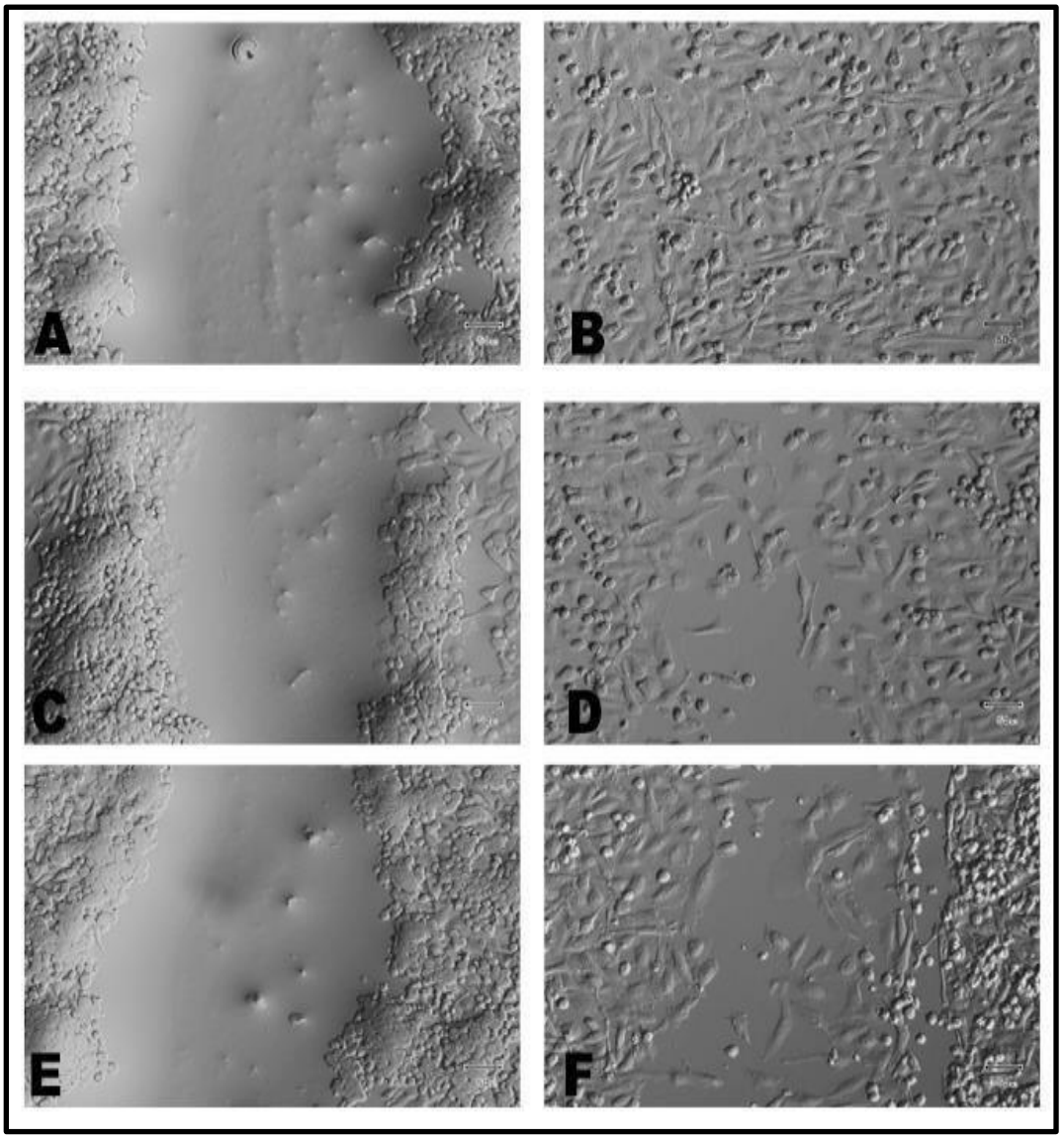


Figure 1a: An *in vitro* migration assay ('scratch assay') shows differences in migration of PaTu 8988t cells treated with control siRNA (A,B), HOXC9 siRNA (C,D), HOXB9 siRNA (E,F). The effects shown in these pictures

were representative for the other HOX genes that show an effect. In pictures A, C and E, the gap is photographed immediately after application of the scratch. In pictures B, D and F, the same area is photographed after 48 hours.

Gene specific siRNAs were used to individually silence all 39 human HOX genes in PaTu 8988t cell line. The results of the *in vitro* migration assay using PaTu 8988t cells showed that knock down of HOXA1, HOXA2, HOXA10, HOXB2, HOXB8, HOXB9, HOXC9, HOXC10, HOXD8, HOXD9 and HOXD10 had a negative effect on *in vitro* migration and led to a decrease in gap closure (Figure 1). These observations suggest a role for these HOX genes in invasion or migration of pancreatic cancer cells.

*HOX genes are involved in metastatic behaviour of pancreatic cancer cells* in vivo

To study the role of the human HOX genes in metastatic behaviour, four pancreatic tumour cell lines were labelled with a carbocyanine dye (CM-DiI) and ectopically transplanted in the yolk sac of 2 dpf zebrafish embryos of the transgenic zebrafish line, Tg(fli1:eGFP), which express GFP under the fli1 promotor (an early endothelial marker) and therefore exhibit a green fluorescent vasculature (Lawson N.D. and Weinstein B.M., 2002). Gene specific and control siRNAs were used to individually silence all 39 HOX genes and the effects in the zebrafish xenotransplantation model were observed.

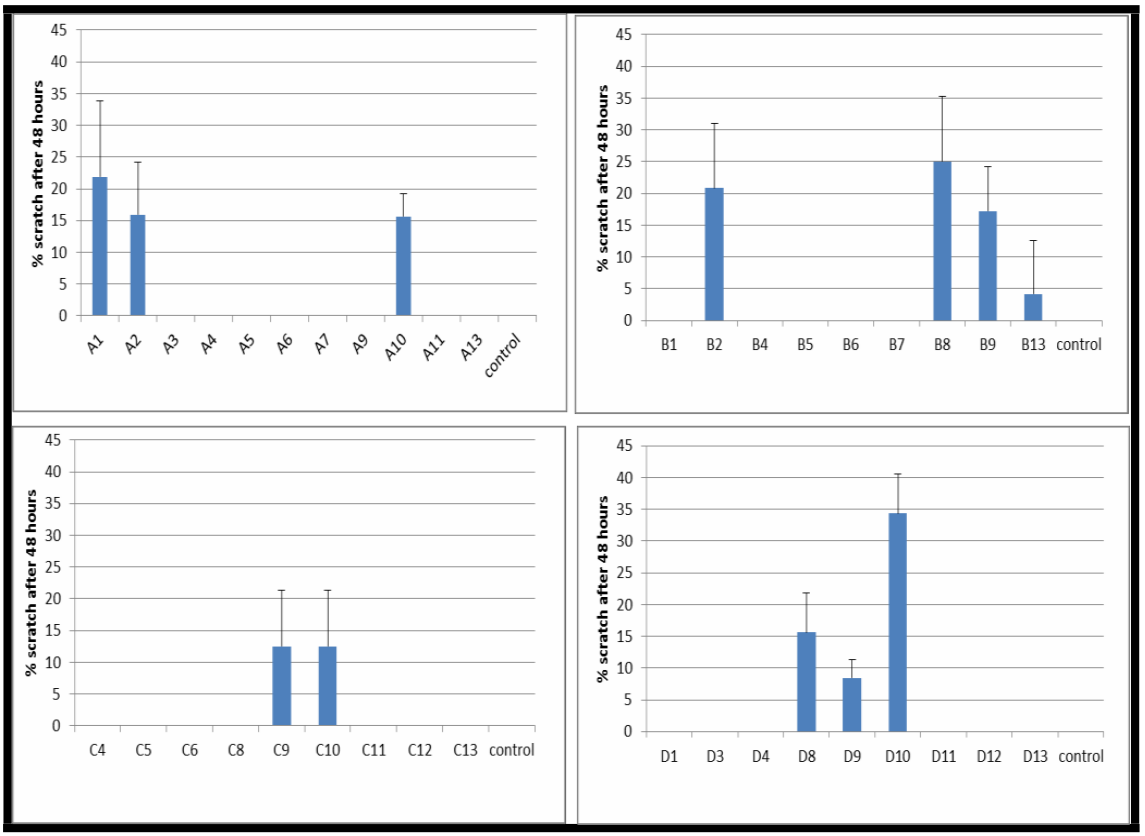


Figure 1b: Gap closure in the migration assay. Gap closure (gap width) over time is shown in percentages compared to the 0h time point (set to 100%). Error bars show standard deviations between three independent experiments.

The results (Table 1 and Figure 2a and b) showed that several HOX genes play important roles in metastatic behaviour either positive or negative. HOXA2, HOXA7, HOXA9, HOXB9, HOXC9 knock downs led to a decrease of metastatic behaviour in all three aggressive cell lines whereas gene silencing of HOXA4, HOXB1, HOXB3, HOXC5 and HOXC13 had a positive effect and led to an increase of metastatic behaviour in PaTu 8988s cells, AsPc-1 showed similar effects for HOXB1,HOXB3, HOXC10 and HOXC13.

The results differed to those obtained with the *in vitro* migration assay using PaTu 8990t cells (Figure 1). However, in contrast to the *in vitro* migration assay where many knock downs had no additional inhibitory effect, metastatic behaviour in the zebrafish *in vivo* model (Figure 2) was completely abolished by an additional set of siRNA knock downs. As observed in an earlier study, the findings indicated significant qualitative and quantitative differences between the two assays and they demonstrate an important role for several additional HOX genes in metastatic behaviour of pancreatic cancer cells.

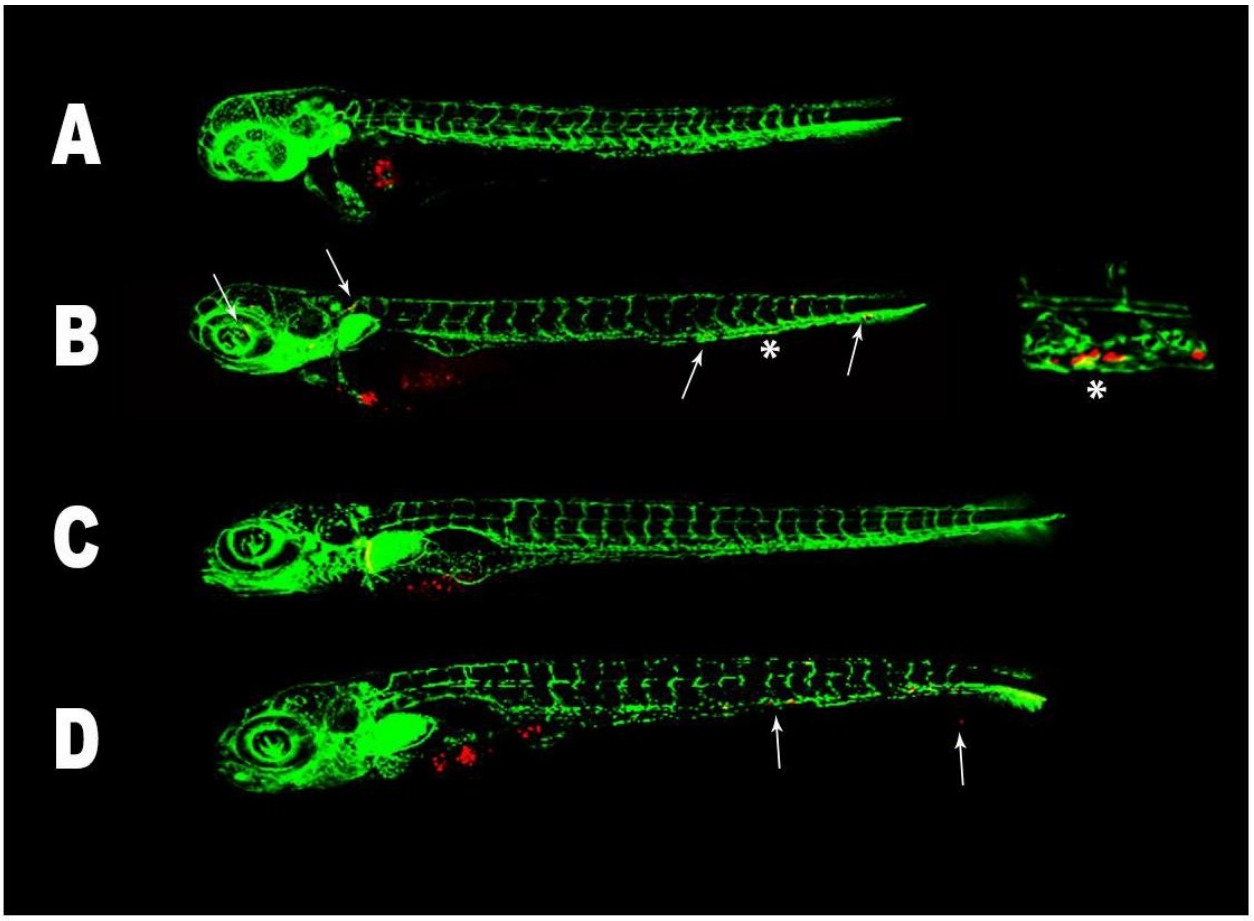


Figure 2: The zebrafish tumour xenograft metastasis assay. Shown are representative pictures taken by confocal microscopy of xeno transplanted. PaTu 8988t cells either treated with the control siRNA (A and C) or treated with the HOX siRNAs (A7 and B7). The pictures show zebrafish embryos 24 hours after transplantation (3 dpf, A, B) and 48 hours post transplantation (5dpf, C,D). In the controls, cancer cells have invaded the embryo and migrated to distant site. This behaviour is blocked by the HOX siRNAs. The area showing many cells with migration behaviour an enlargement is shown (fish B) and here, cancer cells are visible in the vasculature. The arrows show cancer cells which are located in the vasculature. (n=50 embryos per tested siRNA).

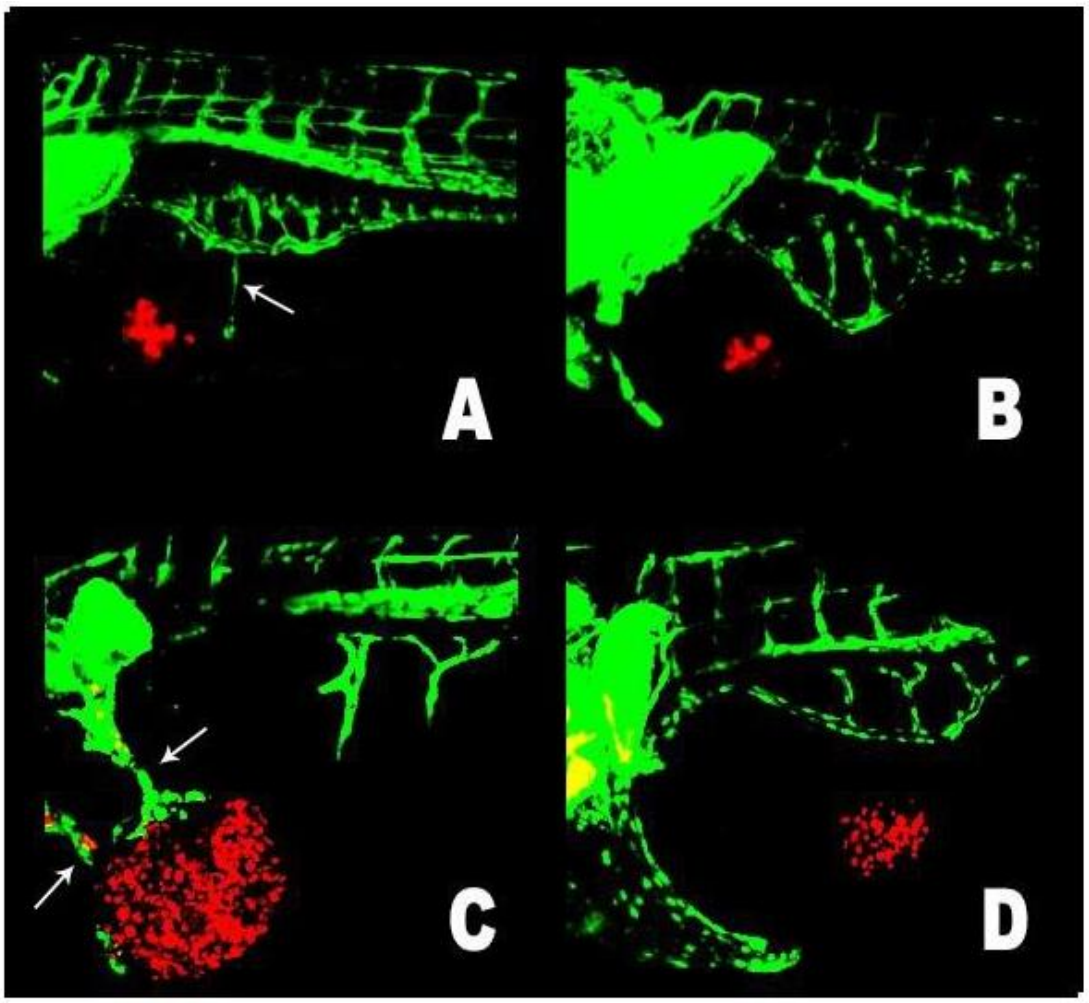


Figure 3: Effects of HOX A2 on angiogenesis in response to tumour cells.

All pictures show PaTu 8990t cells implanted in Tg(:Fli) *Danio Rerio* 36hpf embryos. In Figures A and B similar numbers of cells were injected. Untreated control cells (A and C) cause vessel formation towards the implant. Cells treated with HOXA2 siRNA (and cells treated with siRNAs against other HOX genes showing similar effects listed in Table 1 ) show inhibition of vessel attraction when implanted in zebrafish embryos. In photos C and D, the amount of cells injected is larger than the amount of cells injected in A and B; Pictures A and B were made at one day post injection. Pictures B and C were taken two days post injection. The images shown are examples taken by confocal microscopy of PaTu 8998t cells in which the cells were injected in the perivitelline space of zebrafish embryos. The induction of a new vessel induced by the tumour cells leads from the SIV to the PaTu 8998t cell cluster. Two independent experiments are combined for each treatment and cell type and the number of zebrafish used for each individual treatment was 50.

*Knock down of HOX genes influences tumour cell-induced angiogenesis*

To study tumour angiogenesis *in vivo*, a method is available that is based on the injection of pro-angiogenic mammalian tumour cells into the perivitelline space of zebrafish embryos (Nicoli, S., Presta M., 2007). PaTu 8988t and PaTu 8988s cells, AsPC-1 cells and Panc-1 cells were injected into the perivitelline space of zebrafish embryos at 48h post-fertilization. These pro-angiogenic tumour cell grafts induced an angiogenic response originating from the developing sub intestinal-vessels. In all four pancreatic cancer cell lines, the effects of control siRNA to gene specific siRNA for all 39 human HOX genes were compared.

The results indicate that HOXA1, HOXA2, HOXA5, HOXB1, HOXB3, HOXB7, HOXB9, HOXB8 and HOXD10 knock downs resulted in inhibition of angiogenesis. However, knock downs of HOXA3, HOXC4 and HOXC10 was observed to propagate angiogenesis (Table 2).

**Discussion**

HOX genes regulate cell movement during gastrulation in the developing embryo. Mesoderm cells undergo an EMT as ingression (or involution) takes place. These processes are highly regulated and reflect collinearity: the 3' to 5' sequence of the Hox genes in a Hox cluster matches the sequence in which they act along body axes (HOX1 genes allow involution/ingression earliest). Each Hox gene has a space-time identity on the main body axis of the early embryo. The spatial position of expression and action of these genes is also determined by the developmental age at which they are expressed. This means that HOX1 genes are the first to be expressed and act at the cranial side of the embryo, whereas HOX10 genes determine the difference between thorax and abdomen on the body axis (HOX9 genes work a bit more anteriorly)(Durston AJ. et al., 2011; Durston AJ. et al., 2012).

In the developing embryonic pancreas HOX1 genes, including HOXA1 and HOXD1 are expressed, together with genes from paralogue group 4 (HOXA4, HOXB4, HOXD4) (Slack JM., 1995). These genes seem not to be the most important genes whose signalling affects progression in pancreatic cancer. However, HOXA4 and HOXC4 induced metastasis and angiogenesis respectively (Miller GJ. et al., 2003).

*HOX gene expression varies in different pancreatic cancer cell lines*

Expression of the human HOX complement was examined using PCR on tumour tissue. The results show that not all HOX genes are expressed and from the ones that are expressed, only a part exerts an effect in the performed assays. The last event could be due to redundancy between HOX genes (Meyers PZ., 2007).

HOX genes of the paralogue groups 1 through 9 were previously shown to have the ability of blocking apoptosis (Morgan R. et al., 2014) and it was proposed that up-regulated expression of these genes resulted in a poor prognosis whereas gain of HOX genes of the paralogue groups 10-13 was suggested to result in a higher survival rate (Kachgal S et al., 2012). This is in accordance with our results regarding application of siRNAs against the paralogue group HOX10 and HOXC13 which induced malignant effects in the PaTu 8988s and AsPC-1 cell lines.

*Silencing of HOX genes results in altered migration*

Previously, the behaviour of PaTu 8988t cells in an *in vitro* migration assay was described (Marques I., 2009; Vlecken DH. and Bagowski CP., 2009). The effects of the knock down of HOX genes in PaTu 8988t cells was investigated in an *in vitro* migration assay (Liang CC., 2007).

HOXB2 was shown to be expressed in non-resectable tumours and is associated with a poor prognosis (Alharbi RA., et al., 2013; Gray S. et al., 2011 ) and HOXB2 was previously shown to be involved in metastasis in lung cancer (Boimel PJ., et al., 2007). The data presented here shows that involvement of HOXB2 was merely evident in migration assays performed on PaTu 8988t cells (*in vitro* and *in vivo*).

HOXB5 and HOXB6 were shown to be over expressed in pancreatic tumour tissue (Mizusawa N., et al., 2004) and HOXB6 was shown to be capable of induction of acute myeloid leukaemia as it is able to immortalizing cells (Magli MC., et al, 1997). Additionally, HOXB7 was previously shown to mediate EMT by inducing FGF (fibroblast growth factor) (Wu X., et al., 2006). Also, HOXB7 was shown to be over expressed in pancreatic cancer (AsPC-1 and Panc-1) (Gray S. et al., 2011) and was implicated in metastasis of lung cancer (Chen H., et al., 2008). Our results indicate that HOXB7 is only involved in angiogenesis in PaTu 8988t cells whereas none of the genes of the paralogue group 6 show any reactions to the siRNAs. However, another group of HOX genes was shown to be down-regulated in previous studies of which one is HOXD13, generally involved in development of the digestive and urogenital tract in embryos. HOXD13 (and also HOXB1 and HOXB3) were implied in suppression of metastasis (Gray S., et al., 2011) and therefore loss of HOXD13 is associated with a lower survival rate. Furthermore, it was shown that HOXB1 and HOXB3 are suppressed by miR-10a, which is present in significantly higher concentrations in metastatic pancreatic cancer (Marques I., et al., 2011). This finding confirms our data regarding these two genes on the non-metastable PaTu 8988s cells which disseminate in the absence of RNA of both genes.

The results obtained with the *in vivo* assays differed to those obtained with the *in vitro* migration assay using PaTu 8990t cells (Figure 1). Metastatic behaviour in the zebrafish *in vivo* model (Figure 2) was abolished by an additional set of siRNA knock downs. As observed in an earlier study (Vlecken and Bagowski, 2009), the findings indicated significant qualitative and quantitative differences between the two assays and they demonstrate an important role for several additional HOX genes in metastatic behaviour of pancreatic cancer cells. The differences observed could be a result of embryonic factors that are not present in the media of cultured cells.

*HOX genes play a role in tumour induced angiogenesis*

Previous research showed that HOXA3 increases endothelial cell migration and induces angiogenesis *in vivo* (Mahdipour E., et al., 2011) instead of inhibiting the vessel formation. This is consistent with our results, because the effect that we observed by knocking HOXA3 out was inhibited vessel formation in PaTu 8988t cells. HOXB7 and HOXB9 were also indicated as involved in tumour angiogenesis caused by malignant mammary cells (Hayashida T., et al.,2010; Caré A., et al., 1996),

similar to our results in PaTu 8988t cells where silencing of these genes inhibited vessel formation in the zebrafish embryos.

The pancreas is located in the upper abdomen, near the junction with the thorax. HOX9 and HOX10 are involved in defining this junction during embryonic development. Therefore it can be hypothesized that influence of these genes during carcinogenic processes in pancreatic cancer are location related rather than tumour type specific. Additionally, HOX9 and HOX10 genes are expressed at one of the later stages during embryonic development, due to the space-time identity of HOX genes on the body axes (Durston AJ. et al., 2011 and 2012) and this is in concordance with the age of the pancreatic cancer cells that were used for the experiments.

The pancreatic cancer cells that were used form a representative selection; The PaTu 8988t and PaTu 8988s cell lines used in this study originated from the same primary tumour (Elsässer HP., 1992). PaTu 8988t was observed to disseminate when transplanted into zebrafish embryos while PaTu 8988s cells did not. Using these cell lines, both induction and reduction of malignant processes can be studied and compared. To expand the dataset, the AsPC-1 cell line and the Panc-1 cell line were added because they originate from different types of tumours of the human pancreas. Panc-1 originates from an epithelioid tumour whereas AsPC-1 cells were derived from an adenocarcinoma (Chen WH., et al., 1982).

Focussing on the effects that were similar for all cell lines, some pronounced features emerge. A number of HOX genes were not expressed according to PCR (Tables 1 and 2), while several other HOX genes are expressed but do not seem to have an effect in any of the assays described (Table 2). These were HOXA13, HOXB13, HOXC8, HOXC11, HOXC12, HOXD4 and HOXD13. Also, HOX genes from the paralogue group 6 (HOXA6, HOXB6, HOXC6) do not exert any effects in the assays performed in this study. Unfortunately, no consensus about oncogenecity or tumour suppressive properties was established in the literature.

Whereas the oncogenic potential of certain HOX genes in human cancers has already been defined (Alharbi RA. et al., 2013; Magli MC. et al., 1997; Gray S et al., 2011; Shah N. and Sukumar S., 2010) , their role in neoplasms is still being evaluated. Progress has been hampered by the experimental approach used in many studies in which the expression of small subsets of HOX genes was analysed, and complicated by the functional redundancy implicit in the HOX gene system. Attempts to elucidate the function of HOX genes in malignant transformation will be enhanced by a better understanding of their upstream regulators and downstream target genes. Secondary tumours arising from cancer cell metastasis are the major cause of death in cancer patients, and currently no anti-metastatic drugs are available for therapy. Moreover, the mechanisms that regulate metastatic

behaviour remain so far only partially understood and current models envision metastasis as a highly dynamic multi-step process.

The zebrafish xenograft metastasis model used in this study provides the means to quantitatively study these dynamic processes in high-resolution in real time. Moreover, the zebrafish xenograft angiogenesis model allows further the investigation of tumour cell-induced angiogenesis in living embryos (Marques I., et al., 2009, Nicoli S., 2007 and 2008). It is important to note, that substantial evidence is accumulating that demonstrates functional conservation of human and zebrafish vascular biology and many pro-angiogenic genes have been described in both fish and men (Bussmann J., 2008; Isogai S., 2003). Two zebrafish xenograft assays were used as powerful tools to address and compare the role of the 39 human HOX genes in metastatic behaviour and tumour cell-induced angiogenesis of human pancreatic cancer cells. The zebrafish xenograft metastasis and angiogenesis assays are well suited to help in delineating (e.g. by reverse genetics using siRNAs) the responsible mechanisms and will assist in discovering novel anti-metastatic drugs in the future.

**Conclusion**

Whereas the oncogenic potential of certain HOX genes in human cancers has already been reported, the role of these genes in neoplasms is still being evaluated. Progress has been hampered by the experimental approach used in many studies in which the expression of small subsets of HOX genes was analysed, and complicated by the functional redundancy in the HOX gene system. Attempts to elucidate the function of HOX genes in malignant transformation will be enhanced by a better understanding of their upstream regulators and downstream target genes. Secondary tumours arising from cancer cell metastasis are the major cause of death in cancer patients, and currently no anti-metastatic drugs are available for therapy. Moreover, the mechanisms that regulate metastatic behaviour remain so far only partially understood and current models envision metastasis as a highly dynamic multi-step process. We have shown that several HOX genes are implicated in the process of tumourigenesis in pancreatic cancer cells. Future work will be needed to elucidate the molecular and cellular mechanisms involved.

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**Supplemental Tables**

Supplemental Table 1

RT-PCR of pancreatic cancer cell lines

| HOX gene | PaTu 8988t | PaTu 8988s | AsPC-1 | Panc-1 |
|---|---|---|---|---|
| A1 | Expressed | Expressed | Expressed | Expressed |
| A2 | Expressed | Expressed | Expressed | Expressed |
| A3 | Expressed | Expressed | Expressed | Expressed |
| A4 | Expressed | Expressed | Expressed | No expression |
| A5 | Expressed | Expressed | Expressed | Expressed |
| A6 | Expressed | Expressed | Expressed | Expressed |
| A7 | Expressed | Expressed | Expressed | Expressed |
| A9 | Expressed | Expressed | Expressed | Expressed |
| A10 | No expression | No expression | Expressed | No expression |
| A11 | Expressed | Expressed | Expressed | Expressed |
| A13 | Expressed | Expressed | Expressed | Expressed |
| B1 | Expressed | Expressed | Expressed | Expressed |
| B2 | Expressed | Expressed | Expressed | Expressed |
| B3 | Expressed | Expressed | Expressed | Expressed |
| B4 | Expressed | Expressed | Expressed | Expressed |
| B5 | Expressed | Expressed | Expressed | Expressed |
| B6 | Expressed | Expressed | Expressed | Expressed |
| B7 | Expressed | Expressed | Expressed | Expressed |
| B8 | Expressed | Expressed | Expressed | Expressed |
| B9 | Expressed | Expressed | Expressed | Expressed |
| B13 | No expression | No expression | Expressed | Expressed |
| C4 | Expressed | Expressed | Expressed | Expressed |
| C5 | Expressed | Expressed | No expression | Expressed |
| C6 | Expressed | Expressed | Expressed | Expressed |
| C8 | Expressed | Expressed | Expressed | Expressed |
| C9 | Expressed | Expressed | Expressed | Expressed |
| C10 | Expressed | Expressed | Expressed | Expressed |
| C11 | Expressed | Expressed | Expressed | Expressed |
| C12 | Expressed | Expressed | Expressed | Expressed |
| C13 | Expressed | Expressed | Expressed | Expressed |
| D1 | Expressed | Expressed | Expressed | Expressed |
| D3 | No expression | No expression | No expression | Expressed |
| D4 | No expression | No expression | No expression | No expression |
| D8 | No expression | No expression | No expression | No expression |
| D9 | No expression | No expression | No expression | No expression |
| D10 | Expressed | No expression | No expression | Expressed |
| D11 | Expressed | No expression | Expressed | No expression |
| D12 | Expressed | Expressed | Expressed | Expressed |
| D13 | No expression | No expression | No expression | No expression |

Supplemental Table 2

Overview of HOX gene silencing scores in all experiments

| Not expressed in any cell line | Expressed but no effects | Effect in PaTu 8988t: migration | Effect patut 8988t: dissemination | Effect patu 8988s: dissemination | Effect: AsPC-1: dissemination | Effect Panc-1: dissemination | Effect in PaTu 8988t: angiogenesis |
|---|---|---|---|---|---|---|---|
| | A6, A13 | A1, A2, | A1, A2, A7, *A9* (induced) | [A4] | A2, A5, A7, A9 | A2, A5, A7, A9 | A1, *A2*, (induction), A3 and A5 |
| | B6, B13 | B2, B8, B9 | B2, B8, B9 | [B1, B3] | B9, B5 (induced) | B1, B3 (induced), B2, B8, B9 | B1,B3, B7, B9, B8, |
| | C6, C8, C11, C12 | C9, C10 | C9, *C10* (induced) | [C5, C13] | *C10, C13* (both induction) | | *C4, C10* (both induced) |
| D4,D8, D11, D13 | | D10 | D10 | [D9, D11] | | | D10 |

HOX genes shown in italic were observed to cause an effect in the tested cells when knocked down. The genes that reduced malignant processes are shown in normal text.

Supplemental Table: Scratch assay results

| | absolute distance gap | | | | | | | abs<Jute distance gap | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | day1 | | | | | | | day2 | | | | | | | | | | | |
| | m1 | m2 | m3 | m4 | avg | sd | **gap | ml | m2 | m1 | m4 | avg | sd | **gap day 2 | | | | avg% | sd |
| A1 | 21,875 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 50 | 50 | ISO | 100 | 87,5 | 47,87136 | 12,5 | 12,5 | 37,5 | 25 | 21,875 | 11,96784 |
| A. | 15,9375 | 250 | 300 | 400 | 400 | 337,5 | 75 | 100 | 50 | 75 | 50 | 25 | 50 | 20,41241 | 20 | 25 | 12,5 | 6,25 | 15,9375 | 8,252209 |
| A1 | 0 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| M | 0 | 400 | 400 | 400 | 250 | 362,5 | 75 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f.5 | 0 | 400 | 300 | 300 | 400 | 350 | 57,73503 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f.6 | 0 | 250 | 400 | 400 | 300 | 337,5 | 75 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| A7 | 0 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B9 | 0 | 400 | 275 | 350 | 400 | 356,25 | 59,0727 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| A10 | 15625 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 50 | 75 | 75 | 50 | m1 | 1443376 | 125 | 1875 | 1875 | 125 | 15625 | 3608439 |
| A11 | 0 | 400 | 400 | 300 | 400 | 375 | 50 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| All | 0 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 81 | 0 | ISO | 350 | 175 | 400 | 268,75 | 124,7915 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 82 | 2083333 | 300 | 400 | 400 | 400 | 375 | 50 | 100 | 100 | 100 | 50 | 50 | 75 | 2886751 | 33,33333 | 25 | 12,5 | 12,5 | 20,83333 | 10,20621 |
| B4 | 0 | 270 | 400 | 300 | 400 | 342,5 | 67,51543 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B5 | 0 | 175 | ISO | 175 | 250 | 187,5 | 43,30127 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 86 | 0 | 375 | 400 | 400 | 400 | 393,75 | 12,5 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B7 | 0 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B8 | 25 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 50 | ISO | 100 | 100 | 100 | 40,82483 | 12,5 | 37,5 | 25 | 25 | 25 | 10,20621 |
| B9 | 17,23485 | 400 | 400 | 275 | 300 | 343,75 | 65,74889 | 100 | 50 | 50 | 75 | 50 | 56,25 | 12,5 | 12,5 | 12,5 | 27,27273 | 16,66667 | 17,23485 | 6,974224 |
| B11 | 4,166667 | ISO | 400 | 400 | 400 | 337,5 | 125 | 100 | 25 | 0 | 0 | 0 | 6,25 | 11,5 | 1666667 | 0 | 0 | 0 | 4166667 | 8,333333 |
| C4 | 0 | 300 | 400 | 400 | 400 | 375 | 50 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C5 | 0 | 300 | 350 | 300 | 300 | 312,5 | 25 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C6 | 0 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C8 | 0 | 175 | ISO | 100 | 100 | 111,25 | 37,5 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C9 | 12,5 | 200 | 200 | 400 | 400 | 300 | 115,4701 | 100 | 50 | 25 | 25 | 25 | 31,25 | 12,5 | 25 | 12,5 | 6,25 | 6,25 | 12,5 | 8,838835 |
| C10 | 12,5 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 100 | 50 | 25 | 25 | 50 | 35,3553 | 25 | 12,5 | 6,25 | 6,25 | 12,5 | 8,838835 |
| C11 | 0 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C12 | 0 | ISO | 200 | 350 | 400 | 275 | 119,0238 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C11 | 0 | 300 | 400 | 400 | 400 | 375 | 50 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 01 | 0 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 03 | 0 | 300 | 400 | 200 | ISO | 262,5 | 110,8678 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 04 | 0 | 400 | 350 | 300 | 400 | 362,5 | 47,87136 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 08 | 15,625 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | 50 | 50 | 100 | 50 | 62,5 | 25 | 12,5 | 12,5 | 25 | 12,5 | 15,625 | 6,25 |
| 09 | 8,4375 | 375 | 300 | 400 | 400 | 368,75 | 47,32424 | 100 | 25 | 25 | 50 | 25 | 31,25 | 12,5 | 6,666667 | 8,333333 | 12,5 | 6,25 | 8,4375 | 2,853989 |
| 010 | 34,375 | 400 | 400 | 400 | 400 | 400 | 0 | 100 | ISO | 100 | ISO | ISO | 137,5 | 25 | 37,5 | 25 | 37,5 | 37,5 | 34,375 | 6,25 |
| 011 | 0 | ISO | 275 | 250 | 400 | 268,75 | 102,8247 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

## Chapter 3: LIMK1 and LIMK2 are important for metastatic behaviour and tumour cell-induced angiogenesis of pancreatic cancer cells.

**Vlecken DH, Bagowski CP**

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**Abstract**

The two LIM kinases, LIMK1 and LIMK2, are members of the PDZ/LIM family. These serine/threonine protein kinases are involved in actin cytoskeleton reorganization through phosphorylation and inactivation of ADF (actin depolymerizing factor)/cofilin. Different subcellular localizations of LIMK1 and LIMK2 suggest different functions. Whereas LIMK1 is implicated in microtubule disassembly in endothelial- and cancer cells, activation of LIMK2 plays a role in cell cycle progression. To compare the role of the two LIM kinases in cancer related processes, we used a cell-based *in vitro* migration assay, as well as two zebrafish xenograft assays. We analyzed here the metastatic behaviour and tumour cell-induced neovascularization of pancreatic cancer cells in which both LIM Kinase genes were silenced by siRNAs. Both LIMK1 and LIMK2 single knock down led to a reduction of invasion and metastatic behaviour in the zebrafish xenograft metastasis assay. Interestingly, the double knock down completely blocked invasion and formation of micrometastasis *in vivo*. Moreover, in the zebrafish xenograft angiogenesis assay, we observed a reduction of pancreatic cancer cell-induced angiogenesis for both of the LIMK1 and the LIMK2 knock downs. Our results demonstrate similar functions for the two LIM kinases in pancreatic cancer cells and suggest an important role for both, LIMK1 and LIMK2, in tumour progression and metastasis formation.

**Introduction**

Tumourigenesis involves interactions of cancer cells with each other, neighboring cells and their microenvironments, such as stromal cells or the blood vessels that supply oxygen and nutrients. Cancer cells often induce surrounding endothelial cells to build new vessels in order to balance this supply with the demand of a growing tumour. Blood vessels (as well as lymphatic vessels) are further used by metastasizing cancer cells for their long and short distance traveling. The complex mechanisms that regulate these "nomadic" cancer cells and governs the formation of metastases involves intravasation and extravasation in and out of blood- or lymphatic vessels. In order to successfully form a micrometastasis, cancer cells first need to take advantage of the vasculature and/or the lymphatics, and then after extravasation, have to settle and survive in an often hostile microenvironment. The importance to understand and prevent metastasis formation is underlined by the fact that over 90% of all cancer related deaths are due to secondary tumours arising from metastasizing cancer cells.

The zebrafish has emerged as a novel *in vivo* cancer model to study both tumour-induced neovascularization (Nicoli, S., Presta M., 2007; Nicoli S., Ribatti D., 2007) and the formation of micrometastases of xeno-transplanted cancer cells (Haldi M., et al, 2006; Topczewska JM., et al,2006) and tumour fragments (Marques I., et al, 2009). In the transparent zebrafish embryos, invasion/ migration of tumour cells, their circulation in the vascular system, as well as the formation of micrometastasis and the tumour-induced neovascularization can all be followed with high resolution in real-time (Marques I., et al, 2009). Importantly, these zebrafish models allow to quantitate both metastatic behaviour of transplanted tumour cells and tumour-cell induced neovascularization (Nicoli, S., Presta M., 2007; Marques I., et al, 2009). In this study we use these models to characterize and compare the role of the two LIM Kinases in tumourigenesis of human pancreatic cancer cells.

The LIM Kinases Leucine-rich immunoglobulin-like motif LIMK) are Serine/Threonine Kinases, which regulate actin polymerziation via phosphorylation and inactivation of ADF/cofilin (reviewed in (te Velthuis AJ., et al, 2007)). Actin dynamics contribute to cell motility, morphogenesis, division, differentiation, apoptosis and cancer. Given the importance of the cytoskeleton for most cellular functions, it is not surprising that the LIMKinases are ubiquitously expressed during development, and that disruption of normal LIMK expression and/or signaling is associated with numerous disorders. For example, abnormal expression of LIMK1 in humans has been associated with Williams syndrome (a severe mental disorder with profound deficits in visuospatial cognition) and Alzheimer's disease (Heredia L., et al, 2006; Tassabehji M., et al, 1996). Two LIM Kinase genes are encoded in the human genome. The human LIMK1 gene is located on chromosome 7 (7q11.23) and the LIMK2 gene

is located on chromosome 22 (22q12) (Mao X JT., et al, 1996; Okano I., et al,1995). Both genes encode for proteins with two N-terminal LIM domains, a C-terminal kinase domain and a PDZ domain in between. In addition to their role in regulating the actin cytoskeleton, LIMK1 has also been shown to play a role in microtubule disassembly (Gorovoy M., et al, 2005).

In mice, ablation of LIMK1 leads to abnormalities in dendritic spine morphology and in synaptic function (Meng Y., et al, 2002). In contrast to *Limk1* null mice, *Limk2* knockout mice do not exhibit any impaired neuronal morphology or other phenotypic abnormalities in postnatal growth and development, except for impairment in spermatogenesis (Takahashi H., et al, 2002). However, when a double knockout was applied, the neuronal phenotype appeared more pronounced than in the genetic deletion of *Limk1* alone (Meng Y., et al, 2004). For both LIM Kinases, cell type specific functions have been suggested and their cellular localization was found to differ in vertebrates (Acevedo K., et al, 2006; Ott EB., et al,2007). While LIMK1 is found in focal adhesions, LIMK2 is shown to be localized in endosome-like punctae (Acevedo K., et al, 2006). Recently, it was demonstrated that activation of LIMK2, but not LIMK1, by ROCK (Rho-associated, coiled-coil containing protein kinase) in 3T3 cells led to increased levels of cyclin A and cell cycle progression (Croft DR., Olson MF., 2006). Increased cell cycle progression was also evident in MDA-MB-435 cancer cells overexpressing LIMK1 (Rozita Bagheri-Yarmand AMAASRK, 2006). Thus both LIM Kinases have been shown to play a role in cell cycle progression, but in a cell-type specific manner (Sumi T., et al, 2002).

To date, robust evidence suggests that LIMK1 activity is important for cancer cell metastasis. High expression levels of LIMK1 are observed in invasive breast and prostate cancer cell lines and in malignant human prostate tumours (Wang W., et al, 2006). Overexpression of LIMK1 increases the invasiveness of non-invasive breast and prostate cancer cells *in vivo* and *in vitro*; while expression of dominant-negative kinase-dead LIMK1 or LIMK1 anti-sense RNA greatly reduced the invasiveness of breast and prostate cancer cells, respectively (Wang W., et al, 2006). The mechanism by which LIMK1 increases the invasiveness of cancer cells is not yet clear. It may be due to increased cell cycle progression, increased motility and also as recently suggested to increased expression levels of the serine protease urokinase type plasminogen activator (uPA) and its receptor, known to be involved in metastasis (Rozita Bagheri-Yarmand AMAASRK, 2006). These latter findings suggest an important role for LIMK1 signaling in breast cancer tumour growth, angiogenesis and invasion and a regulatory connection between LIMK1 and the uPA system (Rozita Bagheri-Yarmand AMAASRK, 2006). Whether all these mechanisms are important in tumour progression or if only a few play a part, is not known and needs to be determined in the future. Notably, in contrast to LIMK1, the role of LIMK2 in tumour progression and metastatic behaviour of cancer cells has not been described so far.

In this study, we have investigated the function of LIMK1 in metastatic behaviour of pancreatic cancer cells and compared it to the role of LIMK2. We further tested the hypothesis that both LIM Kinases might play a role in tumour cell-induced neovascularization.

**Materials and Methods**

*Animal care and handling*

Tg(fli1:eGFP) zebrafish were kept at 28°C in aquaria with day/night light cycles (10h dark versus 14h light periods). The developing embryos were kept in an incubator at constant temperatures (which were varied according to the experimental requirements).The zebrafish were handled in compliance with local animal care regulations and standard protocols of the Netherlands.

*Cell culture*

The two human pancreatic cancer cell lines, PaTu 8988t (Elsässer HP LU., et al, 1992) were cultured in DMEM high glucose, with 10% FCS and 1:100 Pen/Strep and Panc-1 (Lieber M MJ., et al, 1975) were cultured in RPMI with 15% FCS and 1:100 Pen/Strep. PaTu 8988t were derived from a liver metastasis over a decade ago (Elsässer HP LU., et al, 1992) and we had earlier described the cell line in detail and its behaviour in an *in vitro* wound healing assay and in the zebrafish xenotransplantation assay (Marques I., et al, 2009).

*siRNA treatment of pancreatic cancer cells and control PCR*

The siRNAs for silencing human LIMK1 and human LIMK2 were purchased from Santa Cruz Biotechnology (LIMK-1: sc-35810 and LIMK-2: sc-35812). The EndoPorter system (Gene Tools, LLC) was used to deliver siRNAs into pancreatic cancer cells. To test for unspecific effects, a control siRNA (Control siRNA-A sc-37007; Santa Cruz Biotechnology) was used, which contains a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. All siRNAs were used at a final concentration of 50 nM. The efficiencies of LIMK-1 and LIMK-2 siRNAs were tested by an RT-PCR, which we described previously including total RNA preparation (van der Meer DLM, et al, 2006). The annealing temperature was set to 55° C and the extension temperature to 72° C. For the amplification, the commercially available primer pairs of human LIMK1 and LIMK2 were used (LIMK1 primer pair: sc-35810-PR fragment size = 419 bp and LIMK-2 primer pair: sc-35812 fragment size = 594 bp; both Santa Cruz Biotechnology).

*Cell staining, transplantation and incubations*

Cells were stained with CM-Dil (red fluorescence) (Vybrant, Invitrogen). Cells were seeded in 6-well plates, grown to confluency and then trypsinized. Subsequently, cells were washed with 67% DPBS (GIBCO, Invitrogen), transferred to 1.5 ml Eppendorf tubes and centrifuged 5min, at 1500rpm. Cells were re-suspended in DPBS (Dulbecco's phosphate buffered saline) containing CM-Dil (4ng/ul concentration). Cells stained with CM-Dil were incubated 4 min at 37$^{o}$C and then 15 min at 4$^{o}$C.

Subsequently, cells were centrifuged 5 min at 1500 rpm, the supernatant discarded and cells re-suspended in 100% FCS, centrifuged again and washed 2 times with 67% DPBS. Cells were now suspended in 67% DPBS for injection into the embryos. 2 dpf zebrafish embryos were dechorionated and subsequently anesthetized with tricaine (Sigma). Using a manual injector (Eppendorf; Injectman NI2), the cell suspension was loaded into an injection needle (15 µm internal- and 18 µm external diameter) and cells were injected in Tg(fli1:eGFP) zebrafish embryos. After injection, embryos were incubated for 1h at 31$^{o}$C and checked for cell presence at 2 hours post transplantation (hpt). Fish with fluorescent cells outside the implantation area at 2 hpt were excluded from further analysis. All other fish were incubated at 35$^{o}$C for the following days.

In vitro *migration assay ("scratch"-assay)*

The *in vitro* migration assay was carried out as previously described (Liang C-C., et al, 2007). Cells were grown to confluency in 6-well dishes. Then the cell monolayer was scraped in a straight line with a 200 µl pipette tip. Pictures of the scratch were taken under a Leica stereomicroscope at 0h, 24h and 48h and the gap was measured and gap closure was calculated in percentage to the initial gap (scratch).

*Zebrafish xenograft angiogenesis model (Tumour-cell induced neovascularization assay)*

The tumour-cell induced neovascularization assay was in general performed as previously described (Nicoli, S., Presta M., 2007; Nicoli S., Ribatti D., 2007). In detail, 48 hpf zebrafish embryos were dechorionated and anesthesized using 0.042 mg/ mL Trincaine (Sigma, St. Luis, MO) and put on a 1,8 % Agarose dish for injections. Cells were prepared as described above, then suspended in 20 µl 12,0 mg / ml Matrigel solution (Cultrex R Basement Membrane Extract, R&D systems, Minneapolis, USA) and kept on ice for a short period until injection. The CellTram Oil injector (Eppendorf, Hamburg, Germany) was loaded by using a manual injector (Eppendorf; Injectman NI2). An aliquot of 5 µl of the cell suspension in Matrigel was loaded into an injection needle, and embryos were injected as described in more detail in previous studies (Nicoli, S., Presta M., 2007; Nicoli S., Ribatti D., 2007). After injection, embryos were incubated at 35°C for 24 h. Embryos were checked and photographed using the Biorad confocal microscope 1024ES (Zeiss).

Imaging

Confocal pictures were taken with the Biorad Confocal microscope 1024ES (Zeiss microscope) combined with a Krypton/Argon laser. For light-microscopy images of pancreatic cells, a Leica MZ16FA stereomicroscope and a Leica DFC 420C camera was used.

**Results**

LIMK1 and LIMK2 are important for migration of human pancreatic cancer cells *in vitro*

Despite their well-documented role in regulation of actin polymerization and microtubule disassembly (Bernard O., 2007), studies comparing other biological functions of LIMK1 and LIMK2 are limited. In contrast to the well described role of LIMK1 in cell migration/invasion e.g. of breast cancer cells (Ding Y., et al, 2008), nothing is known of a role for LIMK2 in these processes.

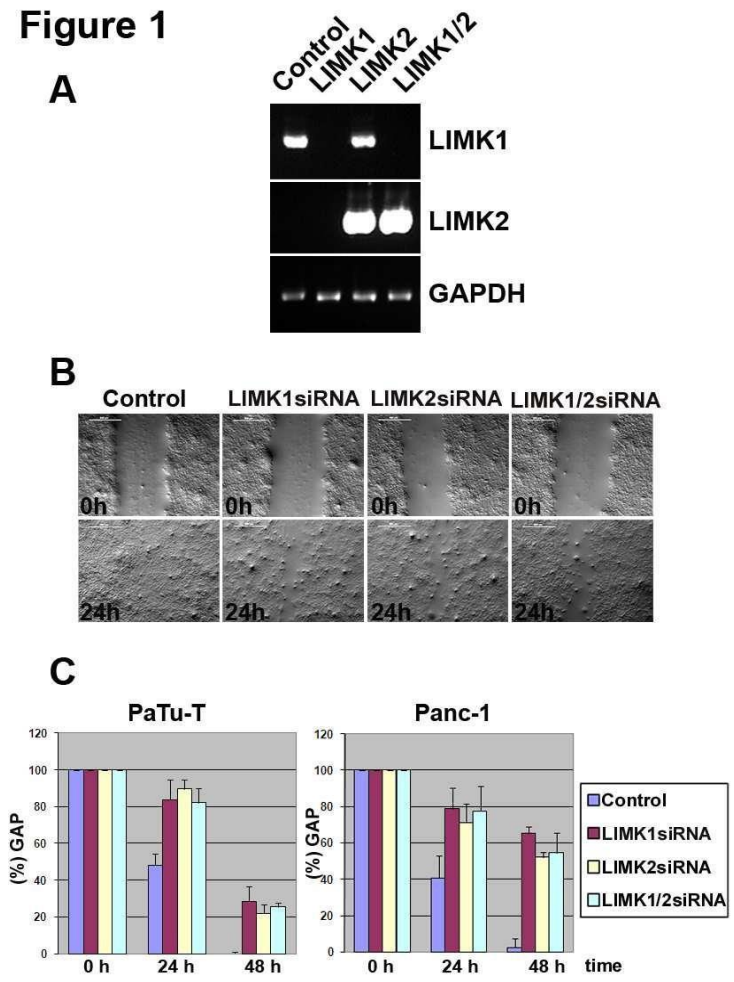


Figure 1: Semi-quantitative RT-PCR and i*n vitro* migration assay of pancreatic cancer cell lines. (A) Semi quantitative RT-PCR shows that LIMK1 and LIMK2 are expressed in PaTu 8988t and in Panc-1 cells and gene specific siRNAs for LIMK1 and LIMK2 silence the respective genes by abolishing mRNA expression. Control GAPDH expression was analyzed by RT-PCR of the same RNA samples. This loading control is shown in the lower panel. LIMK1 specific primers were used in the RT-PCR shown in the top panel and LIMK2 specific amplification is shown

in the middle panel. (B) An *in vitro* migration assay ('scratch assay') shows differences in migration of PaTu 8988t cells treated with control siRNA (Control), LIMK1 siRNA , LIMK2 siRNA or LIMK1 plus LIMK2 siRNA (LIMK1 and 2). Gap closure (gap width) over time is shown in percentages compared to the 0h time point (set to 100%). Similar results were obtained in three independent experiments and error bars show Standard Deviations between the three experiments. On average on day 1 (24 h) for LIMK1 16.4%, LIMK2
10.3%, LIMK1/2 18% and the control 51.8% of the gap was closed. On day 2 (48 h) for LIMK1 53.6%, LIMK2 10.3%, LIMK1/2 18% and the control 51.8% of the gap was found closed. Similar results were obtained in three independent experiments.

Both LIM Kinases had been shown to be expressed in the human pancreas, with LIMK2 showing a relatively high abundance compared to several other tissues (Smolich B., et al, 1997). We found that LIMK1 and LIMK2 are both expressed in the pancreatic cancer cell line, PaTu 8988t (Figure 1A).

In this study, we were interested in the diverse and common functions of LIMK1 and LIMK2 in cancer related processes. In an *in vitro* migration assay (also called *in vitro* scratch assay) (Liang C-C., et al, 2007), we investigated the effects of LIMK1 and LIMK2 knock down in two pancreatic cancer cell lines, PaTu 8988t and Panc-1 cells. We had previously described the behaviour of PaTu 8988t cells in an *in vitro* migration assay (Marques I., et al, 2009). We used gene specific siRNAs to silence the LIMK1 and LIMK2 genes in this cell line and in addition to the respective single knock downs, we performed a LIMK1/2 double knock down. Confirmation of gene silencing in PaTu 8988t cells was obtained by semi-quantitative RT-PCR (Figure1A). The results of the *in vitro* migration assay using PaTu 8988t and Panc-1 cells showed that both, LIMK1 and LIMK2 knock down have an inhibitory effect on gap closure over time (Figure 1B and C). The double knock down had no additive effect and inhibition was comparable to that exerted by both single knock downs (Figure 1B and C). These findings suggest a role for both LIM kinases in invasion/migration of pancreatic cancer cells.

*LIMK1 and LIMK2 both play a role in metastatic behaviour of human pancreatic cancer cells*

In a previous study we have demonstrated that fluorescently labelled PaTu 8988t cells and tumour fragments allow for *in vivo* imaging of metastatic behaviour (Marques I., et al, 2009). PaTu 8988t cells rapidly invaded the zebrafish body, translocated into the vasculature and colonized at distant sites in the zebrafish larvae (5 days post fertilization (dpf)) forming micro-metastases (Marques I., et al, 2009). The sensitivity of the zebrafish tumour xenograft metastasis model allows observation of individual cells and their daughter cells *in vivo* and thereby enables the quantification of metastatic behaviour *in vivo*. To study the role of the LIM Kinases in metastatic behaviour, we labelled two pancreatic tumour cell lines with a carbocyanine dye (CM-DiI) and ectopically transplanted them in the yolk sac of 2 dpf zebrafish embryos. In order to achieve optimal visualization, we utilized the

transgenic zebrafish line, Tg(flil:eGFP) (Lawson ND., et al, 2002), which expresses **GFP** under the *flil* promotor (an early endothelial marker) and therefore exhibits a green fluorescent vasculature (Lawson ND., et al, 2002).

**Figure 2 A**

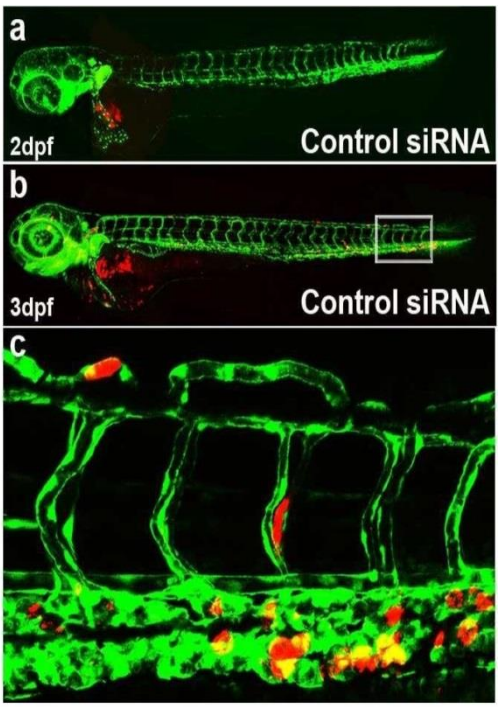


**Figure 2 B**

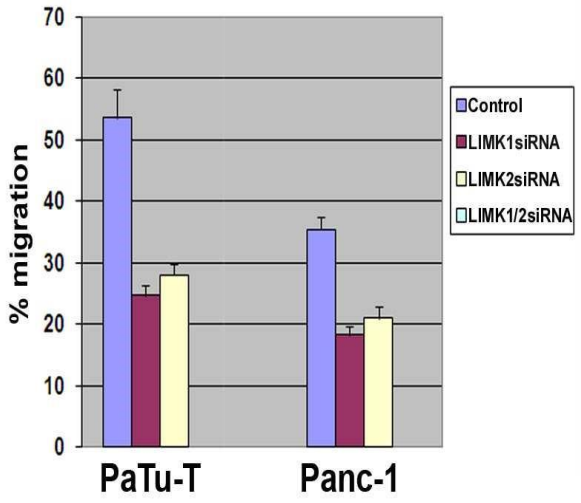


Figure 2:The zebrafish tumour xenograft metastasis assay.

**(A)** Shown are example pictures taken by confocal microscopy of xeno-transplanted PaTu 8988t cells either treated with the control siRNA (a and b) or the LIMK1/2 siRNAs (d and e). The pictures show the zebrafish directly after transplantation (2 dpf) and 24 hours later (3dpf). In the control, cancer cells have invaded the embryo and migrated to distant sites (b). This behaviour is blocked by the double knock down (e). The area marked with a square (b) is shown in an enlargement (c) and here, cancer cells are visible in the vasculature (c). (**B**) Shown are results of the Zebrafish tumour xenograft metastasis assay performed in two different pancreatic cancer cell lines. Two independent experiments are combined for each treatment and cell type. The standard deviations (SD) are shown in brackets. The results are also represented in the graph in Figure 2 and all experiments are shown in Supplemental Table 1 (Vlecken and Bagowski, 2009).

We used once again gene specific siRNAs to silence the LIMK1 and LIMK2 genes individually as well as in combination in the LIMK1/2 double knock down and investigated the effects in the zebrafish xenotransplantation model. Our results, obtained in two different pancreatic cancer cell lines, showed that both LIMK1 and LIMK2 knock down lead to inhibition of metastatic behaviour (Figure 2). These results were not identical but similar to the effects we observed in the *in vitro* migration assay using PaTu 8988t cells (Figure 1B and C). However, in contrast to the *in vitro* migration assay where the double knock down had no additional inhibitory effect, it completely abolished metastatic behaviour in the zebrafish *in vivo* model (Figure 2 A and B). This was observed for both pancreatic cancer cell lines investigated. These findings indicate significant qualitative and quantitative differences between the two assays and they demonstrate an important role for both LIM Kinases in metastatic behaviour of pancreatic cancer cells.

*Knock down of LIMK1 and LIMK2 inhibits tumour cell-induced neovascularization*

Tumour-induced angiogenesis and the recruitment of new blood vessels by solid tumours is an essential component of the process leading to the formation of metastasis. The tumour-induced newly built vessels provide the principal route by which tumour cells exit the primary tumour site and enter the circulation. Recently, it had been shown that human MDA-MB-435 cancer cells xeno-transplanted into athymic nude mice showed not only increased tumour growth but also promoted tumour angiogenesis, when they overexpressed LIMK1 (Rozita Bagheri-Yarmand AMAASRK, 2006). For LIMK2 similar effects had not been described.

Recently, a method has been established to study tumour angiogenesis in zebrafish (*Danio rerio*) based on the injection of pro-angiogenic mammalian tumour cells into the perivitelline space of

zebrafish embryos (Nicoli, S., Presta M., 2007; Nicoli S., Ribatti D., 2007). This zebrafish tumour xenograft angiogenesis assay allows observation of tumour cell-induced angiogenesis in real time.

Here we describe how we used this method to study the role of the two LIM Kinases in tumour cell-induced angiogenesis. We injected two pro-angiogenic human pancreatic cancer cell lines, PaTu 8988t (PaTu 8988t) (Elsässer HP LU., et al, 1992) and Panc-1 (Lieber M MJ., et al, 1975), into the perivitelline space of zebrafish embryos at 48h post-fertilization. These pro-angiogenic tumour cell grafts induce a neovascular response originating from the developing subintestinal vessels. In both pancreatic cancer cell lines, we compared the effects of control siRNA treatment to LIMK1 and LIMK2 gene specific siRNA treatment. We also performed a LIMK1/2 double knock down. Successful gene silencing was controlled by RT-PCR in PaTu 8988t cells (Figure1 A). Our results revealed that both LIMK1 and LIMK2 knock down inhibit tumour cell-induced angiogenesis (Figure 3). The double knock down displayed a slightly additive inhibitory effect but did not lead to complete inhibition of tumour-induced angiogenesis (Figure 3). To the best of our knowledge, this is the first indication that LIMK2 could play a role in tumour angiogenesis.

Figure 3

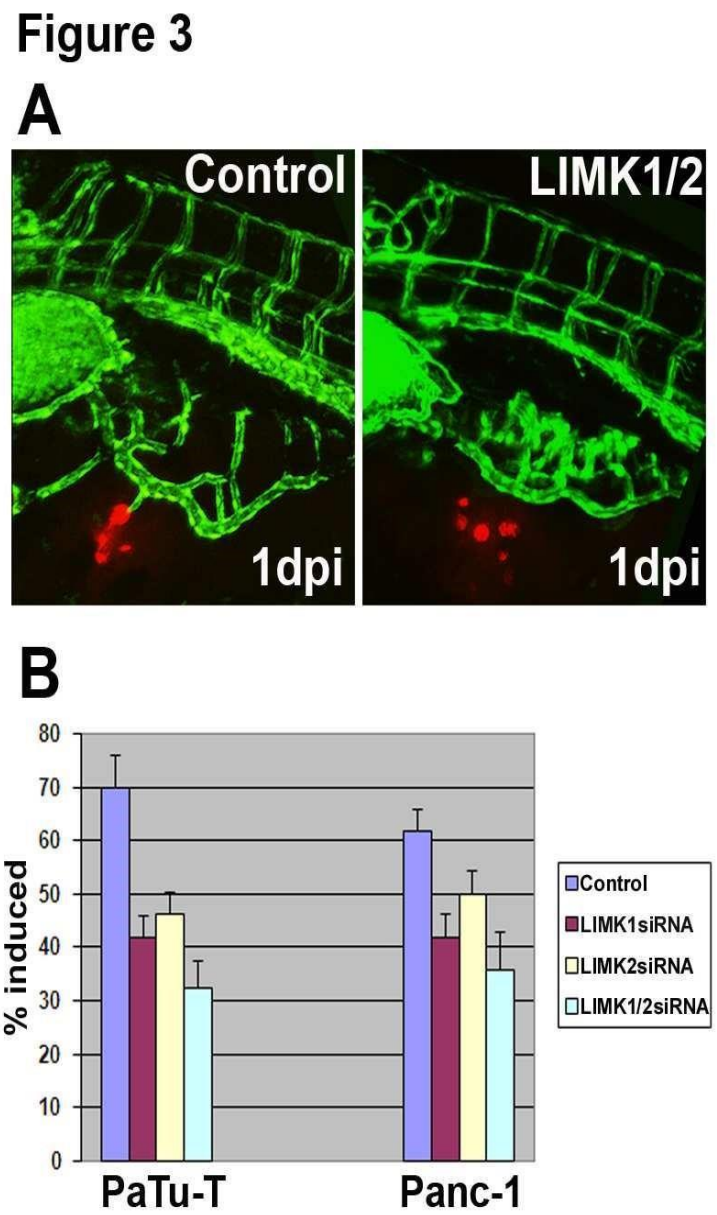


Figure 3: The zebrafish tumour xenograft angiogenesis assay. (A) Images shown are examples taken by confocal microscopy of PaTu 8988t cells 24 hours after cells were injected in the perivitelline space of zebrafish embryos. PaTu 8988t cells were either treated before implantation with the control siRNA (left image) or the LIMK1/2 siRNAs (right image). The picture on the left shows the induction of a new vessel induced by the tumour cells leading from the SIV to the PaTu 8988t cell cluster. This is inhibited by the double knock down shown in the right image. (**B**) Shown are the results of the Zebrafish tumour xenograft angiogenesis assay performed in two different pancreatic cancer cell lines. Two independent experiments are combined for each treatment and cell type and the number of zebrafish used for each individual treatment was 50. Standard deviations (SD) are shown in brackets and the results are also represented in Supplemental Table 1 (Vlecken and Bagowski, 2009.

**Discussion**

Secondary tumours arising from cancer cell metastasis are the major cause of death in cancer patients, and currently no anti-metastatic drugs are available for therapy. Moreover, the mechanisms that regulate metastatic behaviour remain so far only partially understood. The current models envision metastasis as a highly dynamic multi-step process.

The zebrafish xenograft metastasis model used in this study provides the means to quantitatively study these dynamic processes in high-resolution in real time. Moreover, the zebrafish xenograft angiogenesis model allows further the investigation of tumour cell-induced neovascularization in living embryos. It is important to note, that substantial evidence is accumulating that demonstrates functional conservation of human and zebrafish vascular biology and many pro-angiogenic genes have been described in both fish and men (Bussmann J., et al, 2008; Isogai S., et al, 2003).

We used the two zebrafish xenograft assays here as powerful tools to address and compare the role of the LIM Kinases in metastasis and tumour cell-induced angiogenesis of human pancreatic cancer cells. We discovered here that LIMK1, which has been implicated in tumour cell metastasis in various tumour types, is also important for the metastatic behaviour of pancreatic cancer cells. More importantly, our data gives the first evidence that LIMK2 can play a similar role in metastasis formation. Non-overlapping functions of these two kinases in pancreatic cancer cells are demonstrated by the complete abolishment of metastatic behaviour by the double knock down. Interestingly, this differs from our *in vitro* data, where a double knock down had no additional inhibitory effect. A possible explanation is that the results in the *in vitro* migration assay reflect the contribution of the LIM Kinases to the initial step of invasion/migration and in part to cell proliferation, which are both measured in this assay. Cell proliferation, which has been shown to be regulated by both LIM Kinases (Croft DR., Olson MF., 2006; Rozita Bagheri-Yarmand AMAASRK, 2006), certainly plays a partial role in the gap closure times which were measured in the *in vitro* migration assay. Overall, the results point to additional roles (despite regulation of invasion/migration) of the LIM Kinases in later steps of metastasis formation. Similar non-overlapping functions for LIM Kinases have also been implicated in knock-out mice, where double ablation of LIMK1/2 induced a more pronounced neuronal phenotype than in the genetic deletion of *Limk1* alone (Meng Y., et al., 2004). Although LIMK2 ablation alone did not lead to any impaired neuronal morphology, these results revealed redundancies between the two LIM Kinases (Takahashi H., et al, 2002).

Although LIMK1 had been implicated in angiogenesis induced by xeno-transplanted human MDA-MB-435 cells in nude mice (Rozita Bagheri-Yarmand AMAASRK, 2006). No similar function has been

attributed to LIMK2. In our zebrafish xenograft angiogenesis study, we observed a reduction of pancreatic cancer cell-induced angiogenesis for both, the LIMK1 and the LIMK2 single knock downs. However, the double knock down did not exert a complete inhibition and only had a slightly more pronounced inhibitory effect compared to the single knock downs.

The mechanisms by which LIM Kinases increase the invasiveness of cancer cells and their metastatic behaviour and tumour-induced angiogenesis have not been identified yet. The zebrafish xenograft metastasis and angiogenesis assays are well suited to help in delineating (e.g. by reverse genetics using siRNAs) the responsible mechanisms and will assist in discovering novel anti-metastatic drugs in the not so distant future.

In summary, we find here that in addition to LIMK1, LIMK2 is also very important for metastasis of pancreatic cancer cells. A LIMK1/2 double knock down is necessary to completely block metastatic behaviour *in vivo*. We further show that both LIM Kinases play an important role in tumour cell-induced angiogenesis. Our findings propose that simultaneous targeting of both LIM Kinases might be a practical therapeutic approach to inhibit tumour progression and metastasis.

*Acknowledgements*

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*Competing interest statement*

The authors declare that they have no competing interests.

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## Chapter 4: Iridium Complex with Antiangiogenic Properties

**Alexander Wilbuer, Danielle H. Vlecken, Daan J. Schmitz, Katja Kräling, Klaus Harms, Christoph P. Bagowski and Eric Meggers***

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**Abstract**

Substitutionally inert metal complexes are promising emerging scaffolds for targeting enzyme active sites. (Meggers E., 2007) Our group has demonstrated over the last several years that inert ruthenium (II) complexes can serve as highly selective nanomolar and even picomolar inhibitors of protein kinases. (Meggers E., et al., 2007) Octahedral metal coordination geometries in particular offer new gateways to design rigid, globular molecules with defined shapes that can fill protein pockets such as enzyme active sites in a unique fashion (Figure 1) (Maksimoska J., et al., 2008). However, the large number of possible stereoisomers does not only provide new structural opportunities (e.g. the complex illustrated in Figure 1 can form up to 24 stereoisomers if the ligands A-D differ), but also poses a formidable challenge due to the limited ability to control stereochemistry in the course of ligand exchange reactions (Coe B. J. and Glenwright, S. J., 2000). A continued progress in this area of inorganic medicinal chemistry therefore requires the development of strategies for the stereo-controlled synthesis of octahedral metal complexes. Although most of our previous efforts were focused on ruthenium (II) complexes, we envisioned that octahedral iridium(III) (Ir(III)) complexes might be attractive scaffolds for two reasons: First, coordinative bonds with Ir(III) tend to be very inert (J. Burgess, 1972) and therefore Ir(III) complexes should be able to serve as stable scaffolds for the design of enzyme inhibitors (Kwon T.H. et al., 2008; Yu M. et al., 2008; K. K. W. Lo et al., 2008). Second, octahedral Ir(III) complexes can be accessed from square planar Ir(I) complexes by stereospecific oxidative addition reactions (Mondal J. U. and Blake D. M., 1982. This provides a powerful tool to control the stereochemistry of octahedral Ir(III) complexes. In this study, we present the discovery of a bioactive octahedral iridium (III) complex, synthesized through oxidative addition as the key synthetic step. The organometallic compound functions as a nanomolar and selective inhibitor of the protein kinase Flt4 (Fms-related tyrosine kinase 4), also known as VEGFR3 (vascular endothelial growth factor receptor 3) (Karkkainen M. J. and Petrova T., 2000). Flt4 is involved in angiogenesis and lymphangiogenesis (Karkkainen M. J. and Petrova T., 2000) and we demonstrate that this nontoxic organoiridium compound can indeed interfere with the development of blood vessels *in vivo* in two different zebrafish angiogenesis models.

**Experimental procedures and results**

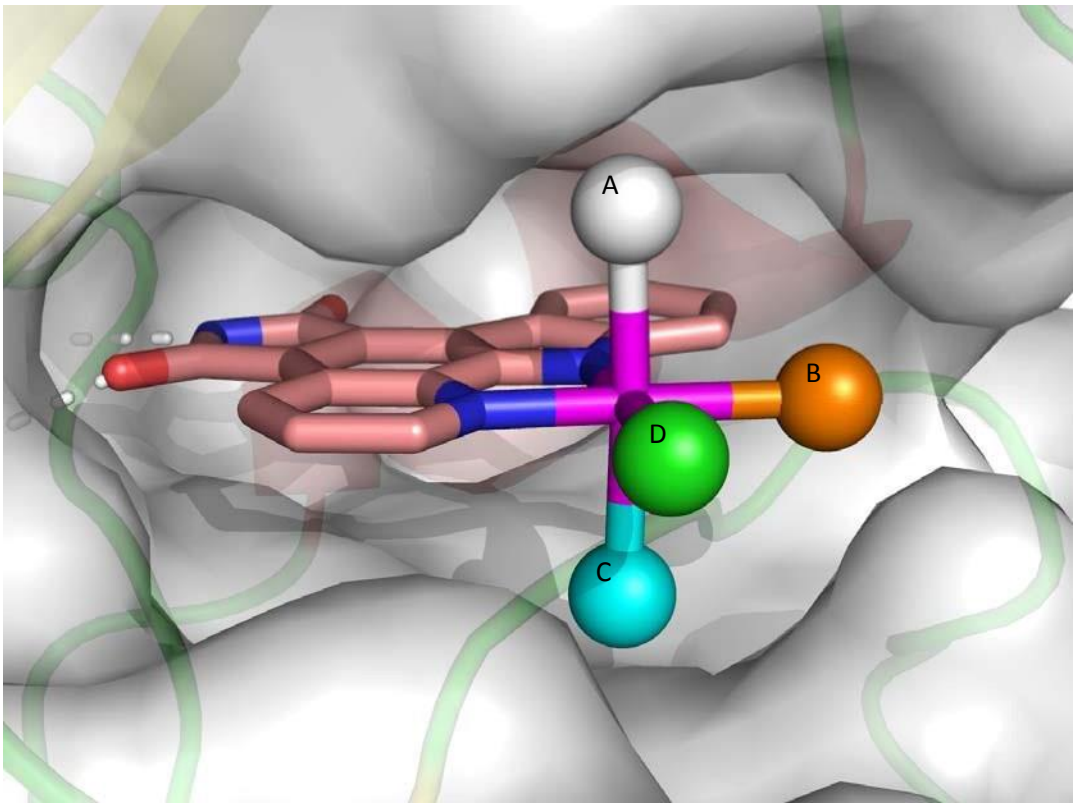
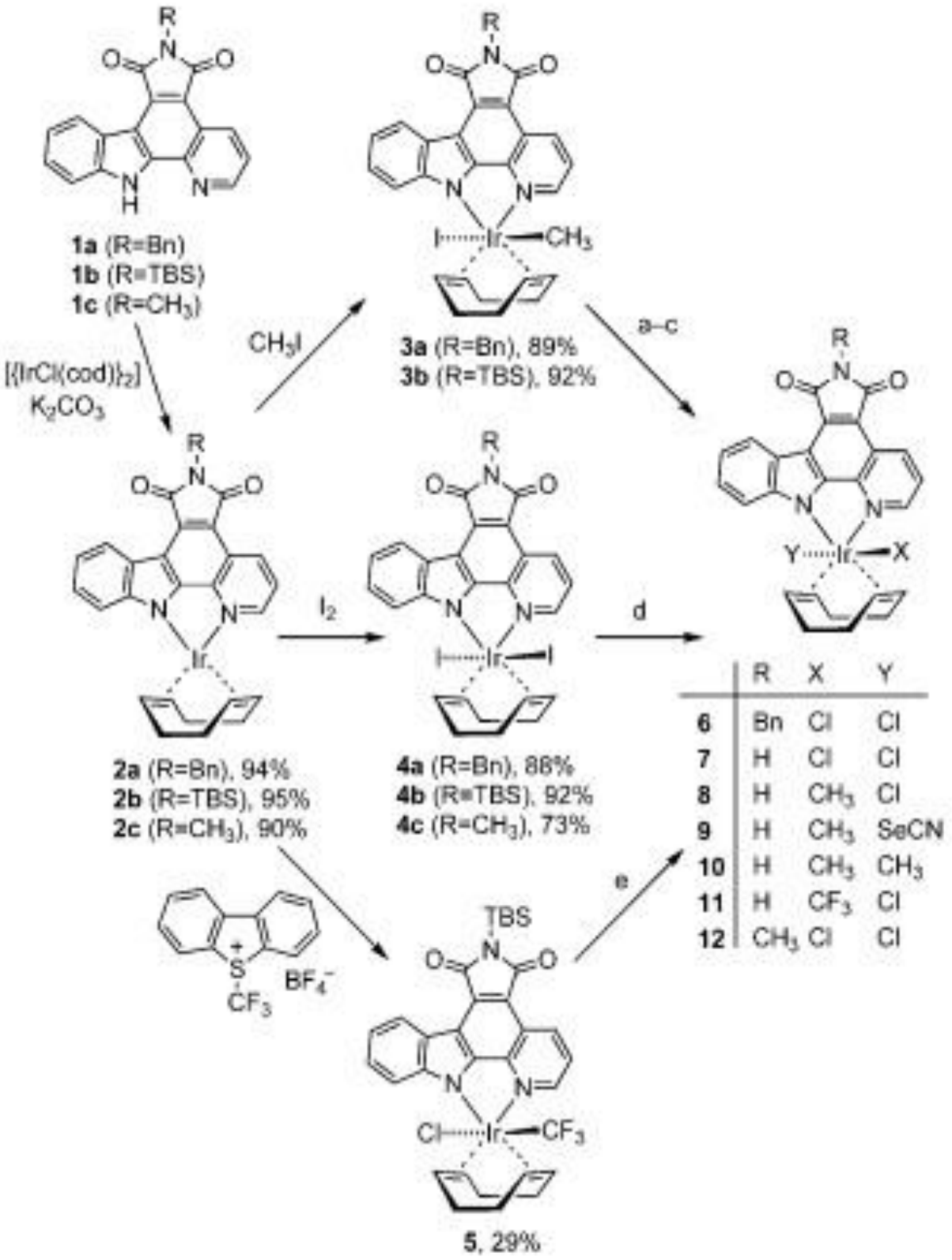



Figure 1. Illustration of an octahedral pyridocarbazole metal complex bound to the active site of a protein kinase. The coordinating ligands A-D are capable of controlling kinase affinities and selectivities, if arranged properly.

We started with iridium complexes containing our recently developed pyridocarbazole pharmacophore bidentate ligand that targets the compounds to the ATP-binding site of protein kinases (Figure 1). For initial synthetic studies we used the benzyl protected ligand **1a** and reacted it with $[IrCl(COD)]_2$ (COD = 1,5cyclooctadiene) in MeCN/MeOH (2:1) in the presence of $K_2CO_3$ to afford the iridium (I) complex **2a** in 94% (Scheme 1). This slightly air sensitive complex efficiently undergoes oxidative addition. For example, the reaction of **2a** with freshly distilled $CH_3I$ in the dark provided the stable octahedral complex **3a** (89%) stereospecifically as the *trans* oxidative addition product (G. Mestroni, 1974). Similarly, the reaction of **2a** with $I_2$ afforded the octahedral complex **4a** in 88%. A crystal structure of complex **4a** is shown in Figure 2 and reveals the *trans* coordination of iodine.

Importantly, the iodide ligands in **4a** can further be subjected to substitution chemistry, as exemplified by the conversion to the dichloride complex **6** upon treatment with tetrabutylammonium chloride (TBAC) (88%).

Encouraged by these results, we synthesized in an analogous fashion, starting with the TBS (Tris buffered saline)-protected pyridocarbazole **1b**, a small library of iridium complexes **7**-**11** bearing unprotected maleimide moieties essential for undergoing hydrogen bonds with the hinge region of the ATP- binding site of protein kinases (Scheme 1 and Supplementary Information).


1a (R=Bn)
1b (R=TBS)
1c (R=CH3)
[{IrCl(cod)}2]
K2CO3
CH3I
3a (R=Bn), 89%
3b (R=TBS), 92%
a–c
2a (R=Bn), 94%
2b (R=TBS), 95%
2c (R=CH3), 90%
I2
4a (R=Bn), 86%
4b (R=TBS), 92%
4c (R=CH3), 73%
d
BF4−
e
5, 29%
R X Y
6 Bn Cl Cl
7 H Cl Cl
8 H CH3 Cl
9 H CH3 SeCN
10 H CH3 CH3
11 H CF3 Cl
12 CH3 Cl Cl

Scheme 1**.**Synthesis of octahedral iridium complexes through oxidative addition as the key step. Reaction conditions a-c: a) TBAC, THF, 79% of **8**; b) KSeCN, DMF, 83% of **9**; c) MeMgBr, CuI, THF, 63%; TBAF, $CH_2Cl_2$, 73% of **10**; d) TBAC, THF, 88% of **6**, 59% of **7**, and 71% of **12**; e) TBAC, THF, microwave (70 °C, 17 min, 20 watts), 50% of **11**. TBAC = tetrabutylammonium chloride.

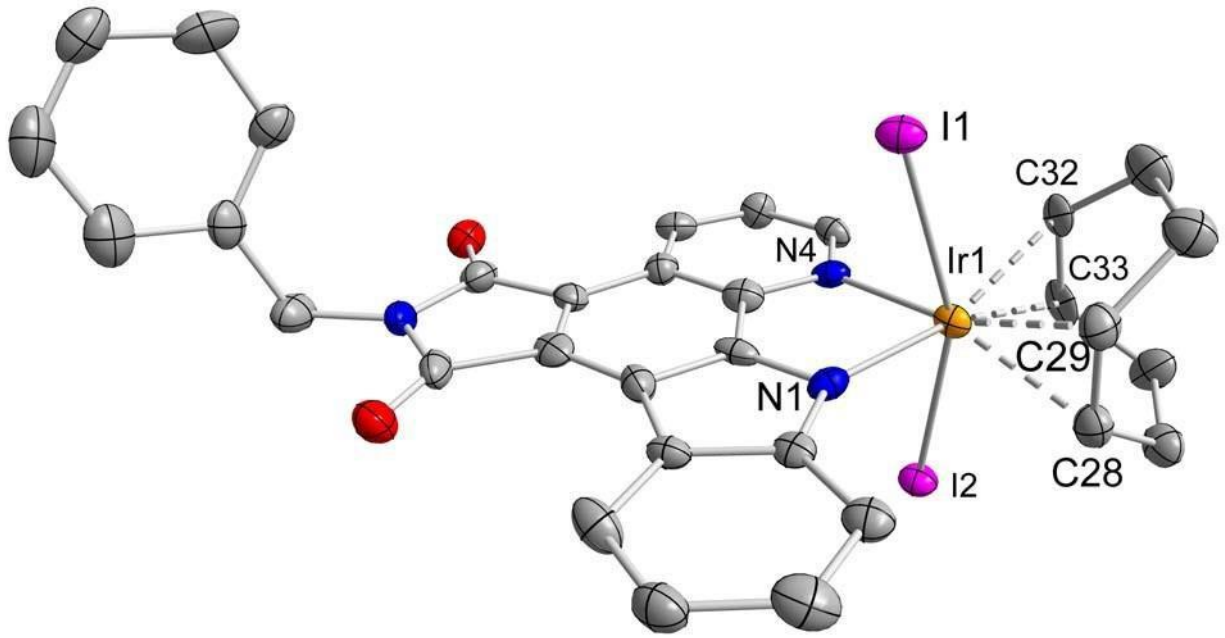



Figure 2. ORTEP representation (50% thermal ellipsoids) of the crystal structure of complex **4a**. Selected bond distances: N1-Ir1. 2.070(6), N4-Ir1 2.122(6), C28-Ir1 2.240(8), C29-Ir1 2.251(7), C32-Ir1 2.222(7), C33-Ir1 2.252(7), I1-Ir1 2.733(6), I2-Ir1. 2.714(6) Å. Selected bond angles: N1-Ir1-I2 82.29(16), N4-Ir1-I2 81.44(16), N1-Ir1-I1 80.42(16), N4-Ir1-I1 81.15(16), I2-Ir1-I1 157.12(2)°.

In order to quickly evaluate the kinase inhibition properties of this class of iridium complexes, we selected compound **8** as a representative member and screened it against a panel of protein kinases. As a result, at a concentration of 1 µM **8** and 10 µM ATP only 12 out of 263 kinases showed activities below 25% (see supporting information). Intriguingly, the receptor tyrosine kinase Flt4 (Fms-related tyrosine kinase 4), which was not selectively inhibited by any of the previously reported ruthenium scaffolds, (Meggers E., 2007; Meggers E., et al., 2007) displayed under these conditions only an activity of 2%. We therefore selected Flt4 for further studies and tested the small library of iridium

compounds **7**-**11** against this kinase by determining the concentrations of **7**-**11** at which Flt4 kinase activity was reduced to 50% ($IC_{50}$) at an ATP concentration of 100 µM. As expected, ligands X and Y of compounds **7**-**11** (Scheme 1) turned out to have a profound effect on the inhibition properties. Whereas compound **8** displayed an $IC_{50}$ value of 211±23 nM, the more hydrophobic dimethyl derivative **10** had a significantly lower potency ($IC_{50}$ = 1007±129 nM). Replacing the $CH_3$ group in **8** against $CF_3$ also reduced affinity with an $IC_{50}$ value of 369±68 nM for **11**. Furthermore, as seen for compound **9**, substituting the chloride for selenocyanate decreased the inhibition potency slightly compared to complex **8** ($IC_{50}$ = 326±52 nM for complex **9**). However, complex **7** with X = Y = Cl showed a significantly improved activity with an $IC_{50}$ value of 123±14 nM, thus being almost an order of magnitude more potent against Flt4 than the related dimethyl derivative **10**. From these data it becomes apparent that the ligands X and Y have a profound effect on the kinase inhibition properties in this class of compounds and the small library of organoiridium complexes **7**-**11** already spans across an activity range of almost one order of magnitudes. This observation is consistent with previous studies of organometallic protein kinase inhibitors which revealed the importance of the ligands oriented perpendicular to the pyridocarbazole moiety (ligands A and C in Figure 1) for kinase binding, especially the ligand pointing towards the glycine-rich-loop (ligand A in Figure 1) (Bregman H., 2006). Our results thus demonstrate that oxidative addition provides a convenient synthetic tool to quickly scan ligands in the positions perpendicular to the pyridocarbazole moiety, which to our opinion is a unique feature of this iridium scaffold.

For the purpose of designing compounds as molecular probes for chemical biology, the selectivity of a compound is one of its most important single features. This is especially a challenge for members of large enzyme families such as protein kinases with more than 500 putative protein kinase genes encoded in the human genome. Nevertheless, iridium complex **7** displays a remarkable degree of selectivity for Flt4 over other protein kinases. Figure 3 shows the results of a screening of complex **7** against a panel of 229 human wild type protein kinases at a concentration of 100 nM (10 µM ATP). Out of the 229 protein kinases, 224 kinases remained an activity of more than 50%, including other members of the VEGFR family such as VEGFR1 (also known as Flt1, 62% activity at 100 nM **7**, 10 µM ATP) and VEGFR2 (also known as KDR, 95% activity at 100 nM **7**, 10 µM ATP). On the other hand, only 5 kinases, including Flt4, were inhibited by more than 50% under these conditions. $IC_{50}$ measurements with some of these kinases at the biologically more relevant concentration of 250 µM ATP confirmed the exquisite selectivity of **7** for Flt4: Pim1 ($IC_{50}$ = 560±8 nM) and GSK3 (measure for the more potent α-isoform: $IC_{50}$ = 338±42 nM) are inhibited with potencies which are lower compared to the inhibition of Flt4 by **7** ($IC_{50}$ = 125±23 nM at 250 µM ATP). Thus, it can be concluded

that the high selectivity of the organometallic complex **7** renders it a promising tool for investigating biological processes related to Flt4.

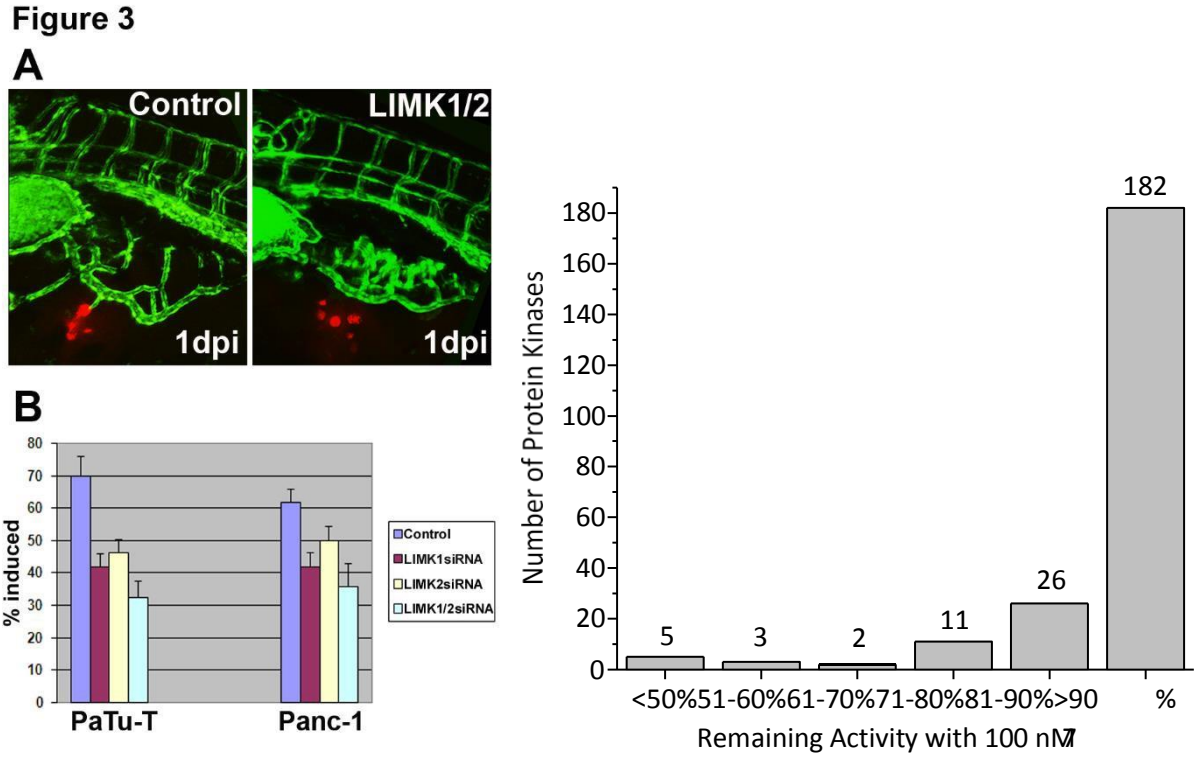



Figure 3. Selectivity profile of iridium compound **7** in a panel of 229 human wild type protein kinases. Determined by Millipore (KinaseProfiler) at a concentration of 10 µM ATP. Remaining activities are mean values from duplicate measurements. Selectivity comparison within the VEGFR family (remaining activities at 100 nM **7**): Flt1 (VEGFR1) = 62%, KDR (VEGFR2) = 95%, Flt4 (VEGFR3) = 32%. Protein kinases which were inhibited by more than 50% at 100 nM **7** are Flt4, Pim1, GSK3α, GSK3β, and MKK7β.

The receptor tyrosine kinase Flt4 plays essential roles in the development and maintenance of lymphatic vessels as well as in the development of the embryonic cardiovascular system (Karkkainen M. J. and Petrova T., 2000). In early mouse embryos it has been shown that a targeted inactivation of the Flt4 gene results in defective blood vessel formation (Dumont D.J., 1998). Similarly, it has been demonstrated that Flt4 signalling is required for vasculogenesis and angiogenesis in the developing zebrafish (Ober E. A. et al., 2004). We therefore tested the effect of complex **7** on angiogenesis and tumour-induced neovascularization in two zebrafish models (Bergamo A. et al., 2002; Ott I. et al., 2009; I. Ott et al, 2009). Accordingly, transgenic zebrafish (*Danio rerio*) embryos in which the vascular

system exhibits green fluorescence due to the expression of the green fluorescent protein (GFP) under an early endothelial promoter, were exposed to the organometallic compound **7**. The subsequent analysis with confocal fluorescence microscopy demonstrated that compound **7** induces a significant damage to the vessel formation with 79% and 100% of the zebrafish embryos being affected 3 days post fertilization at concentrations of 1 µM and 5 µM, respectively (Table 1). Figure 4 gives an example of vessel defects in zebrafish embryos 3 days post-fertilization induced by treatment with 5 µM of **7**.

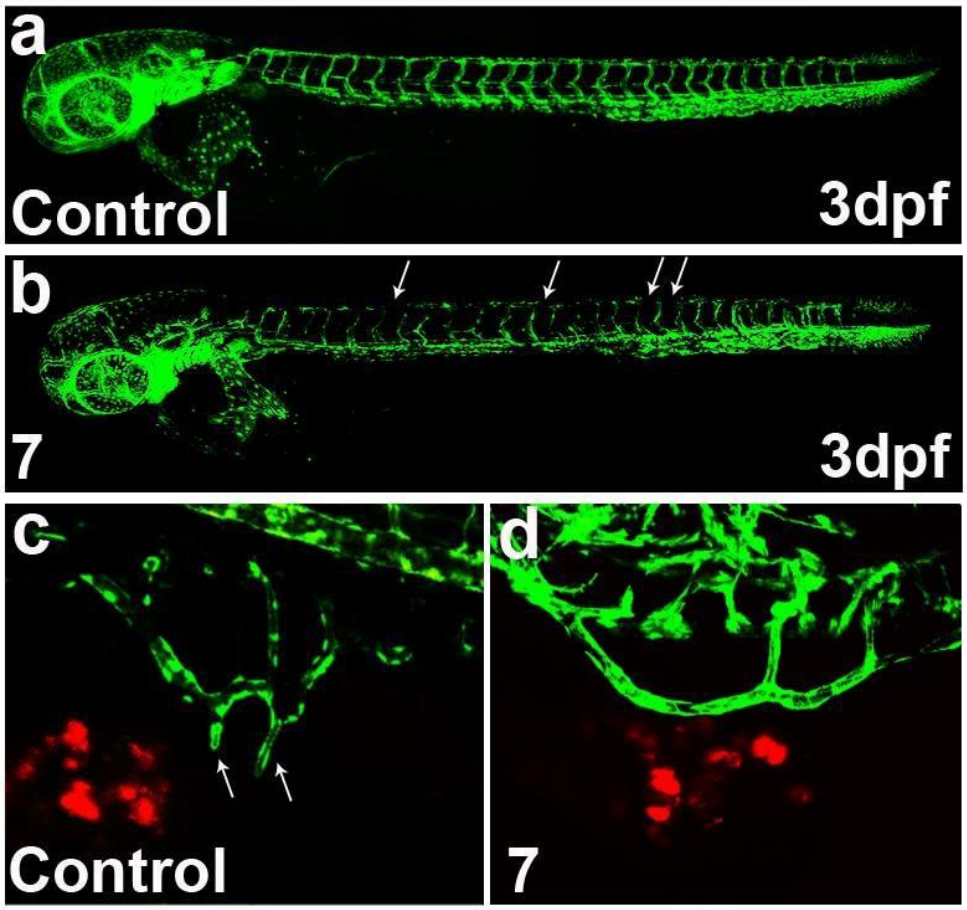



Figure 4. Effect of iridium compound **7** on angiogenesis and tumourcell-induced neovascularization in transgenic zebrafish embryos exhibiting a green fluorescent vascular system. a, b) Angiogenesis assay: Examples of laser scanning confocal microscopy images shown at 3 days post fertilization of zebrafish embryos treated with DMSO (a) or compound **7** at 5 µM (b). Vessel defects are marked with white arrows. c,d) Tumour xenotransplantation angiogenesis assay: Embryos were transplanted with human pancreatic cancer cells (red fluorescent due to CM-DiI staining) and treated with DMSO control (c) or compound **7** at 5 µM and images taken by dual laser scanning confocal microscopy at 24 hours post transplantation. The outgrowth of the subintestinal vein (SIV) in the control experiment is marked with two white arrows and suppressed in presence of compound **7**.

Table 1

Inhibition of angiogenesis and tumour-cell-induced neovascularization in developing zebrafish embryos.[a]

| Compounds | Inhibition of angiogenesis (% defects / % survival)[b] | Tumour-induced neovascularization (% positive)[c] |
|---|---|---|
| DMSO (di-methyl sulfoxide) | 0/91±2 | 78±4 |
| **12** (5 µM) | 0/86±5 | 75±3 |
| **7** (1 µM) | 79±5/90±2 | 36±1 |
| **7** (5 µM) | 100±0/91±3 | 28±3 |

[a] See Supporting information for experimental details. [b] Given are the percentages of embryos with defects in dorsal longitudinal anastomic vessels (DLAV), intersegmental vessels (IV) or/and subintestinal veins (SIV) formation after 72 hours post fertilization. Percentages of surviving embryos under the experimental conditions are also indicated. Three independent experiments were peformed with 100 embryos each. No phenotypic differences were observed for the solvent control and the Nmethylated compound compared to untreated embryos. [c] Given are the percentages of embryos with induced vessel formation at 24 hours post injection of tumour cells. Two independent experiments were performed and 80 embryos were investigated for each concentration and compound. Non-transplanted zebrafish embryos do not show a similar formation of microvasculature from the subintestinal veins (data not shown).

**Discussion and conclusion**

New blood vessel development is an important process in tumour progression (Kerbel R. S., 2008).

Next, in order to evaluate the inhibitory effect of compound **7** on tumour-cell-induced angiogenesis, we used an *in vivo* tumour xenograft angiogenesis assay in zebrafish embryos (Nicoli, S. and Presta, M., 2007; Nicoli S. et al., 2007). This neovascularization assay allows to follow the induction of blood vessel formation by xeno-transplanted pro-angiogenic human cancer cells, in this study transplanted human pancreatic tumour cells (PaTu 8998T cells), in real-time in living zebrafish and enables the quantification of neovascularization *in vivo* and in high resolution. Revealingly, our results show inhibitory effects of **7** on tumour-cell-induced angiogenesis. For example, compound **7**, when added to the water containing the zebrafish embryos at two nonlethal concentrations, strongly inhibited the induction of new microvasculature in the zebrafish neovascularization assay (Table 1). Figure 4 visualizes the effect of compound **7**: Whereas zebrafish embryos treated with DMSO show the typical tumour-cellinduced outgrowth of the subintestinal vein (marked with two arrows in Figure 4c), the formation of new blood vessels is suppressed successfully in the presence of 5 µM of iridium compound **7** (Figure 4d).

Taken together, these data show that compound **7** has antiangiogenic properties *in vivo* and inhibits both, angiogenesis in developing zebrafish embryos as well as tumour cell induced angiogenesis. Importantly, the related organometallic complex **12** (Scheme 1), having an identical coordination sphere around the iridium(III) center but lacking the ability to inhibit protein kinases effectively due to a methylated maleimide moiety (Flt4: $IC_{50}$ = 1520 nM at 100 µM ATP), does not show any effects at similar concentrations in these two zebrafish assays (Table 1). This pronounced difference in bioactivities between the organometallics **7** and **12** strongly suggests that it is the inhibition of protein kinases, most likely Flt4, which is responsible for the *in vivo* bioactivity of the organometallic compound **7**.

Organometallic compounds are traditionally disregarded as molecular scaffolds for the design of drug-like molecules or molecular probes due to the fear of toxicity resulting from an incompatibility of metal-carbon bonds with the biological environment. However, as shown in Table 1, the organoiridium Flt4 inhibitor **7**, containing multiple Ir-C bonds, does not affect the survival of the zebrafish embryos. Furthermore, cellular proliferation experiments with Hela cells confirm the low cytotoxicity of **7** with Hela cells being not affected significantly in the presence of 1 µM **7** over 24 hours (see Supplementary Information).

In conclusion, we herein report the first example of a protein kinase inhibitor based on an octahedral iridium complex scaffold, synthetically accessed in a stereo-selective fashion through oxidative

addition. The high *in vitro* selectivity, presumable due to the overall scaffold rigidity, combined with a lacking cytotoxicity and the distinct *in vivo* biological activity in two zebrafish model systems indicates that organoiridium complexes such as **7**, containing an octahedral metal center and multiple metal-carbon bonds, are falsley neglected as scaffolds for the development of enzyme inhibitors.

*Acknowledgements*

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# Chapter 5: Whole snake venoms: Cytotoxicity, anti-metastatic and anti-angiogenic properties

**Danielle Vlecken, Christoph Bagowski, Herman Spaink and Michael Richardson**

**Abstract**

Currently, biological and organic substances are globally screened for development of a new generation of therapeutics active against cancer. Amongst the snake venoms, candidates have been identified which exhibit promising properties. In this pilot study, snake venoms from different species (*Naja anulifera*, *Naja kaouthia*, *Ophiophagus hannah* and *Echis carinatus* ) were investigated for their anti-malignant properties on pancreatic cancer cells. To this end, cells were incubated with different venoms and subsequently subjected to *in vitro* cell death and migration assays and *in vivo* dissemination and angiogenesis assays. For the *in vivo* assays, the cells after incubation and labelling were subjected to transplantation into the yolk sac of zebrafish embryos and monitored for motility and angiogenesis. Since there were few effects observed *in the vivo* assay, we soaked fish injected with untreated cells in the different venom solutions. The results showed that strong effects occur in *in vitro* samples treated with venoms derived from *Ophiophagus hannah* and *Echis carinatus*. Conversely, in the *in vivo* assays, venom derived from *N. naja* had the most beneficiary effects with respect to angiogenesis. These venoms might therefore be well-suited candidates for further studies.

**Introduction**

At present, biological and organic compounds are promising candidates for anti-cancer drugs. These include snake venoms which were shown to exhibit multiple therapeutic effects, despite their toxic properties. Snake venoms, which are toxic to most animals, can damage many tissues upon a snakebite, such as the central nervous system (Osipov A. and Utkin Y., 2012), the kidneys (Karthik S. and Phadke KD., 2004) and the blood (haemorrhage) (Kamiguti AS., 1996). Also, an innate immune response can be initiated in which the complement system is activated upon venom injection (Leon G., et al., 2011).

Currently, compounds derived from snake venoms are used for multiple therapeutic applications, such as drugs for cardiovascular purposes, anti-coagulants (Koh CY., and Kini RM., 2012) and anti-cancer drugs (Jain D1, Kumar S., 2012; Selistre-de-Araujo HS., et al., 2010; Markland FS, 2001). In this study, anti-malignant properties of snake venoms were tested on pancreatic tumour cells.

Tumourigenesis involves interactions of malignant cells with each other, neighbouring cells and their microenvironments, hereby often attracting surrounding vasculature to balance the oxygen supply with the demand of a growing tumour. The vascular network is further employed by metastasizing cancer cells for their long and short distance traveling to possible secondary metastatic sites. The importance to understand and prevent metastasis formation is emphasized by the fact that over 90% of all cancer related deaths are due to secondary tumours arising from metastasizing cancer cells (Jemal A., 2007). This is especially true for pancreatic cancer types which have a poor prognosis in 85-90% of the patients due to metastatic complications (Ganeh P., et al, 2007).

Zebrafish and their transparent embryos have been employed in several useful models for therapeutic drug research and preclinical studies (Dooley K. and Zon LI., 2000). High throughput screening (HTS) in zebrafish embryos has been established and is nowadays commonly used for different applications (Jones KS., et al., 2008; Miscevic F, et al, 2014; Pichler FB., et al., 2003). A number of unique features make this animal model very attractive: zebrafish are inexpensive to maintain, they breed in large numbers, develop rapidly *ex vivo*, and can be maintained in small volumes of water (Lieschke GJ., and Currie PD., 2007). Recently, the zebrafish and its transparent embryos have also come into view as a new model system to investigate tumour development, cancer cell invasion and metastasis formation (Nicoli S., et al., 2007).

**Materials and methods**

*Animal care and handling*

Tg(fli1:eGFP) zebrafish were kept at 28° C in tanks with day/night light cycles of 10 hours dark alternated with 14 hours light periods. The zebrafish were handled in compliance with Dutch animal care regulations and standard operating procedures. Zebrafish eggs were harvested at two hours post fertilization (hpf), followed by incubation at 28° C in egg water (sea salt, 60 µg/ml in tap water). The developing embryos were kept in incubators at constant temperatures dependent on experimental requirements.

*Cell culture*

The pancreatic cancer cell line (PaTu 8988t) was cultured at a temperature of $37^0$ C and a $CO_2$ pressure of 5%. The PaTu 8988t cell line was isolated from liver metastases over a decade ago (Elsässer H.P., 1992). Their behaviour in an *in vitro* wound healing assay and in an *in vivo* zebrafish xenotransplantation assay were described in detail before (Marques I., 2009). PaTu 8988t cells were cultured in DMEM (Gibco) high glucose supplemented with 10% FCS (Gibco). ZF4 cells are zebrafish cells (Driever W., 1993) that were cultured in L15 medium (Gibco) enriched with 15% FCS (Gibco). Cells were incubated at $28^0$ C, without $CO_2$ pressure.

*In vitro observation of Cytopathic effects and CCID50 (dose that kills half the cells, by observation)*

Cells were seeded at a concentration of $10^4$ cells per well in 96 wells plates (Greiner) and subsequently incubated at the proper temperature and $CO_2$ saturation. DMEM (Gibco) was used as culture medium for PaTu 8988t cells and L15 medium (Gibco) was used for ZF4 cells. Venom was dissolved in Hank's salt solution (Gibco) at a concentration of 2 mg/ml and diluted with to a concentration of 0.1 mg/ml. Subsequently, this solution was passed through a 0,2 µm filter. Logarithmic venom dilutions of stock solution in PBS were brought onto the cells in the culture plates. Plates were then incubated for three days under the conditions mentioned in 2.2. During these three days, cells were photographed regularly. A representative set of photos is shown in Figure 1 CPE (lysis of the cells, blebbing, pyknosis etc.) was first determined using a microscope (Olympus). Data was confirmed with Trypan blue staining of randomized samples. The LD50 was calculated using the Reed and Muench method (Reed, L.J.and Muench, H., 1938) as was described before for other venoms (Lee K.H. et al., 2006).

In vitro *migration assay*

PaTu 8990t cells were grown in DMEM (Gibco), supplemented with 10% FCS (Gibco) in 24 well glass-bottom culture plates (Invitrosci) and incubated o/n at 37 $^{0}$C and 5% $CO_2$ saturation. Before the addition of snake venom, PaTu 8990t cells were supplied with fresh medium without FCS and subsequently a scratch was made using a pipet tip (Eppendorf). A stock solution of 2mg/ml was made of the venom in Hanks salts (Gibco), subsequently, this solution was diluted ten times in PBS (Gibco) and filtered through a 200 nm filter (Millipore) using a syringe (BD). The highest dose rendered a final concentration of 0.5 mg/ml. At a ratio of 1:1 the venom in PBS and medium, the plate was incubated at 37 $^{0}$C and 5%. $CO_2$ saturation until pictures of the scratch were taken under a Leica stereomicroscope at different times, shown in Figure 2. The gap was measured and closure was calculated in percentage to the initial gap (scratch).

In vitro *apoptosis assay*

All cell lines were screened for expression of annexin V on apoptotic bodies after exposure of the cells to snake venoms, using fluorescently labelled antibodies. Annexin V is detected using a fluorescent label: Mitochondrial Membrane Potential Apoptosis Kit, with Mitotracker™ Red & Annexin V Alexa Fluor® 488, for flow cytometry (Molecular Probes®). Cells were grown in glass-bottom multiwell plates (Invitrosci) until 80% confluence was reached. Cells were washed three times in PBS (Gibco, Bleiswijk, Netherlands) before they were incubated with kit components. After labelling and analysis, cells were fixed in PFA (Sigma, Zwijndrecht, Netherlands) 4% in PBS, pH 7,4 for fifteen minutes, followed by incubation for 1h in the antibody solution containing 0.1% BSA (bovine serum albumin) (Sigma, Zwijndrecht, Netherlands) in PBS at a 1:100 dilution. After removal of the antibody solution, cells were washed in PBS and incubated for 1h at RT with a fluorescently labelled secondary antibody.

*Cytotoxicity (Lactate dehydrogenase)*

PaTu 8988t cells and ZF4 cells were cultured in 96 wells plates (Greiner) as was indicated in 2.2, however, culture media were prepared again without FCS. Cultured cells were incubated with snake venoms inducing CPE and subsequent LDH release. The measurement was performed using a LDH Cytotoxicity Assay Kit (Pierce Protein Biology Products). The reactions were performed according to manufacturer's protocol. In brief: The LDH released into the medium was transferred to a new plate and mixed with reaction Mixture. After a 30 minute room temperature incubation, absorbance was measured at 490 nm and 680 nm using a plate-reading spectrophotometer (Tecan) to determine LDH activity. Each measurement was done in four-fold. The last two rows of each plated contained background- positive- and negative (plain media) controls. Results were statistically analysed using a T-test to determine significance of the results.

*Cell tracker application for* in vivo *experiments*

Cells were plated in 24-well plates (Greiner) at a seeding density of 4,0 *$10^4$ cells and incubated for 48 hours at $37^0$ C and 5% $CO_2$. Snake venom dilutions were added, and the cells were incubated again at $37^0$ C and 5% $CO_2$ for 24 hours. After detaching the cells with trypsin (Gibco), two washing steps were done with DPBS (Dulbecco's PBS, Gibco), after which the cells were transferred to 1.5 ml Eppendorf tubes and centrifuged 5 min, at 1500 rpm. Then, the cells were stained according to manufacturer's protocol after which the cells were suspended in 25µl DPBS for injection into the embryos.

*Cell transplantation of PaTu 8988t cells into zebrafish embryos*

After dechorionation, 2 dpf zebrafish embryos were anesthetised with Tricaine methanesulfonate (TMS, MS-222, Sigma). Using a manual injector (Eppendorf, Celltram), the cell suspension was loaded into an injection needle (15 µm internal- and 18 µm external diameter (Oxford apparatus) and cells were injected in 48 hpf Tg(fli1:eGFP) zebrafish embryos. After injection, the embryos were incubated at $35^o$ C and checked for cell presence at 2 hours post transplantation (hpt). Embryos with fluorescent cells outside the implantation area at 24 hpt were excluded from further analysis. All other embryos were incubated at $35^o$ C for analysis during the following days.

*Tumour cell-induced angiogenesis*

The tumour-cell induced angiogenesis assay was in general performed as was previously described (Nicoli S., Presta M., 2007; Nicoli S., Ribatti D., 2007). The 48 hpf zebrafish embryos were dechorionated and anesthetised using 0.042 mg/ mL Tricaine (Sigma). Then, the embryos were positioned on a 1. 8% agarose dish for injections. Cells were stained as described above, then suspended in 20 µl 12. 0 mg / ml Matrigel® solution (Cultrex R Basement Membrane Extract, R&D systems, Minneapolis, USA) and kept on ice for a short period until injection. The CellTram Oil injector (Eppendorf, Hamburg, Germany) was loaded with an injection capillary (Harvard apparatus) which was filled with 5 µl of the cell suspension in Matrigel®, and embryos were injected as described in more detail in previous studies (Nicoli S., Protocols 2007; Nicoli S., Cancer Res 2007;Vlecken and Bagowski, 2009). For statistical analysis, a student t-test was performed which showed that all changes observed were significant (with p-values <0.01).

*Imaging*

Light-microscopy images of pancreatic cells, an MZ16FA stereomicroscope (Leica) and a DFC 420C camera (Leica) were used. Injected zebrafish embryos (which were incubated in egg-water at 35°C for 24h before screening) were photographed using a Bio-rad confocal microscope equipped with a

1024ES lens (Zeiss) and a Krypton/Argon laser. The data obtained were processed using ImageJ software and Photoshop (Adobe). Annexin V labelled cells were analysed using confocal laser scanning microscopy (Zeiss Observer) and processed afterwards using Photoshop (Adobe).

**Results**

In vitro *venom titration on PaTu 8988t cells and ZF4 cells: Cytopathic effects (CPE) and CCID50*

The pancreatic cancer cells and the zebrafish cells were incubated with snake venoms, which induced CPE (lysis) within 4 hours after application (Figure 1). Viable cell densities decreased the first day after which the populations started to grow again. Compared with the untreated control cells, treated cell samples were showing significantly more CPE. Also, it can be seen in Figure 1 that there is a dose dependent effect in the degree of CPE. As can be seen in photo A: cells treated with a low concentration of venom, do not proliferate as one would expect, however, cells shown in D and E have proliferated as well or better than the controls. Cells with the highest concentrations of venom detach and will likely die or change.

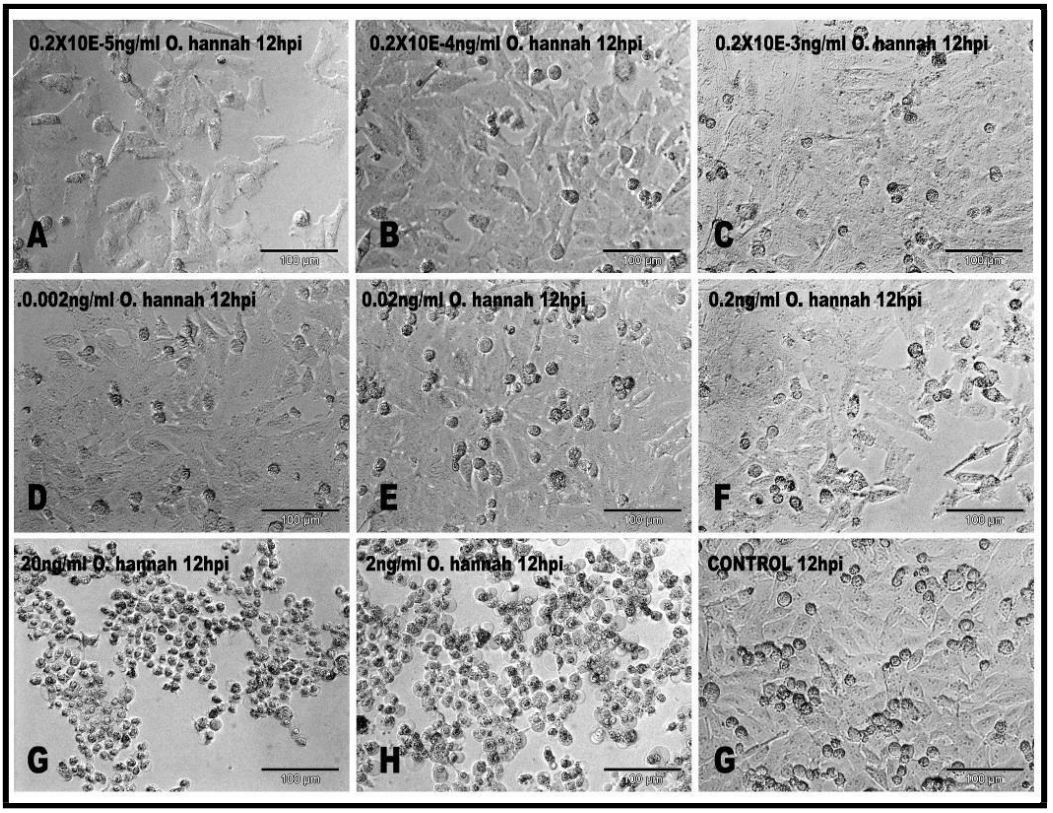


Figure 1: CPE titration after venom treatment. PaTu 8988t cells were treated with king cobra venom and a Trypan blue stain was done to confirm the results. As is shown, many cells have detached from the wells, however not all of those are really dead.

In vitro *migration of pancreatic tumour cells treated with snake venoms.*

Venom treated PaTu 8990t cells tend to migrate slower compared to the cells in the control group. The latter form already after 24 hours a layer with a confluence of 50% while there is not much migration visible at this time in the venom treated cells. However, the amount of CPE, was increased in the venom treated cells. The lower doses, shown in the first column, exhibit a delayed migration pattern, compared with the control cells. After 96 hours, the lowest dose shows to have reached the same level the controls reached after 24 hours. In the medium dose, half of the cells died after 96 whereas in the highest concentration half of the cells were dead after 60 hours and most of the cells were dead after 96 hours. Also, the integrity of the scratch was compromised.

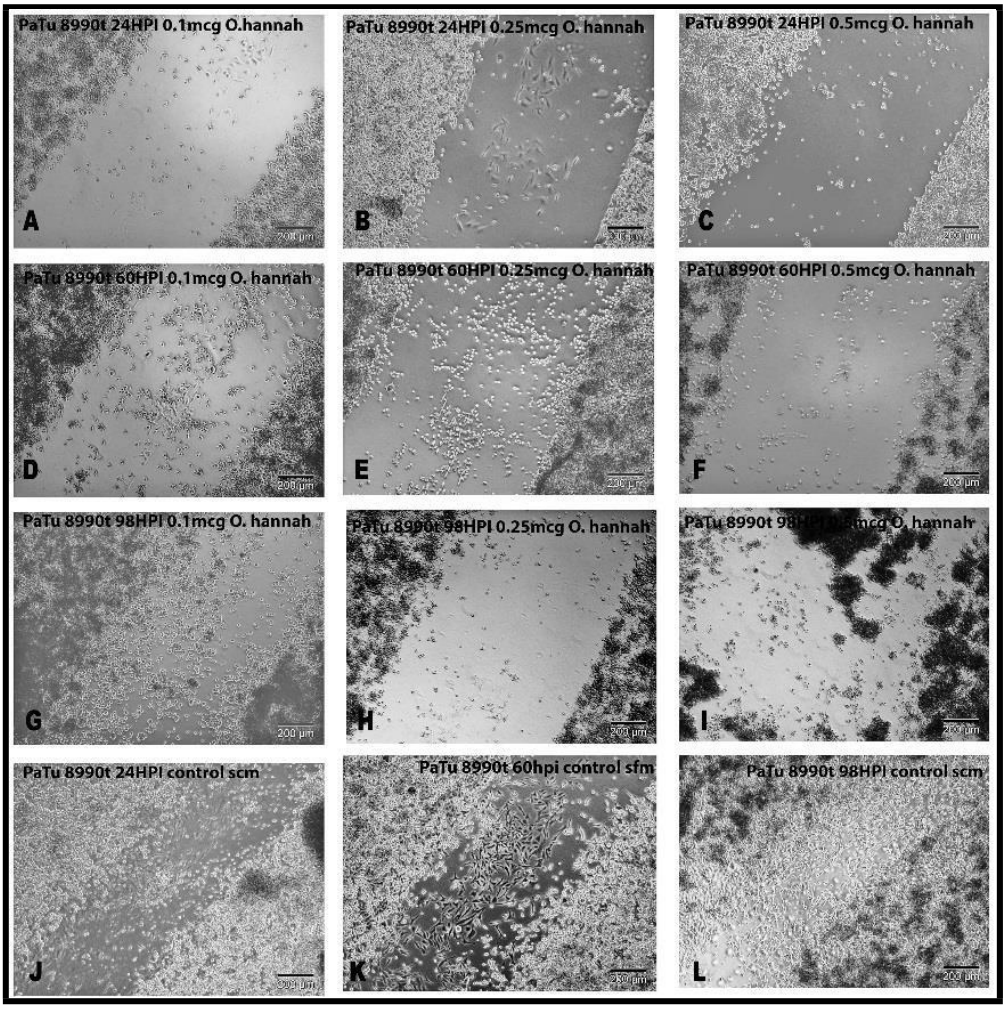

Figure 2: Cell migration of PaTu 8990t cells treated with venom of *Ophiophagus hannah*. Lyophilized *O. Hannah* venom was added to PaTu 8990t cells. In A, D and G 0.1 µg of the venom was added to the cells and in a final concentration of 10E-4 the cells were incubated. In B, E and H, the cells were incubated in a final concentration of 2.5 X10E-4. In the last column a dilution of 5X10E-4 was maintained. The cells were scored at three different times: at 24 hours post infection (24hpi), 60hpi and 98 hpi and the results in time are shown per row. Normal migration of PaTu 8990t cells after the application of a scratch is depicted in J, K and L.

*Apoptosis caused by snake venom*

To investigate whether apoptosis takes place in PaTu 8988t cells after addition of snake venoms immunocytochemistry was performed using an Annexin V antibody, a representative selection is shown for PaTu 8988t cells in Figure 3. Control cells have intact mitochondria and they lack the Annexin V signal (green), whereas cells treated with a low concentration of venom (Second photo, D10000) show apoptotic activity. Cells treated with moderate-high concentrations of venom (Third photo, D1000) show less apoptotic bodies and lysed mitochondria are visible. In the last photo (D100), a high concentration of venom was added and there is no apoptotic activity visible in the sample, although mitochondria lysed to a higher degree than is shown in photo C.

Formation of apoptotic bodies is limited to cells infected with moderate and low concentrations of snake venoms. Also, the integrity of mitochondria proved to be dependent on the concentration of snake venom. Thus, there is a dose related appearance of apoptotic bodies and cell disintegration, mitochondrial lysis and necrosis are observed in the high concentrations of snake venoms.

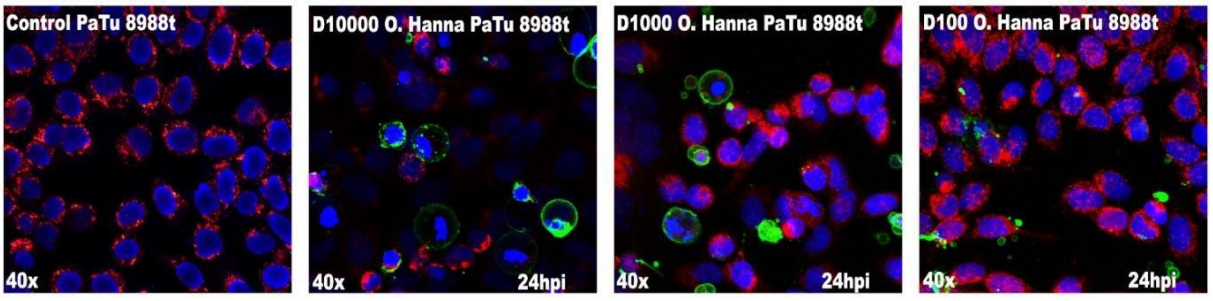


Figure 3: Apoptosis in cells treated with venom of *Ophiophagus hannah* (King cobra). Annexin V is shown using a green fluorescent label while the mitochondria are shown in red Mitotracker™ Red & Annexin V Alexa Fluor® 488, for flow cytometry (Molecular Probes®). In the control picture, PaTu 8988t cells do not show apoptotic

bodies whereas the lower concentrations venom show apoptosis. The degree of mitochondrial integrity decreases when the administered dose increases.

*Cytotoxicity (Lactate dehydrogenase (LDH))*

Lactate dehydrogenase measurement using PaTu 8988t cells and ZF4 cells treated with snake venoms. LDH is an enzyme that is found in damaged tissues in nearly all organisms. It converts lactate to pyruvate and vice versa (and NADH to $NAD^+$). LDH is abundant when tissues are damaged or, in cell culture, when cells are dying.

We used a colorimetric assay to quantitatively measure lactose dehydrogenase (LDH) released into the media from damaged cells as a biomarker for cellular cytotoxicity and cytolysis. An enzymatic reaction that results in a red Formazan product can be measured spectrophotometrically.

As is shown in Figure 4, both cobra venoms do not generate an expected dose dependency, whereas viper and king cobra venoms exhibit a response which is dose dependent. Doses with pronounce CPE, are much higher for zebrafish cells then for human pancreatic cancer cells.

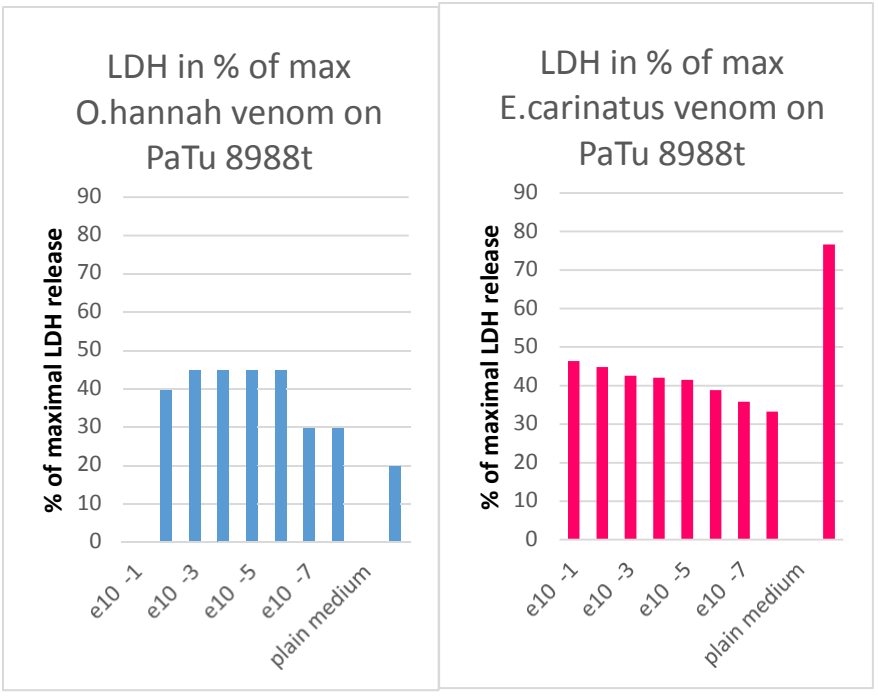

Figure 4: LDH (percentages with respect to LDH max = positive control) released by PaTu 8988t cells treated with snake venoms

The graphs above show LDH production as a reaction on the administered dose of snake venom. In the left graph, measurements with spent medium containing king cobra venom can be seen. The plot on the right also shows a dose response with spent medium containing venom from the Saw-scaled viper. With both venoms dose dependency was observed. For a positive control, three wells with cells were lysed to obtain the LDH-max values and plain medium without cells was used to generate negative controls.

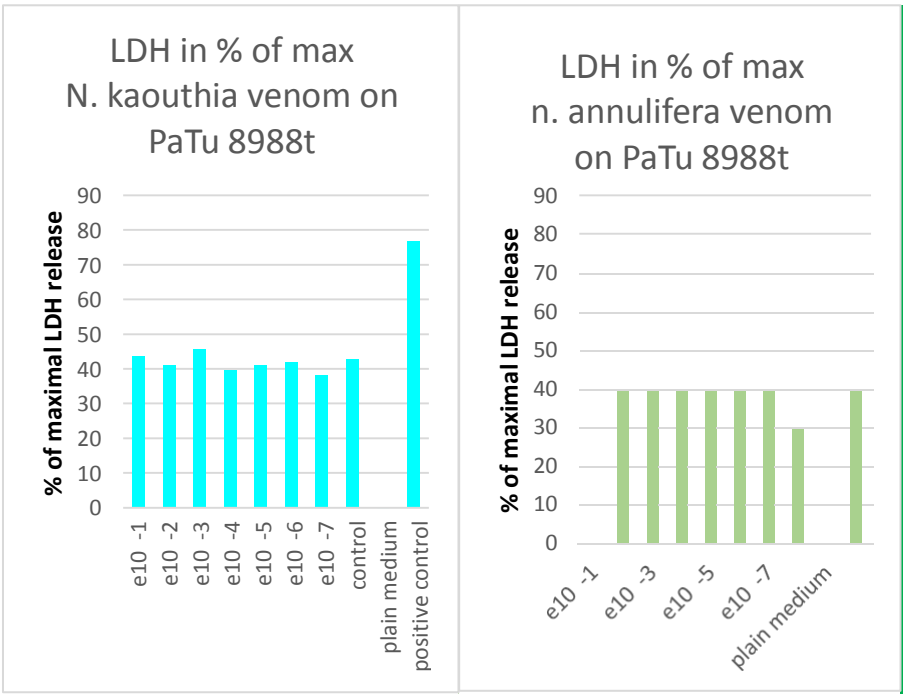


Figure 5: LDH measurements on PaTu 8988t cells treated with snake venoms from Naja species. The graphs how LDH production as a reaction on the administered dose of snake venom. In the left graph, measurements on spent medium treated with Monocled Cobra venom are shown showing that there is no dose dependency observed for these venoms. The plot on the right also shows the results of a dose response experiment on spent medium containing venom from the Snouted Cobra. No dose dependency was observed for these samples.

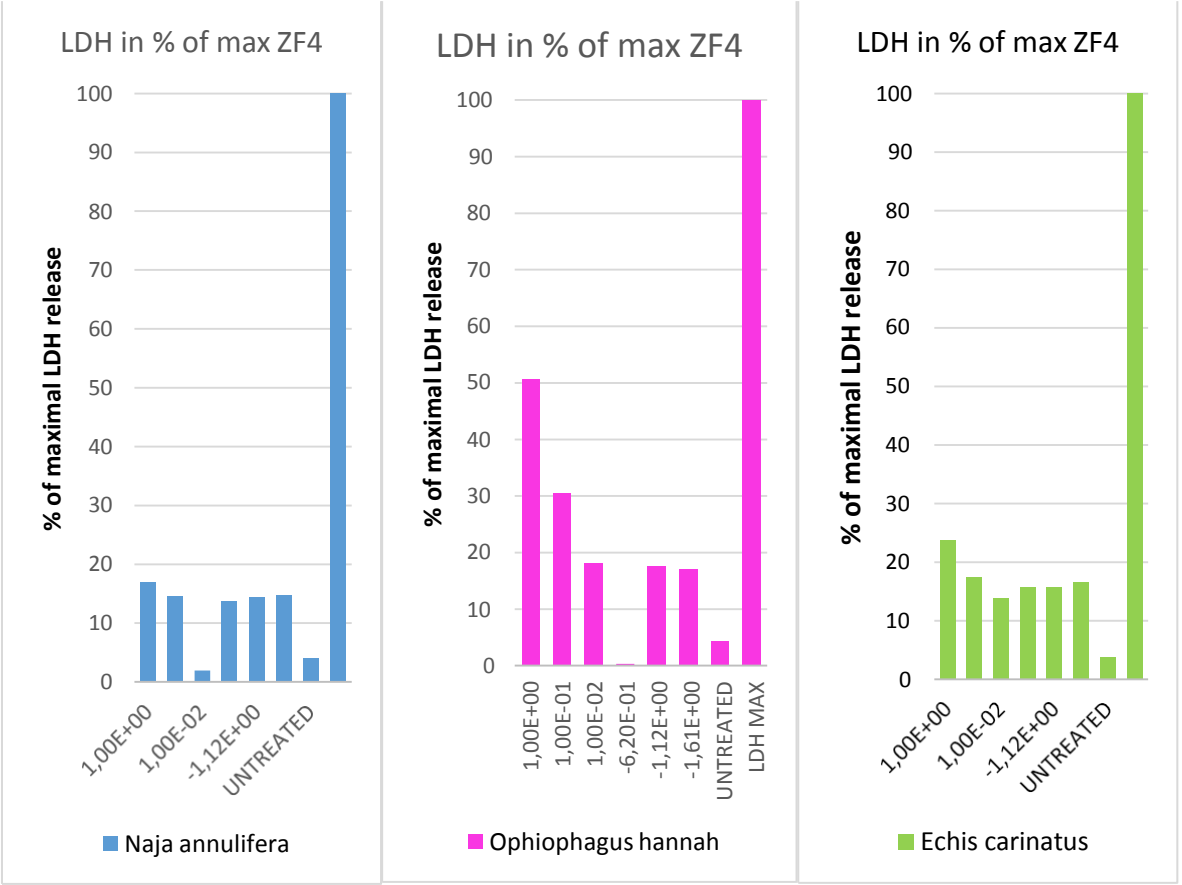


Figure 6: LDH measurements on ZF4 cells treated with snake venoms from different species. In ZF4 cells, the venom of *O. Hannah* generates the expected dose dependent response, whereas the other two (*E. carinatus* venom and *N. annulifera* venom) did not.

*Cobra venom exerts anti-metastatic properties on the behaviour of pancreatic cancer cells* in vivo

To study the role of the snake venoms in metastatic behaviour, PaTu 8988t cells were treated and then labelled with a carbocyanine dye (CM-Dil) and ectopically transplanted in the yolk sac of 2 dpf zebrafish embryos of the transgenic zebrafish line, Tg(fli1:eGFP), which express GFP under the fli1 promoter (an early endothelial marker) and therefore exhibit a green fluorescent vasculature (Lawson N.D. and Weinstein B.M., 2002). As a negative control for the venoms, PBS was used.

The results (Figure 6 and Supplemental Table 1) showed that several venoms (e.g. *O. Hannah* venom and *E. carinatus* venom in this study) have an inhibitory effect on metastatic behaviour, however, not all venoms displayed such effects, for example, *N. annulifera and N. kaouthia* venoms did not cause

significant dose-related effects when applied to the cells. The data obtained in the migration assay *in vivo* differs from the *in vitro* data. Differences exist due to lack of availability of nutrients and growth factors present in an animal. Since no significant effects were observed when cells were treated and implanted into the embryo, a different approach was attempted: Embryos were injected with untreated cells and the fish with the cells was then soaked in an egg water-venom solution. Other venoms that were tested without success were venoms of the copperhead snake and from the rattlesnake. For the angiogenesis assay, the same preconditions as are described in this paragraph are applicable.

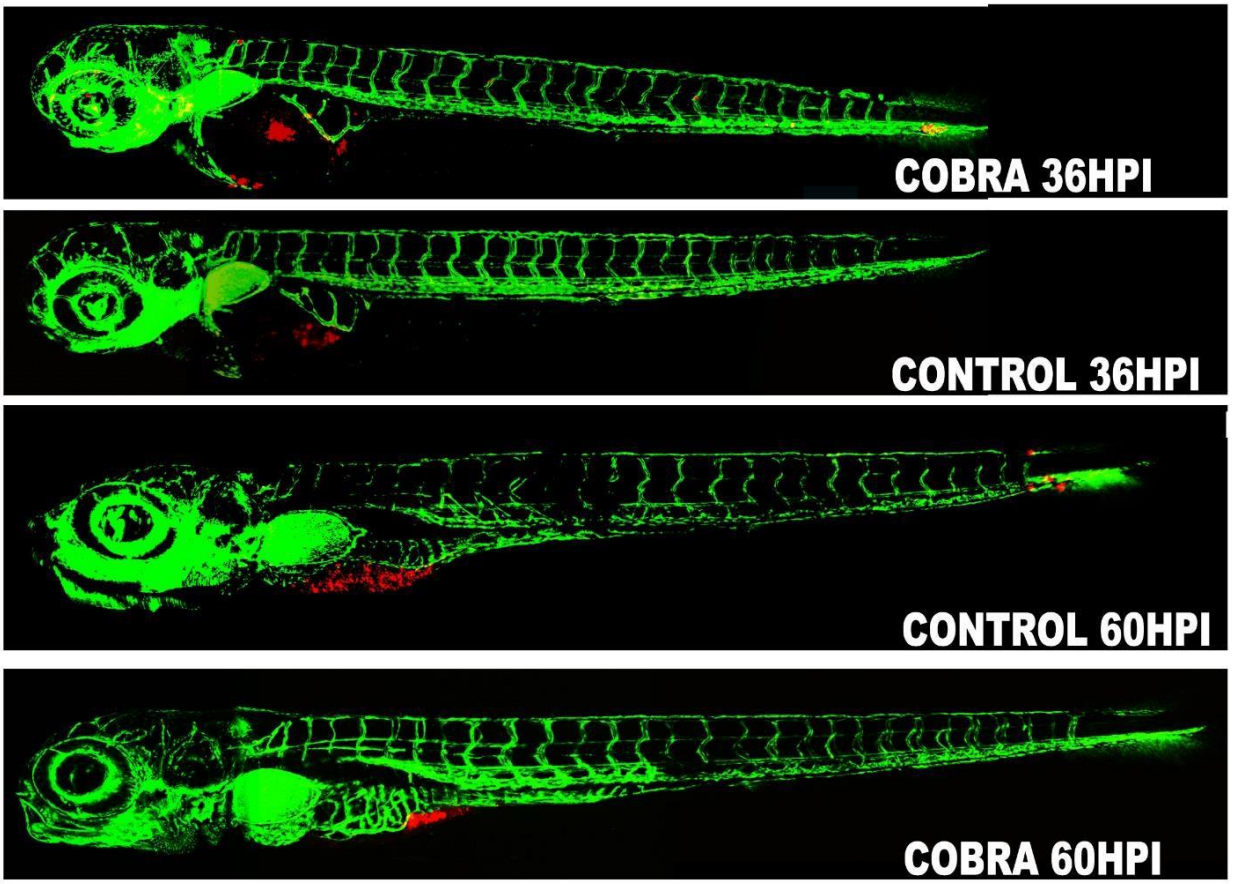


Figure 7: Anti-metastatic effects of cobra venom on fish with pancreatic cancer cell graft containing PaTu 8988t cells. The two control photos show embryos with a transplanted cell mass of which cells have spread via the vasculature. The zebrafish embryos that were treated with cobra venom after receiving the cell mass, did not show migration of malignant cells. (n=20 embryos per tested snake venom).

*Treatment of snake venoms reduces tumour cell-induced angiogenesis*

To study tumour angiogenesis *in vivo*, a method is available that is based on the injection of pro-angiogenic mammalian tumour cells into the perivitelline space of zebrafish embryos (Nicoli, S., Presta M., 2007). PaTu 8988t were injected into the perivitelline space of zebrafish embryos at 48h post-fertilization. These pro-angiogenic tumour cell grafts induced an angiogenic response originating from the developing sub intestinal-vessels. The results indicate shown in Figure 8 and Supplemental Table 1, that treatment with king cobra venom resulted in inhibition of angiogenesis.

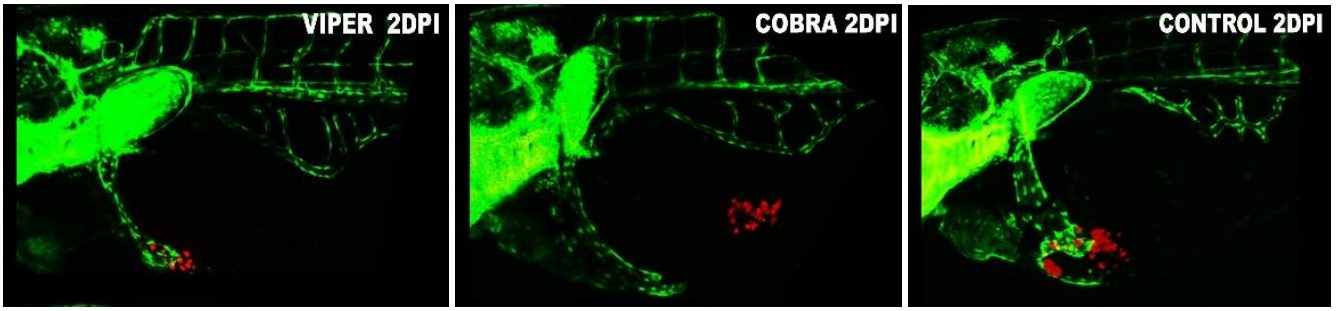


Figure 8: Anti-angiogenic properties of Cobra venom and Viper venom. The figure shows three confocal micrographs of PaTu 8988t cells (red) that were transplanted into 48 hpf fli zebrafish embryos (green). The cells were dispersed in Matrigel, because of which they stay ectopically in the embryos yolk. When Cobra venom was added to the fish carrying the cells, inhibition of angiogenesis was observed, however, treatment of the fish with Viper venom resulted in angiogenesis, like the controls. The magnification of the pictures is 20x. (n=20 embryos per tested snake venom).

**Discussion**

Because public demand continues to be strong and the incidence increased, investigation of candidate anti-cancer drugs is a part of this study. The studied compounds vary from different snake venoms to iridium containing complexes. The compounds that are observed to show an effect are followed up with additional experiments. Not only identification of compounds inhibiting malignant processes must be addressed but also ways to target the malignancies with these compounds must be explored.

Growth inhibition was shown for samples with very low doses, addition of high doses to the egg water in which the zebrafish resided, resulted in cell death (for 20 ng and 2 ng of added venom). However, the moderately higher doses appeared to induce enhanced proliferation. This phenomenon was reported before for venoms from *Bothrops* species which can activate cytokines (Luna K.P.O., et al., 2011; Mora R., et al., 2005) and venom from *Echis* species can cleave TNF (tumour necrosis factor) to its active form for example (VORONOV E., et al., 1999 ). Samples that received moderate doses did not differ much from control cells in the number or morphology of the cells. There might be some sort of equilibrium and deviating from it, could result in altered behaviour of the cells. Cells with high doses snake venom added to them appeared to detach and die. But not all of these cells were dead because upon seeding in fresh medium, cell proliferation was resumed. Damaging by for example snake venoms is known to bring about more mutations resulting in a higher survival rate, therefore prior to experienced trauma the cell and its descendants are not identical to the population that has grown after damage.

Apoptosis caused by snake venom was only induced in moderate doses. In higher doses cells almost lysed and in the controls, no apoptotic bodies could be discerned. This event could be due to the ability of tumour cells to block the apoptotic pathways (Cotter T. G., 2009). The 100x dilution must have been far too toxic for the cells to survive, because no apoptosis was observed, whereas mitochondrial integrity was clearly lost. Also, nuclei became brighter which also indicates that CPE takes place.

Cytotoxicity (LDH) is less severe in ZF4 cells compared to PaTu 8988t cells upon addition of the same dose of the same venom. This suggests that untransformed zebrafish cells are more resilient to snake venoms than tumour cells. This could be explained by a retention of defence mechanisms in untransformed cells that might be able to take action against damage.

Snake venoms were suggested to be effective against metastasis based on the presence of disintegrins (Selistre-de-Araujo H.S., et al., 2010).

Only indications for such an effect were observed when venom from *N. naja* was added to the fish water. Venoms of viperidae (rattlesnake and copperhead snake) did not exert any visible effects in any of the experimental settings used here. Snake venoms exhibit inhibitory properties on tumour cell-induced angiogenesis. This was only observed using venom of *N. naja* on PaTu 8988t cells. Viper venom slightly reduced the response the vasculature had on the cells, but vasculature was formed. Although, it was reported that viper venom contains disintegrins which act against processes such as tumour-induced angiogenesis (McLane M.A., et al., 1998; Markland F.S., 1998).

Snake venoms contain sometimes exosomes which can easily trigger an immune response in the victim, because these particles display MHC molecules on their surface (Ogawa Y., et al., 2008).

**Conclusion**

The results indicate that, if administered in low concentrations, snake venoms display promising anti-malignant properties. However, comparing the results obtained with different venoms it is obvious that venoms derived from the cobra species that were tested, did not have a strong impact on PaTu 8988t cells. Venoms from *Ophiophagus hannah* and *Echis carinatus* might be well suited candidate cancer inhibitors and will be used for more detailed studies wherein whole venom mixtures as well as fractions thereof will be screened.

*In vitro* obtained data is not interchangeable with data generated in *in vivo* assays. This underscores the aspect that the micro-environment of a living being cannot be imitated precisely in a culture flask. The nutrients and other resources which the cells have available in *in vivo* assays, is not present in culture media. Therefore, the response differs between the *in vivo* and *in vitro* assays.

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**Supplemental Tables**

Supplemental table 1:

Quantitation of embryos showing malignant processes

| Russels viper | total | cells out | angiogenesis |
|---|---|---|---|
| 0h | 20 | 0 | - |
| 36h | 16 | >5 in 8 embryos | - |
| 60h | 13 | >10 in 6 embryos, >5 in 1 embryo | - |

| Control | total | cells out | angiogenesis |
|---|---|---|---|
| 0h | 20 | 0 | - |
| 36h | 19 | >5 in 8 embryos | + in 13 embryos |
| 60h | 19 | >20 in 14 embryos, >10 in 2 embryos | ++ in 12, +/- in 2 embryos |

| King cobra | total | cells out | angiogenesis |
|---|---|---|---|
| 0h | 20 | 0 | - |
| 36h | 20 | >5 in 2 embryos | - |
| 60h | 20 | >10 in 2 embryos, <3 in 2 embryos | +/-- in 2 embryos |

## Chapter 6: Discussion

**Danielle Vlecken**

**Causal determinants in Pancreatic cancer**

In this study, two groups of genes were subjected to analysis. Genes that function in motility were assessed as well as genes that function in development were shown to be involved in altering cancer cell behaviour. It is obvious that many more groups of genes are potentially involved in tumour progression. Other genes have already been identified as key players in cancer. All genes, pathways and interactions involved, makes it difficult to predict the patient's disease profile, and the complexity (plus individual differences) makes it even more complicated to generate a custom made cure.

The two most important groups of genes participating in tumour genesis and –progression are the tumour suppressor genes (loss thereof) and oncogenes (overexpression or constitutive activation thereof) (Stan S.D. et al., 2010; Li L. and Leung P.S., 2014). One of these oncogenes is called RAS. In pancreatic cancer especially RAS is upregulated which leads to excessive cell division and tumour growth in the affected cells. Additionally, the Hedgehog (HH) canonical signalling pathway is activated in stromal cells. This results from upregulation of the developmental gene Hedgehog in pancreatic cancer. Also, two additional major pathways important in embryonic development, Wnt-βcatenin and Notch were found to be upregulated in pancreatic cancer (Morris IV JP. et al., 2010; HH. Wong and NR. Lemoine, 2009). All genes mentioned above have functions related to migration or adherence.

Micro-RNAs are approximately ~22 nucleotides in length. They are processed from hairpin forming precursor transcripts and function in posttranscriptional gene silencing by translational repression and promotion of target mRNA decay. These molecules were shown to play an important role in many malignancies (MiR-10b is important for breast cancer for example and MiR-10a is of importance for pancreatic cancer. Targeting these molecules could be feasible strategy to fight malignancies. MiR-10a was identified as a facilitating factor repressing HOX B1 and HOX B3. Repression of MiR-10a was shown to inhibit metastasis and invasion. (Marques I., et al., 2008; Khan S. et al., 2013). If simultaneous retinoic acid receptor antagonists are used, these processes are completely abolished.

**Malignant disease control**

Pancreatic cystic neoplasms are detectable at present day with a biomarker called CEA, on the other hand, pancreatic adenocarcinoma is difficult to detect, while this form accounts for 90% of the cases. For the only biomarker, CA19-9 which is based on ancillary testing, that is available for early detection of pancreatic cancer is not specific for the pancreas but could also point to the bile

bladder, however, this test was reported to be suitable for detection of recurrent adenocarcinoma. Pancreatic cystic cancers can be detected when cystic fluid is taken out (Melton S.D. et al., 2010)

Tumour resection is currently a widely used procedure to treat people with malignant, but also with metastatic disease. This method is generally accepted because the patient acquires then a survival benefit. However for pancreatic metastases is this treatment extremely ill-defined (Adler H., et al., 2013)

**Recent concepts**

Novel treatments and anti-cancer compounds are being evaluated at present as alternative treatments for cancer. One of these concepts was displayed in Chapter 5: biological or organic compounds such as snake venoms. Also herbs are currently under investigation and it was reported that vegetable and fruits for example are able to have a tumour regress (Stan S.D., et al., 2010; Li L. and Leung P.S., 2014; Hendrix M.J.C. et al., 2007)

Recent studies also include assessment of protein trafficking by virus induced nanoparticles into human cells (Mangeot PE. et al., 2011). For instance to target medication to a specific structure in the human body. As is shown in this work, anti-malignant metal compounds are functional against common carcinogenic processes like angiogenesis. However, if such compound would be used by a patient without a proper delivery system, the patient's healthy cells would be the first to die. Nanoscale cancer vaccines are in development presently. These contain exosomes which are loaded with tumour antigen so that antigen presentation takes place through dendrocytes to effector cells. Then NK cells can recognize the tumour by the antigen it expresses, and target it (Tan A. et al., 2012; Zeelenberg IS., et al., 2008). On the other hand, exosome signalling was reported to be abundant in cancer. Such particle is capable of loading itself with oncogenic cargo in order to empty itself after merging with the recipient cell membrane (Lee T.H. et al., 2011).

**Concluding remarks**

As might have been made clear in this work, most cancers originate from damaged or irritated tissue which, over the course of years or decades might adapt to the new (irritable) microenvironment. If such cells do not die or are not removed by the organismal defence mechanismsthey will become adapted and are able to prosper and ultimately will progress into a malignant tumour. It is widely known that nutrition determines physiologic and pathologic processes and recently it was even reported that by altering the tumour microenvironment (by changing diet), the tumour could degenerate (Hendrix MJC. et al., 2007). It is therefore essential to include whole animal models in

cancer studies. In this work I have demonstrated that zebrafish larvae are extremely useful as a model for studying cancer progression and to delineate the properties of potential anti-cancer drugs.

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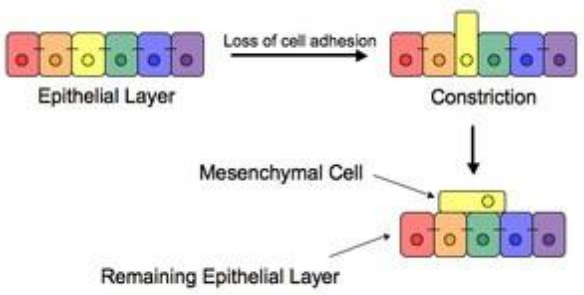
## Beïnvloeding van het gedrag van kwaadaardige pancreas cellen

De pancreas (alvleesklier) is een orgaan in het abdomen dat bestaat uit een exocrien - en een endocrien deel, die verschillende functies hebben. De meest voorkomende vorm van alvleesklier kanker is de adenocarcinoom hoewel ook tumoren bestaande uit andere celtypen voorkomen. Alvleesklier kanker wordt doorgaans in een laat stadium opgemerkt, zodra lichamelijke klachten zich presenteren. Vaak hebben de tumorcellen zich ergens anders in het menselijk lichaam kunnen vestigen waar ze tot een nieuwe tumor kunnen uitgroeien. Als de patiënt zich meldt voordat metastatische ziekte geïnitieerd is, kan chirurgie een optie zijn. Echter door de complexe positionering van de alvleesklier in het abdomen (gedeeltelijk intraperitoneaal, draait om de aorta heen) is resectie niet altijd mogelijk.

*Cellulaire processen in kwaadaardige tumoren*

Voordat er een tumor ontstaat, groeit en zich uitzaait, moet zich eerst een aantal processen in de cel voltrokken hebben, voortkomend uit veranderingen (mutaties) in het DNA. Daarna vinden de drie hieronder beschreven stappen plaat.

1. Epitheel-mesenchym transitie (EMT): Bij EMT heeft een epitheelcel de vorm aangenomen van een mesenchymale cel (Figuur 1). Dit betekent dat de anders zo immobiele epitheelcel los kan breken uit het rigide weefsele en op een invasieve manier omliggende structuren kan binnendringen, waaronder de bloedbaan. Een transformatie is vaak een lange-termijn proces waarbij er adaptatie (aanpassing) plaatsvindt van de cel aan een “veranderde” en/of chronisch-irriterende omgeving.



Figuur 1: Processen die een EMT kenmerken: Een intacte epitheliale laag ondergaat een mutatie waarbij de adhesieve kenmerken van de cel aan de basale laag verloren gaan. Hierdoor wordt de cel eerst uit zijn vorm gedrukt waardoor hij vervolgens los kan komen uit het epitheel weefsel. Langzaam verandert de cel nu van een kubische, vaak gespecialiseerde, in een langerekte cel die in staat is tot migratie, maar hierdoor zijn vermenigvuldigings-integriteit en (secretieve) functies verloren is.

(Overgenomen van http://en.wikipedia.org/wiki/Epithelial%E2%80%93mesenchymal_transition)

2. Zodra er een tumor groeit, moet er, net als bij een orgaan, aanvoer van nutriënten en zuurstof zijn en afvoer van afvalstoffen en koolzuurgas. Zolang de tumor nog klein is, kan deze zichzelf van zuurstof en nutrienten voorzien door gebruik te maken van diffusie. Maar bij een toegenomen omvang kan er zuurstof tekort (hypoxy) optreden in de kern van het tumorweefsel. Hypoxie activeert via een aantal door de tumor afgescheiden signaal moleculen de aanleg van vasculatuur (angiogenese). Dit is een process waarbij er vanuit bestaande bloed- en lymfevaten, een nieuw vaatstelsel aangelegd wordt, in de tumor. Dit proces mag niet verward worden met vascularisatie waarbij er vanuit een enkele haemotpoietische stamcel een bloedvat aangelegd wordt. De tumorcellen werden behandeld met short interference RNA (si-RNA) en aansluitend werden ze gekleurd. Hierdoor konden de cellen gevolgd worden na transplantatie in de perivitelline ruimte van de dooierzak. Voor het angiogenese experimentmwerden de cellen voorafgaand aan transplantatie gesuspendeerd in Matrigel ®. Dit is een stof die lijkt op de basale laag in weefsels, met als eigenschap dat de cellen erin blijven zitten en niet migreren.

3. Op het moment dat er vaten van en naar de tumor zijn aangelegd, kan een van tumorcellen een bloedvat binnendringen (intravasatie) en het vervolgens op een andere locatie verlaten (extravasatie). Op deze plek kan de cel uitgroeien tot een nieuwe tumor. Deze gebeurtenis wordt aangeduid als metastase. Door aanvullende mutaties die de kwaadaardige cellen verworven hebben kunnen ze overleven in vreemde of vijandige omstandigheden. Hiervoor moeten cel-cel contacten en cel-matrix contacten doorbroken worden in de tumor. De metastaserende cel maakt dan gebruik van de aangelegde vasculatuur om door het lichaam te reizen en zich mogelijk elders te vestigen. De overlevingskansen van de patient zijn dan aanzienlijk slechter geworden, omdat operatie niet meer mogelijk is. Dat betekent dat de patient aangewezen is op andere behandelmethoden.

In deze studie is het gedrag van pancreas-tumorcellen onderzocht om meer zicht te krijgen op de impact die verschillende behandelingen hebben. In het eerste deel van de thesis is de verandering in

gedrag ten gevolge van het uitschakelen van genen bestudeerd. In het tweede deel zijn de effecten van diverse experimentele stoffen geanalyseerd op het moment dat ze bij de cellen gevoegd werden.

*Genetische veranderingen in kwaadaardige pancreascellen*

HOX genen zijn van essentieel belang voor segmentatie en patroonvorming van het lichaam tijdens de ontwikkeling van bijna alle dieren. HOX genen hebben vele functies tijdens de embryonale ontwikkeling en zijn sterk geconserveerd tussen soorten en in de tijd. De rol die HOX genen spelen in metastase en angiogenese van alvleesklier-tumorcellen wordt beschreven in het **tweede hoofdstuk**. Alle 39 HOX genen werden onderzocht door middel van *in vitro* en *in vivo* experimenten in zebravis embryo's. Niet alle HOX genen leken betrokken te zijn bij metastase en angiogenese. Daarnaast is het zo dat Hox genen in staat zijn elkaars functie over te nemen, dit is gebleken doordat uitschakeling van meerdere HOX genen resulteerde in gewijzigde mate van metastase en / of angiogenese, terwijl uitschakeling van de afzonderlijke genen niet altijd in een effect resulteerde.

In het **derde hoofdstuk** wordt de invloed van LIM kinase 1 en - 2 onderzocht in alvleesklier-tumorcellen, wederom gebruik makende van zowel *in vitro* als *in vivo* modelsystemen en platforms. Deze assays werden uitgevoerd door LIMK1 en -2 mRNA uit te schakelen met toevoeging van si-RNA's tegen LIMK1 en -2 in de cellen, een dag voor de analyse. De Lim-kinases zijn betrokken bij de motiliteit van de cel doordat ze de celspiertjes (actine) kunnen beïnvloeden. Als de Lim-kinases uitgeschakeld zijn, doen zich er significant minder maligne processen voor. Als beide tegelijk uitgeschakeld werden, konden er geen kenmerken meer ontdekt worden van metastaserende – of angiogenese inducerende cellen in de zebravis embryos.

De impact van natuurlijke en synthetische bio-actieve stoffen op het gedrag van alvleesklier tumor cellen wordt beschreven in het **vierde hoofdstuk**. Dit is gebaseerd op een drugs-screen waarbij gezocht werd naar mogelijke verbindingen die eerder werden geïmpliceerd een remming te hebben in tumor cellen en een effect op angiogenese en vascularisatie. Iridium complexen zijn in staat gebleken kwaadaardige processen te remmen binnen hun therapeutische breedte.

Hoewel om van deze verbinding een succesvol geneesmiddel te maken moeten toedieningsroutes worden onderworpen aan vervolg- onderzoek. Ook moeten adjuvantia worden geoptimaliseerd of moeten ze opnieuw ontworpen worden voor toepassing in de mens.

**Het vijfde hoofdstuk** geeft meer inzicht in de eigenschappen van slangengif als het toegevoegd wordt aan tumorcellen of vissen waarin tumorcellen getransplanteerd zijn. Uit de experimenten die werden gedaan wordt duidelijk dat bepaalde slangengiffen (zoals bijvoorbeeld dat van de koningscobra) als heel mengsel remmende effecten heeft op tumor cel metastase en angiogenese in

de *in vitro* en *in vivo* systemen. Opvallend hierbij is, dat in vergelijking tot de andere onderzoeksprojecten, er een afwijkend resultaat ontstond: Als de cellen werden behandeld met een gif alvorens transplantatie, werd er geen significant effect waargenomen, maar als de zebravis embryos na celtransplantatie in hun water giffen toegediend kregen, werd er wel een effect waargenomen.

De conclusie die hieruit getrokken kan worden is dat alvleesklier kanker alleen terug gedrongen kan worden als er een drastische verandering van de leefstijl doorgevoerd wordt. Het veranderen van de omgeving van de tumor door andere voeding in te nemen, kan resulteren in afname van de celmassa, zoals eerder werd aangetoond.

## Curriculum Vitae

Danielle Helena Wilhelmina Vlecken was born on 12 November 1975 in Ain Aer, Lebanon and grew up in Amstenrade, The Netherlands. She attended secondary education at the Waterland college in Amsterdam-Noord.

In 2004, Danielle started her study in biology at the University of Leiden, where she completed two internships. Both internships were done in Dr. Bagowski's lab which formed a bridge between Integrative Zoology and Molecular and Cell biology. During the first (BSc) internship Danielle studied the role of myoglobin in zebrafish development which became a publication in the International journal of developmental biology. During the second (MSc) internship, Danielle worked on two projects. The first one included studying the expression patterns of LRIG1, LRIG2 and LRIG3 in zebrafish and mouse embryos. The other project involved assessment of the impact of metal compounds on vasculogenesis and angiogenesis in zebrafish embryos resulting in various co-authorships. These projects resulted in several co-author publications. After finishing the thesis on her MSc research projects, she received her Master's degree in 2009 (cum laude, summos honores).

From 2009-2011, two projects were started at the university of Leiden, completed and subsequently published (Chapters 3 and 4 of this thesis). Two projects were started, but not fully completed (Chapters 2 and 5 of this thesis). The *in vivo* experiments were performed, whereas the *in vitro* work was completed in 2014.

From 2009-2013, Danielle also worked on studies for the projects "Platform technology and expression systems for viral vaccines" and "Adaptation patterns in human viruses". These studies were done within the framework of a PhD project at the NVI (Netherlands Vaccine Institute), the RIVM (national institute for public health and the environment), Intravacc and the university of Utrecht.

In the beginning of 2014, Danielle returned to the University of Leiden to complete her PhD thesis based on research on the behaviour of pancreatic cancer cells under gene-knockdown conditions and in drug-screens using biological –and synthetic bio-active compounds.

In September 2014, Danielle was appointed as a scientist at the university of Leiden and she works on a project involving signaling during embryonic limb development in the chick. Additionally, Danielle is working on the setup of four companies involved in production of biological compounds and development of platforms for the manufacture of biomolecules. These companies, one of which will start as a spin-off company of the university of Leiden, are launched in 2015.

**List of publications**

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   The oncogenic potential of HOX genes in pancreatic cancer cell dissemination and tumour-induced angiogenesis

   Danielle Vlecken, Prisca Leferink, Markus Lerch, Christoph P. Bagowski and Ulrich F. Weiss,

2. In Preparation.
   Whole snake venoms: toxicity and anti-angiogenic properties
   Danielle Vlecken, Babette van Soolingen, Sjors Nooteboom, Freek Vonk, Christoph P. Bagowski, Herman Spaink and Michael Richardson

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