DISTINCT RESPONSE OF CIRCULATING microRNAs TO THE TREATMENT OF PANCREATIC CANCER

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By

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ABSTRACT

An early detection and monitoring of pancreatic adenocarcinoma has been very challenging, which makes it one of the deadliest cancers today. This has stimulated research to explore new therapeutic strategies and new ways of following treatment response. We believe that miRNAs play an important role in cancer, and circulating miRNAs from peripheral blood could not only be used as early diagnostic biomarkers, but their expression profile could give us a valuable clue about response to therapy.

We focus on targeting tumor-stroma interaction in a pancreatic cancer xenograft mouse model, with two small molecule inhibitors: (1) a fibroblast growth factor receptor (FGFR) tyrosine kinase inhibitor, PD173074; and (2) an anaplastic lymphoma kinase receptor (ALK) kinase inhibitor, TAE684.

Both drugs reduced COLO357PL pancreatic cancer cell proliferation and disruption of endothelial cell monolayer by cancer cells in vitro. In vivo, initial treatment with both drugs reduced mitosis and angiogenesis similarly. First, we identified serum miRNA
expression changes as a response to the tumor presence. Furthermore, we describe a distinctive set of circulating miRNAs that corresponds to each initial treatment before necrotic changes took place and influenced miRNA expression pattern. More importantly, in our study we distinguish between drug effect on the tumor versus host, based on the miRNA expression changes in tumor tissue and the circulation. Finally, we show a connection between successful treatment of pancreatic cancer xenografts and the circulating miRNA expression pattern.

The approach of determining circulating miRNA expression levels and patterns upon the initial treatment could be of immense importance for pancreatic cancer patients in finding the effective drug (combination) treatment and might be a model to expand to other cancers and treatment evaluations.
DEDICATION

I dedicate this work to my family -
To my mom and dad, Ljiljana and Ive, and my grandmother Ana,
who have always supported me and believed in me; and
To my family and friends for always being there for me.

May this be just the beginning.
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CHAPTER 1: INTRODUCTION
1.1 Epidemiology and etiology of pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer death in the United States with one of the lowest 5-year survival rates of only 6%, which is mainly due to the lack of symptoms during early disease progression and consequently late diagnosis (1). Pancreatic cancer is the 10th most common cancer diagnosed in men, and the 9th in women (1). However, it is more prevalent in men than women, with the median age of patients at diagnosis of 71 (1). Furthermore, pancreatic cancer is more prevalent and has higher mortality rate among the African-American compared to Caucasian and Asian-American population (1). Major risk factors for pancreatic cancer are tobacco use, obesity, family history and certain genetic syndromes like Peutz-Jeghers syndrome or familial atypical multiple mole-melanoma syndrome (1).

1.2 Treatment of pancreatic adenocarcinoma

Pancreatic cancer is typically diagnosed late because early stages are usually not accompanied with clear symptoms. However, symptoms may include abdominal discomfort, back pain, jaundice and weight loss, while nausea and vomiting occur mainly in patients with more advanced disease (1).

In 15-20% of cases, when pancreatic cancer is diagnosed in earlier stages, surgical resection is possible (1,2), but even then disease in many patients tends to recur and unfortunately patients decease from metastatic disease (3). Besides surgery and radiation therapy, standard care of pancreatic cancer patients includes chemotherapy with the nucleoside analogs, gemcitabine or fluorouracil (1). Incorporation of these pyrimidine analogs into the DNA during rapid replication of tumor cells, will prevent further
synthesis of DNA, and consequently it will result in apoptosis (3,4). Adjuvant chemotherapy with the cytidine analog gemcitabine significantly increased recurrence-free survival in treated patients after surgical resection (5). However, gemcitabine did not have an effect on overall survival (5). As opposed to nucleoside metabolic inhibitors, another treatment option is targeted therapy that is directed specifically against tumor cells based on protein (e.g. receptor) expression. One of the commonly overexpressed receptors in pancreatic cancer cells is epidermal growth factor receptor, EGFR that can be targeted by a tyrosine kinase inhibitor erlotinib (1,3). Combination therapy of gemcitabine with erlotinib showed a slight increase in patient survival compared to gemcitabine single agent therapy or placebo (3). However, combination therapy also revealed more side-effects (3).

After all, pancreatic cancer as one of the most therapy resistant cancers, still has very low overall survival time with the median of only 4.4 months (1). This has prompted research to seek new ways to diagnose, monitor disease progression and pave a way to new therapeutic options.

### 1.3 Targeting tumor-stroma crosstalk in pancreatic adenocarcinoma

Pancreatic cancer is a desmoplastic disease, where stroma has a strong impact on tumor progression, angiogenesis, metastasis and therapy resistance (6,7). In the setting of desmoplastic cancer, where targeting of the epithelial component of cancer has not made much impact into disease outcome, we decided to focus on disruption of tumor-stroma interactions.
1.3.1 Targeting of FGFR pathway

One of the most crucial signaling pathways in tumor-stroma crosstalk is the fibroblast growth factor (FGF) signaling (8). The FGF signaling pathway in human and mice consists of 22 growth factors in the FGF family and 4 FGF tyrosine kinase receptors (9). Besides cell proliferation, stemness, anti-apoptosis, epithelial-mesenchymal transition (EMT), invasion and drug resistance, the FGF pathway drives formation of new blood vessels (angiogenesis) (10) and by blocking it, there is a possibility to block tumor growth (11) and spreading of tumor cells into the circulation and to secondary tumor sites, i.e. metastasis. It is known that overexpression of FGF receptors and ligands is associated with advanced stage of pancreatic cancer (12) and we hypothesized that targeting this pathway can be beneficial in the treatment of pancreatic cancer (13). A small molecule drug, PD173074 is a first generation inhibitor developed to target FGFR and it binds into the ATP-binding pocket of the tyrosine kinase domain (10) with IC$_{50}$ of 26 nM (10,11).

1.3.2 Targeting of ALK pathway

Similarly to FGF signaling components, anaplastic lymphoma kinase receptor (ALK) and its ligand pleiotrophin (PTN) are overexpressed in cancer versus normal pancreatic tissue (12,14,15). ALK belongs to the insulin-receptor superfamily of tyrosine kinase receptors and in normal physiological conditions, ALK is expressed during development (16) and in the adult central and peripheral nervous system (17-20). It has also been shown that ALK mRNA is highly expressed in endothelial cells during active angiogenesis (21). A selective small molecule tyrosine kinase inhibitor, TAE684 with IC$_{50}$ of <10 nM is reducing ALK-driven cell proliferation (22) and we hypothesized that we can prevent activation of downstream signaling pathways involved in tumor progression (23) as well.
as prevent formation of new blood vessels within a tumor. Alternative option in targeting ALK receptor signaling is the use of a blocking antibody against the ligand binding domain of the receptor that eventually prevents downstream signaling (23). Moreover, an antibody against ALK receptor could be potentially combined with a small molecule drug, which would have an advantage in specific drug targeting of tumor cells that overexpress ALK receptor (24).

1.4 Progression of pancreatic adenocarcinoma

A progression of pancreatic intraepithelial neoplastic (PanIN) lesions towards cancer is well described in the literature and defined by the number of gene mutations and deregulated proteins characteristic for each disease stage. The most commonly found mutation in pancreatic adenocarcinoma patients is an activating point mutation of Kras, which is detected in about 30% of patients with early PanIN lesions (25). With the disease progression, the occurrence of mutated Kras gene increases and is found in 80-100% of human pancreatic adenocarcinomas (25-27). The next group of deregulated proteins is involved in an autocrine loop that potentiates tumor growth and includes overexpression of epidermal growth factor (EGF) family ligands and receptors like EGFR and HER2/neu that are overexpressed in pancreatic cancer tissue compared to healthy pancreatic duct epithelium (25,27,28). A Kras gene mutation is usually followed by a CDKN2A/p16 tumor-suppressor loss of function through gene mutation, deletion or epigenetic changes through promoter hypermethylation (1,25,29). In the late PanIN lesions, inactivation of p53 occurs and is detected in 50-75% of patients (1,25-27,29,30). Loss of heterozygosity in this locus leads to genetic instability (1,25). Along with the p53
inactivation in advanced lesions, there is a frequent inactivation of SMAD4/DPC4 (1,25,27,31,32) and occasional mutations of BRCA2 found only in late PanIN lesions and invasive cancer (1,25,27,33).

1.5 Modeling pancreatic adenocarcinoma

The well-defined pancreatic cancer progression at the genomic level has provided a solid basis for the development of mouse models that closely mimic the human disease.

1.5.1 Transgenic mouse model of pancreatic adenocarcinoma

There are several transgenic mouse models that successfully recapitulate pancreatic adenocarcinoma progression in humans, from early to late PanIN lesions and ultimately to cancer development. However, there is a one major distinction between human disease and mouse models, i.e. a multifocal disease found in mouse models as opposed to focal disease developed in humans.

The transgenic mouse model LSL-Kras$^{wt/mut}$LSL-p53$^{wt/mut}$PDX1$^{wt/Cre}$, which was first described in 2005 by Hingorani SR et al. (1,34), in many aspects resembles pancreatic cancer development in humans. This model has one mutated $Kras$ and one mutated $p53$ allele that are expressed only in pancreas after Cre recombinase from pancreatic-specific promoter $PDX1$ or $p48$, removes transcriptional stop cassette in front of the mutated alleles (1,2,34). LSL-Kras$^{wt/mut}$LSL-p53$^{wt/mut}$PDX1$^{wt/Cre}$ model has median survival of 5 months compared to the median survival of more than 12 months in the previously described mouse model LSL-Kras$^{wt/mut}$PDX1$^{wt/Cre}$ or LSL-Kras$^{wt/mut}$p48$^{wt/Cre}$ with mutation in the $Kras$ gene only (3,35). Even though the described models are great tools for studying pancreatic cancer biology and treatment effects, they have a limitation in
monitoring of disease stage in each particular mouse. This is especially true for the transgenic model with two mutated genes, \textit{Kras} and \textit{p53} because of the shorter median survival time.

1.5.2 \textbf{Xenograft mouse model of pancreatic adenocarcinoma}

An alternative option for studying pancreatic cancer \textit{in vivo} is the use of xenograft mouse models. A human pancreatic ductal adenocarcinoma cell line, COLO357PL was used in xenograft mouse models because of its positive Kras mutation status and highly aggressive phenotype (1,36). Parental COLO357 cells are moderately differentiated pancreatic cancer cells that were isolated from a lymph node metastasis of a pancreatic cancer patient (3,4,36-38). COLO357PL cells were \textit{in vivo} selected for more aggressive phenotype after parental COLO357 cells went through several cycles of metastasizing from pancreas to liver in nude mice (5,37). This mouse model has advantages because its subcutaneous tumors can be easily followed, but the weakness lies in the immuno-compromised host environment.

1.5.3 \textbf{Allograft mouse model of pancreatic cancer}

The third option is the use of a allograft mouse model in which pancreatic cancer cells are isolated from the transgenic mouse model (e.g. LSL-Kras$^{wt/mut}$ LSL-p53$^{wt/mut}$ p48$^{wt/Cre}$), cultured \textit{in vitro} and possibly cloned out, followed by injection into immuno-competent mice that are usually wild type littermates of the transgenic mice from which initial primary cancer cells were isolated. The latter described model still retains the advantage of monitoring the tumor size if cells are injected subcutaneously, but it also has the advantage of studying pancreatic cancer in an immuno-competent environment \textit{in vivo}. 
1.6 Circulating microRNA in pancreatic adenocarcinoma

Since its discovery in 1993 (5,39,40), analysis of microRNA has become an expanding research area. miRNAs are about 22 nucleotides long untranslated RNA molecules that prevent protein synthesis primarily by degrading mRNA (1,3,41) based on the complementarity of the seed sequence in miRNA and 3’ untranslated region (UTR) or open reading frame (ORF) of target mRNA (3,42,43).

miRNAs were first discovered in *C. elegans*, as short non-coding RNA molecules that have complementarity with the targeted mRNA whose translation gets inhibited via RNA-RNA interaction (3,39,40). Later they were also discovered in other organisms and were shown to be highly conserved across species, indicating a unique mechanism of posttranscriptional gene regulation throughout evolution (1,44).

1.6.1 miRNA biogenesis

miRNAs are partially coded from genes that have their own promoter (6,7,45), or from exonic or intronic regions of protein-coding and non-coding genes (8,46,47). Primary miRNAs (pri-miRNA) are transcribed mainly by RNA polymerase II (9,48,49) and are characterized by a 5’ methylated cap and 3’ polyadenylated tail (10,46,49,50). It is also common that several miRNAs get transcribed as one unit, a cluster. miRNAs from the same cluster may belong to the same family if they share nucleotide sequence in the seed region (11,46,51,52). In the nucleus, long pri-miRNA transcript is cleaved by Drosha into approximately 70 nucleotides long stem-loop precursor RNA (pre-miRNA) (12,53,54). Pre-miRNA is then exported from the nucleus into the cytoplasm by Exportin 5 (13,45,55), where it is further processed by Dicer (10,51,53,56). Dicer cleaves the pre-miRNA hairpin into an approximately 22 nucleotides long double stranded duplex of
mature miRNA and its complementary strand (12,14,15,45,48,53). Finally, the RNA duplex is bound into the RNA Induced Silencing Complex (RISC) that includes Argonaute2 (Ago2) protein as its major component (16,45,53,57). Based on thermodynamic stability, one RNA strand stays bound with Ago2, while the other strand gets degraded (17-20,48,58-60). A final step in the process of posttranscriptional regulation of gene expression is recognition of the target RNA by the RISC complex. Single-stranded RNA target recognition depends on the mRNA availability, and its nonspecific and transient binding into the RISC complex (21,61). However, only target RNA with the thermodynamically favorable binding based on the high complementarity with the miRNA seed sequence stays in the RISC complex and ultimately gets cleaved (22,61). Other, less common mechanism of posttranscriptional gene regulation mediated by miRNA with a lower level of complementarity is translational repression (23,41,53).

1.6.2 miRNA in cancer

In the decade following miRNA discovery, research in this field drastically expanded and already in 2002, miRNA was associated with cancer for the first time (23,62-64). Moreover, in 2008 a first paper was published on miRNA detected in the plasma of pregnant women (24,65). Soon after, Mitchell PS et al., suggested a use of circulating miRNAs as diagnostic biomarkers for cancer detection (25,66). In 2011 an extensive study was done on the potential use of circulating miRNA as diagnostic biomarkers of different disease conditions including cancer (25-27,67). It is fascinating that already ten differentially expressed circulating miRNAs can distinguish between patients with different diseases and healthy individuals (25,27,28,67). Interestingly, Rottiers V and Naar AM proposed endocrine and/or paracrine role of circulating miRNAs, suggesting
that miRNAs are not only being shed into the circulation, but they as well might be functional and have a signaling role in distant recipient cells, tissues or organs (68,69).

1.7 Hypothesis

We hypothesize that miRNAs play an important role in cancer, and circulating miRNAs from peripheral blood could not only be used as early diagnostic biomarkers, but their expression profile could be also used for monitoring treatment response. In 2011 we have shown a differential drug effect through changes of circulating miRNA after gemcitabine treatment of control animals and LSL-Kras\textsuperscript{wt/\textit{mut}p48\textsuperscript{wt/Cremut}} transgenic mice with pancreatic cancer (70) that encouraged further studies. Here, we describe a set of circulating miRNAs that correlates with a distinct treatment response to pathway-targeted drugs in a pancreatic cancer model. A panel of four serum miRNAs related to one of the drugs, PD173074, was also confirmed in a breast cancer xenograft mouse model in addition to the pancreatic cancer model. We also distinguished between drug effect on the tumor versus host, based on the miRNA expression changes in tumor tissue and serum.

This approach of determining circulating miRNA expression levels and patterns after the treatment initiation could be of importance for identifying an effective drug or drug combination early on, based on this pharmacodynamics readout. Moreover, by targeting the tumor-stroma crosstalk, as opposed to targeting cancer cells only, we hypothesize that we could cut off the blood supply to tumor tissue, which would be beneficial in pancreatic cancer treatment by ultimately leading to tumor regression.
CHAPTER 2: METHODS
2.1 Cell culture

Human pancreatic cancer cells COLO357PL (37), obtained from Dr. Isaiah Fidler (University of Texas, M. D. Anderson Cancer Center, Houston, TX), human breast cancer cells, MDA-MB-231, obtained from the Tissue Culture Shared Resource at Georgetown University, and metastatic breast cancer cells MDA-MB-231-BrM2, kindly provided by Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY), were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Life Technologies) with the addition of 10% fetal bovine serum (FBS).

2.2 Treatment compounds

An FGF receptor tyrosine kinase inhibitor, PD173074 (Calbiochem) was initially dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific) to the concentration of 10 mg/mL, aliquoted and stored at -20°C protected from light. A small molecule ALK inhibitor, TAE684 (Selleck Chemicals) was also initially dissolved in DMSO to the concentration of 10 mg/mL, aliquoted and stored at -20°C. For in vitro studies both drugs were further diluted in 1x phosphate buffered saline (PBS, Invitrogen), whereas for in vivo studies TAE684 was diluted in peanut oil (Spectrum Chemical Mfg. Corp.).

2.3 Cell cycle analysis (Vindelov staining)

COLO357PL cells were treated with 100 nM PD173074 and 32 nM TAE684 drug, including the appropriate vehicle controls. Forty-eight hours later cells were harvested after trypsinization and the cell number was adjusted to 1-2 million cells per condition, followed by a wash with 1x PBS. Cells were then centrifuged at 1000 rpm for 5 min, the
supernatant was aspirated and the cell pellet gently vortexed. 100 µL of citrate buffer was added to the cell suspension, mixed well, and stored at -80°C until analysis.

2.4 Apoptosis analysis (Annexin V assay)

COLO357PL cells were treated with 100 nM PD173074 or 32 nM TAE684 drug, including the appropriate vehicle controls. The apoptosis assay based on annexin V, PI and FITC staining was done 48 h from the treatment start, according to the manufacturer’s instructions (Trevigen, Gaithersburg, MD).

2.5 Electrical cell-substrate impedance sensing (ECIS) assay

To monitor cell growth and cancer cell disruption of an endothelial cell monolayer in in vitro studies (“endothelial invasion”), we used the electric cell-substrate impedance sensing system. In the growth assay, 8,000 cells per well were plated in duplicates in an xCelligence E-plate array (Roche Applied Biosciences) and impedance was measured until confluency was reached. Cells were plated in DMEM + 10% FBS with addition of DMSO as a vehicle control or with different concentrations of PD173074 (6 nM, 25 nM and 100 nM) or TAE684 drug (2 nM, 8 nM and 32 nM). For the endothelial cell invasion assay, 30,000 human umbilical vein endothelial cells (HUVEC, obtained from Cambrex Biosciences, Walkersville, MD) per well were plated in duplicates in EGM-2 media (EBM-2 medium with supplements and growth factors; Lonza). Approximately 24 h later, after HUVEC cells formed a monolayer, 6,000 pretreated COLO357PL cells per well were plated in DMEM + 10% FBS in addition of DMSO as a vehicle control or 100 nM
PD173074 and 32 nM TAE684. Disruption of the endothelial monolayer was followed during next 6 h as indicated by a drop of impedance relative to controls.

2.6 Western blot

At least one million cells per 10 cm cell culture dish were harvested for Western blot analysis. Cells were washed twice with cold 1x PBS, lysed with cold lysis buffer in the presence of proteinase inhibitor and sodium orthovanadate and collected by using a cell scraper. Following the incubation on ice for 30 min, cells were centrifuged at the maximum speed for 15 min at 4°C and the supernatant was transferred to a new chilled tube. Alternatively, tissue lysates were prepared by homogenizing tissue in 2x NP40 lysis buffer in the presence of proteinase inhibitor and sodium orthovanadate in the MagNA Lyser machine twice at maximum speed for 10 s. The supernatant was then transferred to chilled tube, and centrifuged at the maximum speed for 15 min at 4°C followed by a transfer of the supernatant to a new chilled tube. Protein concentration was measured by the Bradford assay (Bio-Rad, Life Science). A protein standard (Precision Plus Protein Dual Color Standard, Bio-Rad, Life Science) and equal amounts of protein lysates were loaded on NuPAGE 4-12% Bis-Tris gel (Novex, Life Technologies) and electrophoresis was run in 1x MOPS SDS running buffer (Novex, Life Technologies) at 120V. After proteins were separated, they were transferred onto a polyvinylidene flouride (PVDF) membrane by using the iBlot system (Novex, Life Technologies). Next, the membrane was blocked for 2 h at room temperature in 5% non-fat dry milk prepared in 1x PBST, washed with 1x PBST (0.1% Tween20 solution in PBS), and incubated with primary antibody overnight at 4°C. The following day, the membrane was washed with 1x PBST
and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. After washing the membrane with 1x PBST, the signal was developed by using chemoluminescent HRP substrate (Immobilon Western Chemoluminescent HRP Substrate, Millipore).

Primary antibodies used were: phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9101, Cell Signaling), p44/42 MAPK (Erk1/2) (#9102, Cell Signaling), and actin (#MAB1501 Millipore).

### 2.7 Immunohistochemistry

Formalin fixed paraffin embedded tumor tissue samples were histochemically stained with hematoxylin and eosin (H&E) or stained immunohistochemically (IHC) for von Willebrand factor (vWF) (#AB7356, Millipore) as an endothelial marker and for phospho-FGFR1 (#PAB16969, Abnova).

Slides were first deparaffinized at 60°C for 1 h, and rehydrated: incubation in xylene twice for 8 min, 100% ethanol twice for 5 min, 95% ethanol twice for 5 min, 70% ethanol for 15 s and rinsed with double distilled water (ddH2O) three times. Antigens were retrieved by boiling in citrate buffer, pH 6.0 (Invitrogen) for 20 min. After slides cooled down, they were rinsed three times with ddH2O. Slides were blocked in 3% H2O2 solution for 10 min at room temperature, rinsed three times in 1x PBS for 2 min, followed by avidin block (Vector Laboratories) for 15 min at room temperature and rinsed again three times in 1x PBS for 2 min. Biotin block (Vector Laboratories) was applied for 15 min at room temperature and slides were rinsed three times with 1x PBS for 2 min followed by protein block with goat serum (Vectastain Elite ABC Kit, Vector
Laboratories) for 10 min at room temperature. Tumor tissue slides were incubated in a humid chamber overnight at 4°C with rabbit vWF polyclonal antibody (#AB7356, Millipore) diluted 1:150 in antibody diluent for IHC (BD Biosciences) or with rabbit FGFR1 (phospho Y154) polyclonal antibody (#PAB16969, Abnova) at a dilution of 1:150. Antibody diluent (BD Biosciences) alone was used as a negative control. Slides were rinsed three times in 1x PBS for 2 min followed by 30 min incubation with secondary anti-rabbit biotinylated antibody (Vectastain Elite ABC Kit, Vector Laboratories) at room temperature in a humid chamber. Slides were washed 3 times in 1x PBS for 2 min and incubated with Vectastain Elite ABC Reagent (Vectastain Elite ABC Kit, Vector Laboratories) for 30 min at room temperature, followed by rinsing 3 times in 1x PBS for 2 min. Diaminobenzidine (DAB+; Dako) in DAB+ substrate buffer (Dako) was used as a chromogen and was incubated for 20 s for vWF staining or about 1.5 min for pFGFR1 staining. Reaction was stopped in 1x PBS and slides were rinsed three times in ddH2O. Sections were counterstained with hematoxylin for about 55 s, rinsed three times with tap water, once in 1x PBS for 30 s, again three times in tap water and three times in ddH2O. Slides were then dehydrated in 70% ethanol for 1 min, twice in 90% ethanol for 10 s, once in 100% ethanol for 10 s, and for 3 min and twice in xylene for 2 min. Sections were mounted in Cytoseal 60 mounting media (Richard-Allan Scientific).

2.8 Mature miRNA analysis

In the xenograft study equal volumes of serum samples from the mice that belonged to the same treatment group were pooled together, followed by the miRNA isolation with the miRNeasy Mini Kit (Qiagen) in addition of the RNeasy MiniElute Cleanup Kit
(Qiagen). miRNA was reverse transcribed to cDNA by using RT² miRNA First Strand Kit (SABiosciences) on an Eppendorf Thermal Cycler (Eppendorf). A human genome wide microRNA array for mature miRNAs (RT² miRNA PCR Array: Human Genome V2.0 (Set 1); SABiosciences) or mouse cancer microRNA array for mature miRNAs (SABiosciences) based on quantitative polymerase chain reaction (qPCR), was performed on an iCycler (BioRad) by using RT² SYBR Green/Fluorescein qPCR master mix (SABiosciences).

Top hits miRNAs were confirmed by running qPCR reactions with separate primers. Furthermore, serum versus tumor miRNA expression levels were analyzed.

In the transgenic mouse model study, equal volumes of serum samples from the mice that belonged to the same treatment group were pooled together, followed by miRNA isolation with miRCURY RNA Isolation Kit for Biofluids (Exiqon). miRNA was reverse transcribed to cDNA by using miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit II (Exiqon). A Serum/Plasma Focus microRNA PCR Panels (Exiqon) based on qPCR was performed by using ExiLENT SYBR® Green master mix (Exiqon).

2.9 Statistical analysis

Data were analyzed with software available on the SABiosciences website, GraphPad Prism (GraphPad Software, La Jolla California USA, www.graphpad.com) and IPA software (Ingenuity® Systems, www.ingenuity.com).
2.10 Xenograft mouse model

All animal studies were done with the Georgetown University Institutional Animal Care and Use Committee (IACUC) approval. One million of COLO357PL cells were injected bilaterally into athymic nude mice subcutaneously. Tumors were measured daily at two perpendicular axes and after they reached measurable size, 7 days of treatment with PD173074 or TAE684 was initiated. Six mice per drug treatment group and 7 mice in vehicle treatment group were used. PD173074 drug was administered daily intraperitoneally at a concentration of 1 mg/kg. This dose was previously established as effective for *in vivo* studies by Dr. Elena Tassi (71). TAE684 was administered daily by oral gavage at a concentration of 10 mg/kg as previously described (72). The control group was injected daily intraperitoneally with DMSO diluted in saline.

In the other xenograft mouse model 0.75 million of MDA-MB-231 cells were injected subcutaneously bilaterally into nude mice. Two days later, 11-day treatment of mice with PD173074 was initiated. The dose and drug administration was the same as in the pancreatic cancer xenograft mouse model described above. Each treatment group consisted of 3 mice per drug treatment group and 4 mice per vehicle treatment group.

After 7-day or 3-week treatment in the pancreatic cancer model and 11-day treatment in breast cancer model, mice were euthanized, blood samples and tumors were collected.

In the metastatic breast cancer mouse model, we injected one million MDA-MB-231-BrM2 cells into the heart of nude mice. MDA-MB-231-BrM2 cells metastasized to different organ sites (brain, lung and bone). At the experimental endpoint, 4 weeks after cardiac injection, blood samples were collected.
2.11 LSL-Kras\textsuperscript{wt/mut} LSL-p53\textsuperscript{wt/mut} \textit{p48}\textsuperscript{wt/Cre} transgenic mouse model

All animal studies were done with the Georgetown University IACUC approval. At about 3 months of age, treatment of mice with LSL-Kras\textsuperscript{wt/mut} LSL-p53\textsuperscript{wt/mut} p48\textsuperscript{wt/Cre} or control genotype (p48\textsuperscript{we/Cre}) was initiated. Mice were treated for 6 weeks according to the following treatment schedule: 3 times during the first week of treatment (on Monday, Wednesday and Friday), and twice a week during the next 5 weeks (on Mondays and Thursdays). A few hours after the last dose was administered, mice were euthanized, blood samples, tumor tissue, lungs and livers were collected for subsequent analysis. Each group contained at least 6 mice. PD173074 drug was administered intraperitoneally at a concentration of 1 mg/kg, while TAE684 was administered by oral gavage at a concentration of 10 mg/kg. The control group was injected intraperitoneally with DMSO diluted in saline.

2.12 Primary pancreatic cell isolation and culturing

Prior to primary cell isolation, sterile working surface inside of a laminar flow hood and two sets of sterile tools were prepared. After euthanizing a mouse with LSL-Kras\textsuperscript{wt/mut} LSL-p53\textsuperscript{wt/mut} \textit{p48}\textsuperscript{wt/Cre} genotype, the skin of the mouse was cut with the first set of sterile tools, and after that proceeded with the second set of sterile tools for cutting peritoneal membrane and isolating the pancreas. A piece of pancreas with the macroscopically apparent cancer mass was isolated and immediately transferred to the warmed up DMEM/F12 media (Invitrogen, Life Technologies) with the addition of 1:100 volume parts of penicillin + streptavidin and 1:200 volume parts of gentamicin. Tumor tissue was then minced on a clean glass surface with sterile scalpels or razor blades, and
afterwards transferred into the filter sterilized and warmed up collagenase solution (35 mL of DMEM/F12 + antibiotics, 70 mg of collagenase, 140 mg of trypsin powder, 1.75 mL of FBS, 175 µL of gentamicin). After incubation of 1-2 h at 37°C with shaker set at 150 rpm, tissue was spun at 600 rpm for 10 min. Supernatant was aspirated carefully, and was added to 15 mL of fresh DMEM/F12 media + antibiotics. Tissue was then 4 times spun at 600 rpm for 2 s, supernatant was aspirated and 10 mL of fresh DMEM/F12 media + antibiotics was added. After the final spin, primary cells were plated on a collagen coated 10 cm cell culture dish in 10-15 mL of primary cell media (500 mL of F12 media (Invitrogen, Life Technologies), 50 mL of FBS, 8 mg of insulin, 5 µg of EGF, 200 µg of hydrocortisone, 2 µg of cholera toxin, 25 mg of gentamicin, 1:100 volume parts of penicillin + streptavidin). At least 30 min later, the supernatant from the cell culture dish was transferred to a 6-well plate and primary cells were incubated at 37°C for 48 h without disturbing them.

2.13 Allograft mouse model

All animal studies were done with the Georgetown University IACUC approval. One million of mouse pancreatic cancer cells isolated from a mouse with LSL-Kras\textsuperscript{wt/mut}LSL-p53\textsuperscript{wt/mut}p48\textsuperscript{wt/Cre} genotype were injected subcutaneously and/or intraperitoneally into wild type or p48\textsuperscript{wt/Cre} littermates. Tumor growth was checked daily and tumor size was measured at two perpendicular axes.
2.14 BrdU labeling

Four hours before euthanasia and collecting tissues, mice were injected intraperitoneally with the BrdU solution in concentration of 5 mg/mL (Invitrogen, Life Technologies). The volume injected in µL was 20x the body weight in grams (e.g. 500 µL for a 25 g mouse).

2.15 Antibody production

Hybridoma cells were grown in T175 flasks in 25 mL of RPMI media (Invitrogen, Life Sciences) + 10% FBS until confluent. Hybridoma cells were also grown for 1-3 weeks in a CELLine CL-1000 flask Device (BD Biosciences) with the cultivation chamber filled with 15 mL of RPMI + 10% FBS, while there was 1 L of RPMI media in the nutrient supply chamber. Media with floating hybridoma cells was harvested and centrifuged for 5 min at 1000x g at 4°C. The supernatant was then filtered and stored at 4°C, while hybridoma cells were split.

2.16 Antibody purification

For antibody purification HiTrap Protein G HP 5 mL column (GE Healthcare, Life Sciences) was used. First, the column was reconstituted by washing it with 50 mL of filtered binding buffer, 1x PBS at the rate 5 mL/min. Then, the collected filtered supernatant from hybridoma cells was applied to the column at the slower rate of 1 mL/min, and “flow through” (F.T.) was collected. The column was then washed with 50 mL of 1x PBS to wash out all of the unbound material that was collected as “wash”. Fast protein liquid chromatography (FPLC) was performed at the rate of 3 mL/min, and 1 mL fractions were collected during elution with filtered 0.1 M glycine HCl, pH 2.8 elution
buffer, as follows: gradient reaching 100% of elution buffer during first 3 mL and
keeping 100% of elution buffer for next 20 mL. Each fraction was pH neutralized with
100 µL of 1 M Tris HCl, pH 7.5 buffer. Fractions were stored at 4°C until subsequent
experiments.

2.17 Dot blot
To determine a concentration of the purified antibody fractions, dot blots were performed.
Fifteen µL of each elution fraction from the FPLC, including starting material
(supernatant from hybridoma cells), flow through and wash, was spotted onto a
nitrocellulose membrane. As a standard a serial dilution of normal mouse IgG (Santa
Cruz Biotechnology) was used. After the membrane dried, it was blocked with 5% non-
fat dry milk in 1x PBST for 2 h. The membrane was washed with 1x PBST and HRP-
conjugated secondary anti-mouse IgG antibody was added. One hour later, the membrane
was washed with 1x PBST and the signal was developed by using chemoluminescent
HRP substrate (Immobilon Western Chemoluminescent HRP Substrate, Millipore).

2.18 Maltose binding protein (MBP) fusion protein isolation
Bacteria expressing MBP fusion protein were plated on a Luria-Bertani (LB) + ampicillin
agar plate and incubated at 37°C overnight. The next day, a single colony was transferred
into the 15 mL tube with 5 mL of LB + ampicillin media and incubated overnight in a
shaker at 37°C. The following day, 250 mL of LB + ampicillin media was inoculated
with 5 mL of overnight bacteria culture and incubated in a shaker at 37°C. At first every
60 min and later every 30 min optical density at 600 nm (OD600) was measured until it
reached 0.6-0.8. Protein synthesis was induced in logarithmically grown bacterial culture with the addition of 0.1 mM isopropyl-1-β-D-1-thiogalactopyranoside (IPTG). Final OD600 was measured after 3 h of incubation at 28°C. The bacterial culture was spun at 5000 rpm for 5 min, and the bacterial pellet was frozen overnight at -20°C. The following day, the pellet was thawed on ice and resuspended in 25 mL of binding buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) with the addition of 1:200 volume parts of phenylmethanesulfonyl fluoride (PMSF) protease inhibitor and 1:1000 volume parts of β-mercaptoethanol. Bacterial cell suspension was sonicated on ice until it cleared with cycles of 10 s sonication and 5 s break, with the amplitude of 10%. The suspension was then spun down at 10,000 rpm for 30 min to pellet the cell debris. Twenty-five mL of supernatant were loaded on a 2 mL amylose column that was previously washed with 5 mL of column buffer, and “flow through” was collected. The column was afterwards washed with 20 mL of binding buffer and the “wash” was collected. The MBP fusion protein was eluted from the amylose column with 5 mL of elution buffer (binding buffer + 10 mM maltose) per fraction. All of the steps of protein purification on the amylose column were performed at 4°C and the elution fractions were kept on ice. To the fraction, 1:200 volume parts of PMSF and 1:1000 volume parts of β-mercaptoethanol were added. Protein concentration was measured with the Bradford assay (see above) and purified protein was kept at -20°C.

2.19 Enzyme-linked immunosorbent assay, ELISA

The antigen was diluted in 1x PBS to a final concentration of 5x10^{-3} µg/µL (=0.5 µg/100 µL), aliquoted in triplicates on a 96-well Maxisorb plate (NUNC) and incubated
overnight at 4°C. The following day antigen was aspirated and wells were blocked with 5% non-fat dry milk in 1x PBST for 2 h at room temperature, and then washed once with 1x PBST. Primary antibody was diluted in 1x PBS and incubated for 2 h at room temperature, followed by 2 times wash with 1x PBST. Secondary antibody (anti-mouse HRP) was diluted 1:1000 in 1x PBS and incubated for 1 h at room temperature. Wells were then washed 2 times with 1x PBST and once with water. 100 µL/well of 1-Step Turbo TMB substrate (Pierce Biotechnology, Rockford, IL) was added until a blue color appeared, and the reaction was immediately stopped with 100 µL of 1 M H₂SO₄. Absorbance was measured at 450 nm on the microplate reader.
CHAPTER 3: DISTINCT RESPONSE OF microRNAs TO THE TREATMENT OF PANCREATIC CANCER XENOGRAFTS WITH FGFR AND ALK KINASE INHIBITORS
3.1 Inhibition of proliferation and invasion of pancreatic cancer cells *in vitro*

In this study, we focused on targeting pancreatic cancer and its crosstalk with the microenvironment by two small molecule drugs that inhibit kinase domain of FGFR receptor, PD173074; and ALK receptor, TAE684. The human pancreatic cancer cell line, COLO357PL with wild type FGFR and ALK receptors was tested for proliferation and invasion in the presence of PD173074 or TAE684. Both drugs showed a trend in reducing proliferation rate in a dose dependent manner (Fig. 1A and B). This suggests a partial dependence of these cells on autocrine signaling.

Furthermore, we were interested in the drug effect on tumor-stroma crosstalk. To investigate this, we performed an endothelial monolayer disruption assay. In this experimental set up, PD173074 and TAE684 reduced disruption of the HUVEC monolayer by cancer cells by 50 and 37%, respectively (Fig. 1C). Neither, PD173074 nor TAE684 showed an effect on the endothelial cell monolayer without added cancer cells.

In addition, cell cycle analysis by Vindelov staining showed a slight increase in the percentage of cells that entered G1 cell cycle phase arrest after PD173074 and TAE684 treatment (Fig. 2A and C). Annexin V assay showed increase in the cell population that undergoes early apoptosis after treatment with either of drugs (Fig. 2B and D).

Cell cycle and apoptosis analysis shows that PD173074 and TAE684 are not particularly toxic for COLO357PL cells in concentrations used in the experiments, but they rather influence tumor-stroma crosstalk by reducing invasive potential of cancer cells.
Figure 1. Proliferation and invasion assay of COLO357PL cells with the inhibitors, PD173074 and TAE684 using the electric cell-substrate impedance sensing (ECIS) system. In the proliferation assay, COLO357PL cells were plated in duplicates with a treatment compound and measurements were made every 15 min. COLO357PL cells were treated with three concentrations of PD173074: 6 nM, 25 nM and 100 nM (A), or TAE684: 2 nM, 8 nM or 32 nM (B). As a vehicle control in both experiments cells were treated with DMSO diluted in cell growth media at a concentration of the highest treatment compound. Proliferation curves in A and B are shown as a mean of experimental results from the duplicate wells +/- SEM. In the invasion assay (C), HUVECs formed a stable monolayer prior to addition of pretreated COLO357PL cells together with drug. The cell index was measured every 5 min and it was followed for the first 6 h after addition of the cancer cells. The percentage of the endothelial monolayer disruption was calculated for the 6 h time point. The experiment was done in duplicate wells with shown mean +/- SEM. Data were statistically analyzed with t-test and p-values are indicated.
Figure 2. Cell cycle progression and apoptosis analysis of COLO357PL cells treated with PD173074 or TAE684. COLO357PL cells were treated with 100 nM PD173074 (A and B) or 32 nM TAE684 (C and D) for 48 h and subsequently analyzed for cell cycle progression (Vindelov staining) (A and C) and for apoptosis (Annexin V) (B and D) by FACS analysis. Vehicle treatment consisted of DMSO diluted in cell growth media. Experiments were done in duplicates and repeated twice, and mean +/- SEM is shown.
3.2 Reduction of tumor cell proliferation and angiogenesis in vivo

After determining the effect of PD173074 and TAE684 on the growth rate and invasion ability of COLO357PL cells in vitro, we examined the drug effects on tumor in vivo, in a xenograft model. Three groups of nude mice were injected bilaterally with COLO357PL cells. After tumors reached measurable size, mice were treated for 7 days with 1 mg/kg of PD173074, 10 mg/kg of TAE684 or vehicle.

The tumor sizes measured by caliper were not different after this treatment period. However, treatment with both drugs impacted mitosis when compared to vehicle treated group, and was quantified on H&E slides (Fig. 3A and B). In addition, the microvasculature was quantified on H&E stained tumor slides (Fig. 3C). The number of mitotic figures and capillaries was significantly reduced in tumors from PD173074 and TAE684 treated mice compared to the control group. The effect of the two drugs on mitosis and capillary density was indistinguishable from one another.

Furthermore, we performed immunohistochemical staining of tumor samples for von Willebrand factor (vWF) as a marker of endothelial cells (Fig. 4). vWF staining corroborated findings on H&E tumor slides in reduction of blood vessels after PD173074 treatment. However, IHC staining for vWF stains bigger blood vessels and not necessarily capillaries, which might be the reason for non-significant reduction in number of blood vessels after initial treatment with TAE684.
Figure 3. H&E stained COLO357PL xenografted tumor tissue accompanied by quantification of mitotic figures and capillary number per field. Formalin fixed paraffin embedded COLO357PL tumors were H&E stained. Ten pictures of different fields per tumor sample were taken for subsequent analysis. Representative pictures of tumor samples from different treatment groups (vehicle, PD173074 and TAE684) are shown at 20x magnification (A). Average number of mitotic figures (B) and capillaries (C) per picture was assessed in each group. Minimum of 7 tumor sections per treatment group were analyzed. Mean +/- SEM is shown followed by statistical analysis with t-test. ** p<0.01; *** p≤0.0002. White arrowheads in panel A represent capillaries that were counted during the analysis.
Figure 4. Immunohistochemistry for von Willebrand factor (vWF). Ten pictures of different fields per tumor sample were taken for analysis. Representative pictures of tumor samples from different treatment groups (vehicle, PD173074 and TAE684) are shown at 20x magnification (A). Minimum of 5 tumor sections per treatment group were analyzed. The average number of capillaries per picture was assessed (B) and statistical significance was determined by t-test. ** p<0.01.

To assess the FGFR1 kinase inhibitory activity of PD173074 in vivo, we also performed IHC staining of tumor samples for phosphorylated receptor FGFR1. Tumors in the PD173074 treated group of mice showed a significant reduction of activated FGFR1 compared to control or TAE684 treated group (Fig. 5A-C). Interestingly, TAE684
treatment significantly increased phosphorylation of FGFR1 in the tumor sections, suggesting that the activation of an alternative pathway (FGFR pathway in this case) after the inhibition of ALK signaling could provide a compensatory effect. Moreover, to assess the impact of drug treatments on downstream signaling, western blots of tumor samples for downstream effectors were performed. Staining of phosphorylated ERK was decreased after both, PD173074 and TAE684 treatment (Fig. 5D) indicating drug efficacy on downstream signaling. This is of particular interest since the cancer cells carry activated Kras that can drive ERK phosphorylation independent of upstream activators. This suggests a possible feedback loop that is interrupted.

Figure 5. Efficacy of PD173074 and TAE684 treatment analyzed by immunohistochemical staining for phospho-FGFR1 and Western blot for phospho-ERK. After immunohistochemical staining for pFGFR1, pictures of 10 fields per tumor
sample were taken for analysis. Representative pictures of tumor samples from different treatment groups (vehicle, PD173074 and TAE684) are shown at 20x magnification with zoomed-in inserts to show localization of staining (A). Immunohistochemical staining of tumor tissues for pFGFR1 was quantified according to the staining intensity divided into 4 categories from very light (+/-) to strong staining (+++) (B). Statistical significance was determined by using chi-square test for trend. *p<0.05; ** p<0.01; *** p<0.0001 (C). Protein lysates from frozen tumor samples were analyzed by Western blot for pERK and compared to the levels of total ERK protein expression (D).

3.3 Serum miRNA analysis in pancreatic cancer xenograft mouse model

Serum samples were collected at the experimental endpoint, i.e. after the 7-day treatment period, and pooled from the mice that belonged to the same experimental group. Pooled serum samples were analyzed for miRNA expression by q-PCR and data were normalized for the median Ct value of a panel of 352 miRNAs.

A cutoff of miRNA expression in serum with the Ct value of 30 cycles was used. That left us with 108 out of 352 miRNAs above the provisional detection limit (Fig. 6).

Serum miRNA expression was analyzed further as depicted in Fig. 6 in a schematic miRNA analysis flow chart.
Figure 6. Flow chart of serum miRNA analysis. The expression levels of 352 miRNAs in serum samples of 4 different treatment groups of mice were analyzed by q-PCR. The criteria used to select differentially expressed serum miRNAs are given.

Intriguingly, in a hierarchical clustering analysis, the vehicle treated group of cancer bearing mice grouped with PD173074 treated mice, suggesting that there might be a smaller effect of the PD173074 compared to TAE684 treatment (Fig. 7).
First, serum miRNA levels in COLO357PL xenografted mice and non-tumor bearing mice were compared. This panel of deregulated miRNAs is solely related to the COLO357PL tumor presence. Significantly deregulated serum miRNAs were considered those miRNAs that were ± 2 fold up- or down-regulated. Tumor related serum miRNAs include more than 2 fold up-regulated miR-16 and miR-19b, and more than 5 fold down-regulated miR-22, miR-128, miR-222, miR-296-5p, miR-486-5p and miR-574-5p (Fig. 8).
Figure 8. Serum microRNAs impacted by the presence of COLO357PL xenograft tumors (tumor related) and by treatment of xenograft tumor bearing mice with PD173074 or TAE684. The expression levels of 352 miRNAs were analyzed by q-PCR and normalized for the median Ct value. Here, the most up- and down-regulated serum miRNAs are shown relative to control for the presence of xenografts (“tumor related”), while changes due to the treatment (“PD treated” or “TAE treated”) are relative to vehicle treated mice with xenografts.

Similarly, in the comparison of serum miRNA expression levels from xenografted vehicle treated mice with the xenografted PD173074 or TAE684 treated mice, we got treatment related serum miRNAs (Fig. 8). From there we identified a list of mainly up-regulated serum miRNAs that are related to the PD173074 treatment response. miR-18a, miR-22, miR-93, miR-101, miR-186, miR-296-5p and miR-361-3p were more than 2.5 fold up-regulated. Correspondingly, we identified a list of serum miRNAs that is related to TAE684 treatment. miR-1 and miR-192 were approximately 2 fold up-regulated,
whereas miR-17, miR-29a, miR-140-5p, miR-320a and miR-425 were more than 2.5 fold down-regulated in the serum of tumor bearing mice upon the treatment.

Subsequently, we confirmed the data obtained from the miRNA panel with separate q-PCR reactions (ran in duplicates) for 10 miRNAs that showed the largest deregulation. Additionally, we analyzed tumor- and drug-related series of serum miRNAs for impacted pathways using the Ingenuity software (Fig. 9). The analysis is based on the published literature and provides an output of pathways, diseases and cellular functions known to be associated with each set of miRNAs.

![Figure 9. Pathways, diseases and cellular functions associated with serum microRNAs related to the tumor presence and to PD173074 or TAE684 treatment. The analysis was done with the Ingenuity software and is based on the available published literature.](image)

This analysis showed that miRNA changes in three sets (tumor related, PD173074 and TAE684 treatment related) overlap in pathways related to cancer and cell cycle, as one would expect since all three sets of miRNAs were from tumor bearing mice. The cancer related list of miRNAs included pathways involved in gastrointestinal disease, while PD173074 and TAE684 treatment related lists overlap in cell signaling pathways, likely
due to altered downstream signaling expected from receptor kinase inhibitors. The TAE684 related list of miRNAs also includes pathways involved in drug metabolism and small molecule biochemistry, possibly due to a broader range of effects of TAE684 versus PD173074.

3.3.1 Drug effect on serum miRNAs in non-tumor bearing mice

In addition to PD173074 and TAE684 treatment of mice with COLO357PL xenografts, we also analyzed two additional groups of mice (n=5) that were not injected with cancer cells, but treated for 7 days with either of the two drugs. At the experimental endpoint, we collected serum samples and ran q-PCR on each (non-pooled) serum sample for the panel of 6 miRNAs that were chosen based on the most deregulated miRNAs related to COLO357PL tumor presence and PD173074 or TAE684 treatment (Fig. 8). Data were normalized for U6 small nuclear RNA. All six serum miRNAs showed down-regulation after either of the treatments compared to non-treated non-tumor bearing nude mice. Since cancer bearing mice have distribution of serum miRNAs after the treatment in both directions, this suggests the impact of tumor tissue contribution to the serum miRNA readout upon the treatment.
Figure 10. Serum microRNAs related to short-term treatment with PD173074 and TAE684 of non-xenografted mice. A set of six serum miRNAs was analyzed in serum samples of nude mice without xenografts that were treated for 7 days with PD173074 or TAE684. Data from COLO357PL xenografted mice from Fig. 8 are included here as well.

3.4 Comparison of serum miRNA from pancreatic and breast cancer xenograft mouse models

To evaluate a different tumor type, we also included a study with the breast cancer MDA-MB-231 xenograft mouse model treated with PD173074. Serum samples from the breast cancer xenograft mouse model were collected at the experimental endpoint, similarly to the pancreatic cancer model. Here, we analyzed a panel of 88 mature miRNAs by q-PCR. Nineteen of 88 miRNAs in the panel were ≥2 fold up- or down-regulated upon the initial PD173074 treatment of breast cancer bearing mice. Furthermore, 4 of these 19
deregulated serum miRNAs (let-7c, miR-30c, miR-103 and miR-122) were more than 2 fold up-regulated after the treatment in both xenograft models of breast and pancreatic cancer (Table 1). Interestingly, two of four miRNAs: let-7c and miR-103, were down-regulated in both cancer types relative to healthy controls, and PD173074 treatment brought their levels up. Moreover, a group of mice with primary MDA-MB-231 xenografts treated with PD173074 clustered with healthy mice rather than with cancer bearing vehicle treated mice, suggesting that treatment might have pushed serum miRNA expression towards the pattern seen in healthy animals (Fig. 11A).

Additionally, we defined a set of serum miRNAs related to breast cancer in comparison with non-treated MDA-MB-231 xenografted mice with the non-tumor bearing mice (Table 1). In a comparison of serum miRNAs that are deregulated in pancreatic cancer mouse model with the miRNAs relevant to breast cancer model, we obtained two distinct lists specific for each cancer type. However, we found 7 miRNAs that were deregulated in both models. It is an interesting observation that 4 of these miRNAs (let-7c, miR-103, miR-15a and miR-222) move in the same direction in both tumor types, but the other 3 miRNAs (miR-16, miR-191 and miR-195) move in opposite direction.
Table 1. Changes in serum microRNAs due to the MDA-MB-231 xenograft tumors and initial PD173074 treatment. The expression levels of 88 mature miRNAs were analyzed by q-PCR. Serum miRNAs shown in the table are the most deregulated miRNAs related to the presence of MDA-MB-231 xenograft tumors when compared to the non-xenografted mice. A list of PD173074 related serum miRNAs was obtained in comparison to the vehicle treated MDA-MB-231 xenografted mice. In bold are those serum miRNAs that are deregulated in both xenograft mouse models of breast and pancreatic cancer after PD173074 treatment. In parentheses is included the fold change from the pancreatic cancer xenograft model study for comparison.

<table>
<thead>
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<th>miR</th>
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<th>fold change over primary tumor bearing mice (COLO357PL for comparison)</th>
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<td>let-7c</td>
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<td></td>
<td>+2.0</td>
</tr>
<tr>
<td>miR-195</td>
<td>-10.7</td>
<td>+2.3</td>
</tr>
<tr>
<td>miR-20a</td>
<td>-7.6</td>
<td></td>
</tr>
<tr>
<td>miR-212</td>
<td></td>
<td>+2.0</td>
</tr>
<tr>
<td>miR-214</td>
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<td>+2.9</td>
</tr>
<tr>
<td>miR-221</td>
<td></td>
<td>-2.3</td>
</tr>
<tr>
<td>miR-30c</td>
<td>-10.7</td>
<td>+2.2 (+2.4)</td>
</tr>
<tr>
<td>miR-98</td>
<td>-6.6</td>
<td>+2.0</td>
</tr>
</tbody>
</table>
3.5 Metastasis related serum miRNA in MDA-MB-231 breast cancer xenograft mouse model

In the breast cancer model we also sought to identify serum miRNAs related to metastasis rather than the primary tumor. A set of 23 out of 26 deregulated serum miRNAs found in primary tumor bearing mice, was also deregulated in the serum of mice with metastases (Fig. 11B).

Figure 11. Serum expression analysis of 88 miRNA in healthy, MDA-MB-231 primary tumor-bearing and metastatic mice. Clustogram depicts genetic distance between listed 4 groups of mice, based on serum miRNA expression (A). Schematic overlap of serum miRNAs related to the presence of primary and metastatic MDA-MB-231 breast cancer xenografts (B).
However, 6 serum miRNAs (miR-1, miR-126-3p, miR-126-5p, miR-130a, miR-146a and miR-205) were unique to metastatic disease and were not deregulated in mice with localized primary xenograft tumor (Table 2). This suggests their potential use as serum biomarkers of metastatic breast cancer in addition to the diagnostic biomarkers of the primary cancer.

Table 2. Serum microRNA related to the presence of MDA-MB-231 metastasis.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>fold change over healthy mice</th>
<th>fold change over primary tumor bearing mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MDA-MB-231 metastasis related miRs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>that were not changed in serum of mice with primary tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-126-3p</td>
<td>-8.0</td>
<td>-3.9</td>
</tr>
<tr>
<td>miR-1</td>
<td>-8.6</td>
<td>-3.4</td>
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<tr>
<td>miR-126-5p</td>
<td>-4.6</td>
<td>-2.8</td>
</tr>
<tr>
<td>miR-146a</td>
<td>+2.1</td>
<td>+2.3</td>
</tr>
<tr>
<td>miR-130a</td>
<td>+2.8</td>
<td>+2.9</td>
</tr>
<tr>
<td>miR-205</td>
<td>+2.8</td>
<td>+3.8</td>
</tr>
</tbody>
</table>

Table 2. Serum microRNA related to the presence of MDA-MB-231 metastasis. The expression level of 88 miRNAs was analyzed by q-PCR. Metastasis related serum miRNAs were compared to the expression levels in both groups: primary xenografts and non-xenografted mice. The serum miRNAs deregulated in metastatic disease and not in mice with localized MDA-MB-231 tumors are listed.

Interestingly, mice with metastatic MDA-MB-231 xenografts were separated from healthy and primary xenograft bearing mice in the clustogram that was based on serum miRNA expression (Fig. 11A).
3.6 Tumor miRNA analysis

Following the serum miRNA analysis, we determined miRNA expression levels in COLO357PL tumor samples. Due to the different cellular sources, serum and tumor miRNA expression levels are expected to show distinct patterns (Fig. 12). For example, miR-126 or miR-192 have higher expression levels (lower Ct values) in COLO357PL tumor samples, whereas miR-486-5p is detected in serum samples at higher levels relative to tumor tissue (Fig. 12).

Figure 12. Comparison of the impact of drug treatment on the serum and tumor microRNA expression. Baseline expression level of each miRNA in vehicle treated COLO357PL tumor-bearing mice is represented by an open circle, and expression level of the same miRNA after the treatment with PD173074 (A) or TAE684 (B) is represented by an arrowhead of the same color. The direction of vectors indicates the change in miRNA expression upon treatment. q-PCR for miRNAs in serum samples were run in duplicates on previously pooled serum samples from 6-7 mice per treatment group, while 7-8 tumor samples per treatment group were analyzed for miRNA expression separately. Mean Ct values are represented, while SEM for serum samples is in the range 0.00 – 0.25, and for tumor samples 0.24 – 2.13.
The treatment affects pathways in tumor and host differently, depending on the microenvironmental context, that eventually leads to the changes in the miRNA readout. To compare drug effect site, i.e. tumor versus host, the changes seen after drug treatment are presented relative to control in Fig. 13.

![Figure 13. PD173074 (A) and TAE684 (B) effect on miRNA expression in the serum and tumor tissues.](image)

The baseline expression levels of miRNAs in vehicle treated COLO357PL tumor-bearing mice was set as 1 in serum and in tumor samples (represented by an open black circle). The arrowheads represent the fold changes in the serum (on the x-axis) and in the tumor (on the y-axis) after the treatment. q-PCR for miRNAs on serum samples were run in duplicates on previously pooled serum samples of mice that belonged to the same treatment group (n=6-7 serum samples per treatment group), while tumor samples were analyzed separately for miRNA expression (n=7-8 tumors per treatment group).

The PD173074 and TAE684 treatment effect is shown relative to the miRNA expression levels in the serum and tumor tissue of vehicle treated cancer bearing mice. To quantify
treatment effect site preference for each miRNA, we measured the angle that each vector from Fig. 13 displays relative to the ordinate (y-axis). When treatment impacts miRNA expression levels only in tumor tissues, meaning that there is only a vertical shift, the angle would be 0° or 180°. In other words, the closer to the vertical axis, the more the miRNA reflects drug effects in the tumor rather than the host. On the other hand, if the vector points along the horizontal axis with a 90° or 270° angle, the respective miRNA reflects drug effects on the host tissues that are not part of the tumor. In most cases, treatment triggered changes in tumor tissue as well as in serum samples, giving the range of angles between -18° and +163° in case of PD173074 treatment; and between -58° and +21° for TAE684 treatment (Table 3.). Interestingly, most of the observed miRNA changes point to tumor tissue versus serum, suggesting that the respective drug has a greater impact on the tumor than on the host. The exception is miR-1 after TAE684 treatment, and miR-223 change after PD173074 treatment, since those miRNAs change in both compartments almost equally.

<table>
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<th>miRNA</th>
<th>angle</th>
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<th>distance in cycles</th>
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</thead>
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</tr>
<tr>
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</tr>
<tr>
<td>miR-126</td>
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<td>0.32</td>
<td>1.48</td>
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<td>miR-16</td>
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<td>0.12</td>
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</tr>
<tr>
<td>miR-18a</td>
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<td>-0.31</td>
<td>1.44</td>
</tr>
<tr>
<td>miR-192</td>
<td>21.8</td>
<td>0.37</td>
<td>1.20</td>
</tr>
<tr>
<td>miR-22</td>
<td>-11.0</td>
<td>-0.19</td>
<td>2.08</td>
</tr>
<tr>
<td>miR-223</td>
<td>36.2</td>
<td>0.59</td>
<td>1.73</td>
</tr>
<tr>
<td>miR-486-5p</td>
<td>-1.2</td>
<td>-0.02</td>
<td>1.16</td>
</tr>
<tr>
<td>miR-93</td>
<td>-3.6</td>
<td>-0.06</td>
<td>1.63</td>
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</table>

<table>
<thead>
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<th>miRNA</th>
<th>angle</th>
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<th>distance in cycles</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>miR-101</td>
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<td>0.11</td>
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</tr>
<tr>
<td>miR-126</td>
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<td>miR-18a</td>
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</tr>
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<tr>
<td>miR-22</td>
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<td>miR-223</td>
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<td>miR-486-5p</td>
<td>6.5</td>
<td>0.11</td>
<td>2.57</td>
</tr>
<tr>
<td>miR-93</td>
<td>21.0</td>
<td>0.36</td>
<td>4.24</td>
</tr>
</tbody>
</table>

Table 3. Quantification of tumor versus serum microRNA expression level changes as an angle between vehicle and treatment group. The angle demonstrates whether the changes in miRNA expression after treatment occur preferentially in the serum (host) or
the tumor sample. The arrow length represents an overall shift in expression after treatment, and it is presented here as a distance in Ct values.

For instance in Fig. 13A, miR-223 changes almost equally in serum and tumor samples after PD173074 treatment. Conversely, miR-486-5p changes almost exclusively in the tumor after PD173074 treatment, while miR-192 changes only in the tumor after TAE684 treatment.

Additionally, from the type of analysis shown in Fig. 13 (length of the vectors) and in Table 3 (distance in cycles), it appears that TAE684 has a greater effect on miRNAs relative to PD173074.

In Fig. 14, the sinus values of different vectors of miRNA changes after PD173074 or TAE684 treatment are plotted. PD173074 has a preferential effect site in the tumor compared to TAE684 when affecting pathways where miR-1 or miR-93 are involved. In contrast, miR-192 or miR-223 are changed preferentially in the tumor after treatment with TAE684, compared to the effect of PD173074. Finally, both drugs affect pathways where miR-16, miR-22, miR-101, miR-126 and miR-486-5p, are involved in a similar manner.
Figure 14. Tumor versus host effect site preference of the treatment. The direction of each vector from Fig. 13 was described by an angle relative to the ordinate (y-axis), and it is a measurement of treatment effect site preference between tumor and host (serum). Here, in Fig. 14, the sinus values of the vectors of miRNA changes after the PD173074 or TAE684 treatment are plotted. The area of the tumor effect site preference of the treatment is encircled.

Nonetheless, if we take a sine of 0.30 as a cutoff, there are 8 of 10 miRNAs in the TAE684 treated group that follow the criteria, and 5 of 10 miRNAs in the PD174073 treated group. This suggests that compared to the PD173074 drug, TAE684 affects pathways and causes miRNA changes in tumor tissues to the greater extent relative to the host.

3.7 The effect of prolonged treatment on tumor growth

To determine the effect of prolonged treatment on COLO357PL tumor growth, we treated mice for 3 weeks. Since the initial treatment of 7 days with PD173074 or TAE684 showed similar histological effects in reduced formation of microvasculature and reduced
cancer cell proliferation in xenograft tumors (see Fig. 3), we could not predict the outcome of the longer treatment.

When we continued daily treatment of mice with PD173074 or TAE684, there was a significant delay in tumor growth in the group of TAE684 treated mice compared to control. Median survival of TAE684 treated group of mice was 24 days versus 12 days in vehicle treated group (Fig. 15).

![Figure 15. Tumor size after prolonged treatment with PD173074 and TAE684 of COLO357PL xenografted mice.](image)

Figure 15. Tumor size after prolonged treatment with PD173074 and TAE684 of COLO357PL xenografted mice. Treatment started at day 5, after the subcutaneous injection of COLO357PL tumor cells, and tumor size was measured daily. In a Kaplan-Meier curve of tumor growth, each event was an increase in tumor size of 100 mm$^2$ or more relative to the size of 26 mm$^2$ at the treatment initiation day. Kaplan-Meier curves were compared by a Gehan-Breslow-Wilcoxon test: **,p=0.0091; and Mantel-Cox (log-rank) test **,p=0.0052.

Moreover, in the 3-week treatment experiment, we assessed the tumor cell proliferation rate by BrdU staining. All three groups of mice showed similar staining of tumor sections within 3 cell layers from the blood vessels (Fig. 16 A and C). However, vehicle treated tumors showed staining that was more prominent between the blood vessels (Fig. 16 A and B). This data complement the results obtained after short-term treatment, when we
found less actively dividing cells in tumors of drug treated mice compared to controls (see Fig. 3B). This finding can also be correlated with the higher number of capillaries in vehicle treated mice versus PD173074 or TAE684 treated groups found in the 7-day treatment study (see Fig. 3C). In this case, control mice might have more capillaries accompanied by the higher number of dividing tumor cells. However, these capillaries are much smaller than the blood vessel that were taken into account while quantifying BrdU positive cells between versus away from the vessels in Fig. 16.
Figure 16. BrdU staining of COLO357PL xenografted tumor sections in prolonged vehicle, PD173074 or TAE684 treated mice. Localization of proliferating tumor cells immunostained for BrdU in COLO357PL xenograft tumor sections at 10x magnification (A). Quantification of BrdU staining of tumor sections between blood vessels (B) and within 3 cell layers from vessels (C) is represented. A minimum of 22 fields per group (total of 71 fields) was made from tumors that belonged to 4 vehicle treated mice, and to 3 mice per each drug treatment group. The average number of BrdU positive cells per mouse was calculated and the mean +/- SEM per group is shown in panels (B) and (C). Statistical significance was determined by t-test. n.s. – non significant.
Furthermore, we stained COLO357PL xenograft tumor sections from the mice in the prolonged treatment experiment for apoptotic cells with a TUNEL assay (Fig. 17A). We observed more apoptotic cells in the group of PD173074 treated mice and statistically significant increase in number of apoptotic cells in TAE684 treated group of mice (Fig. 17B).

Figure 17. TUNEL staining of COLO357PL xenografted tumor sections in prolonged vehicle, PD173074 and TAE684 treated mice. Localization of the TUNEL stained apoptotic tumor cells in COLO357PL xenograft tumor sections at 40x magnification (A). Apoptotic cells were quantified in 5 pictures per tumor, the average number of TUNEL positive cells per tumor in each mouse was calculated, and the mean +/- SEM is shown in panel (B). Statistical significance was determined by t-test. n.s. – non significant.
An important observation during the dissection was a presence of the large necrotic regions in the subcutaneous tumors of TAE684 treated mice, where tumors consisted of only thin layer of tumor cells around large empty or fluid filled area in the middle of tumor (Fig. 18). Necrosis in COLO357PL xenograft tumor tissue was quantified, and a trend in increased tumor necrosis in TAE684 treated group was shown in Fig. 19.
Figure 18. Histology of the COLO357PL subcutaneous tumor tissues (H&E)
staining) from mice in the prolonged treatment study. A mouse identification number with the tumor site, final size of the tumor in mm², and a day post tumor injection when the mouse was sacrificed is indicated in each panel. Pictures were taken at 4x magnification.

Figure 19. A quantification of necrosis in COLO357PL xenograft tumor after prolonged treatment. Between 3 and 5 tumors were evaluated using H&E stained sections. The necrotic areas were identified and quantitated by image analysis and are shown as percent of the total tumor (mean +/- SEM). Statistical significance was determined by one sample t-test.

In conclusion, the TAE684 treatment effect was indicated by the serum miRNA profile after the initial treatment, even though the full effect in delaying tumor growth became obvious only later, during the prolonged treatment.
3.8 Discussion

A response of the whole organism to a disease and/or treatment can be followed at the serum miRNA expression level. As every tissue and organ system is vascularized in order to receive nutrients and oxygen and to discard waste metabolic material, each organ can as well shed miRNA molecules into the circulation. Furthermore, cancer can be considered as a systemic disease once changes in circulating molecules such as miRNAs are observed either due to a release from the primary tumor site or as a response to the presence of cancer cells. An interesting observation is a comparison of drug effects on miRNA expression in the tumor tissue itself and in serum as a response of the whole organism to the drug exposure. Correspondingly, we consider the serum miRNA profile changes as the host response. Obviously, tumor response indicated by miRNA changes does not correlate with host response because many other tissues besides the tumor are affected by the treatment. Therefore, we can follow a particular miRNA to understand how it changes within the tumor and host as a response to a specific drug treatment.

It is worth noting that a drug in a defined class or mechanism of action or target, will display a specific response profile of serum miRNA expression changes. While the PD173074 drug in the pancreatic cancer model up-regulates most of the serum miRNAs compared to vehicle treatment, TAE684 impacts serum miRNAs in both directions equally and it does it with a greater overall shift. It appears that already subtle changes in miRNA expression levels, caused by the initial treatment, can lead to the fine-tuning of the system because we found that smaller changes in miRNA expression levels caused by PD173074 treatment are associated with a reduced number of mitotically active tumor
cells and reduced angiogenesis. However, the 3-week treatment study revealed that TAE684 drug had a better effect in impeding tumor growth that resulted in longer survival time of mice in the TAE684 treated group. This result might suggest that the overall shift in miRNA expression levels in combination with the treatment associated miRNA pattern, actually indicates treatment success. Clustogram (Fig. 6), miRNA pattern (Fig. 8) and overall miRNA shift (Fig. 13) upon the treatment, including tumor versus host preference (Fig. 14), all imply that in the setting of COLO357PL xenograft mouse model, the TAE684 drug causes changes in miRNA expression patterns that can be used as an indicator of effective treatment as far as tumor growth is concerned. However, it is possible that a higher dose of PD173074 drug would drive bigger changes in miRNA expression that could result in a more favorable outcome, since the dose used here showed some favorable anti-tumor effects similar to the TAE684 treatment, e.g. reduced angiogenesis and cancer cell proliferation, as well as reduced downstream signaling. Nonetheless, the dose adjustment has to be done in a way to avoid introduction of unwanted side effects.

The ability to monitor a treatment response based on the circulating miRNA profile can be of immense importance for therapy modification in patients that do not show obvious effects of the treatment measured by conventional means (tumor size, metastasis by imaging).

Several deregulated serum miRNAs found in this study were interesting in the context of cancer. The tumor related miR-22 was more than 3-fold down-regulated in the serum of pancreatic cancer bearing mice compared to healthy controls, and the same miRNA got
up-regulated after PD173074 treatment. The same miRNA has already been known as a senescence-associated miRNA up-regulated in human senescent fibroblast and epithelial cells (73), and its overexpression inhibits cancer cell growth by inducing cell cycle arrest (74-76).

Serum miRNAs of particular importance related to PD173074 treatment are miRNAs significantly up-regulated (≥2.0 fold) upon the treatment in both xenograft models of pancreatic and breast cancer: let-7c, miR-103, miR-122 and miR-30c. let-7c has been down-regulated in metastatic prostate cancer compared to the high grade localized prostate cancer (77) and in hepatocellular carcinoma compared to adjacent normal tissue (78,79). It has also been published that let-7c gets up-regulated during hypoxia (80) and to promote apoptosis of endothelial cells through direct targeting of the anti-apoptotic protein Bcl-XL (81). PD173074 treatment possibly causes up-regulation of serum let-7c due to hypoxic state that might be caused by the reduced number of capillaries after the treatment, and pushes its level towards the one detected in healthy mice. miR-103 also gets up-regulated in hypoxia (80) and its over-expression causes reduction in the migratory ability of neuroblastoma cells (82). miR-122 is considered a tumor-suppressor miRNA (83) that is down-regulated in hepatocellular carcinoma and metastatic disease (79,84) and its over-expression up-regulates apoptosis of hepatoma cells (85,86). miR-30c was found down-regulated in hepatocellular carcinoma (79) and colorectal cancer (87), and can promote cell cycle arrest by direct targeting of cyclin D2 (88). It is very encouraging that all of these 4 miRNAs with tumor suppressor properties get up-regulated after PD173074 treatment in both, the pancreatic and the breast cancer xenograft mouse models.
Another interesting observation is that two of these four serum miRNAs: let-7c and miR-103, were down-regulated in both cancer types when compared to healthy controls, and the PD173074 treatment brought their levels up (Table 1).

In the metastatic breast cancer mouse model we found 6 serum miRNAs highly relevant for this aggressive phenotype. miR-1, miR-126-3p and miR-126-5p got down-regulated, while miR-130a, miR-146a and miR-205 got up-regulated in the mice with metastatic tumors compared to primary breast cancer bearing mice or compared to healthy controls. miR-1 has showed decreased expression comparing normal to primary to metastatic tissues in prostate cancer (89), which would fit with the model of metastatic breast cancer where miR-1 is also reduced in serum samples of mice with metastatic lesions. Furthermore, miR-126-3p inhibits proliferation of mouse mammary epithelial cells (90), and in the metastatic breast cancer xenograft model, we found the same miRNA down-regulated, which could support cancer cell proliferation. Also, down-regulated serum miR-126-3p was found associated with a higher risk of lung adenocarcinoma progression and poor prognosis (91). Low expression of miR-126-3p/miR-126-5p (miR-126/miR-126* in the older miRNA nomenclature) is correlated with higher levels of pro-angiogenic factors and angiogenesis (92-94). On the other hand, serum miR-130a is up-regulated in the metastatic breast cancer model, which matches with reports that show that increased miR-130a expression in NSCLC tissues is associated with lymph node metastasis (95). miR-146a was found up-regulated in invasive breast cancer cell lines, including MDA-MB-231, compared to normal or less invasive breast cancer cell lines (96), corroborating our model. However, miR-146a and miR-205 (both up-regulated in
serum samples of mice with metastatic breast cancer) were also described as metastasis suppressors (97,98).

In addition to monitoring drug effects, an earlier diagnosis of pancreatic cancer could have significant impact. By identifying pancreatic cancer related sets of circulating miRNAs, the diagnosis of cancer could be facilitated. We identified serum miRNAs that are related to pancreatic cancer with 3 fold up-regulated miR-16 and more than 3 fold down-regulated miR-1, miR-22 and miR-486-5p when compared to mice without tumor xenografts. miR-1 and miR-22 are known tumor-suppressor miRNAs (75,89,99) and we found them down-regulated in the serum samples of pancreatic cancer bearing mice. miR-486-5p was also published to be down-regulated in colon cancer compared to normal tissue, as well as in lung cancer tissue (87,100,101). miR-16 has been shown to be down-regulated in cancer tissues (102), that is quite the opposite of the up-regulation observed in serum samples here. However, serum sample will contain contribution of all the organs shedding miRNAs into circulation and not exclusively tumor miRNAs, and could reflect the response of the host to tumor presence.

The profile of pancreatic cancer related serum miRNAs differs from the set of serum miRNAs related to breast cancer. However, four serum miRNAs (let-7c, miR-103, miR-15a and miR-222) are shared between two cancer types and all of them are down-regulated in cancer-bearing mice compared to controls, suggesting a broad-spectrum role in cancer. For example, miR-15a has been shown to have a pro-apoptotic role in human cancer cell lines including MDA-MB-231 (103) and has been found down-regulated in different tumors (102,104). Similarly, let-7c was shown to be down-regulated in prostate
and hepatocellular carcinoma, and it directly targets the anti-apoptotic protein Bcl-XL (78,81,105).

In summary, PD173074 and TAE684 slightly reduce pancreatic cancer cell proliferation in vitro and prevent disruption of an endothelial monolayer by cancer cells in vitro. Moreover, both drugs reduced mitosis and angiogenesis, as well as downstream signaling in xenografted pancreatic tumors. We identified two different sets of serum miRNAs that are related to each treatment response, where TAE684 had a greater overall impact on serum miRNA expression level changes. Additionally, we confirmed a set of four serum miRNAs related to PD173074 treatment in the pancreatic and the breast cancer xenograft model. Moreover, different miRNAs indicate treatment effect sites impacted in the tumor and host. Finally, the circulating miRNA expression profile indicates a successful prolonged treatment. Thus, circulating miRNA expression levels and patterns generated after treatment initiation could signify an effective drug, a combination or reveal the lack of efficacy or resistance.
CHAPTER 4: DISTINCT RESPONSE OF CIRCULATING microRNAs TO THE TREATMENT OF TRANSGENIC PANCREATIC CANCER MICE WITH FGFR AND ALK KINASE INHIBITORS
4.1 Histopathology of pancreatic cancer in the LSL-Kras\textsuperscript{ wt/mut }LSL-p53\textsuperscript{ wt/mut }p48\textsuperscript{ wt/Cre } transgenic mouse model

Here we used an established pancreatic cancer model (LSL-Kras\textsuperscript{ wt/mut }LSL-p53\textsuperscript{ wt/mut }p48\textsuperscript{ wt/Cre }) where mice show a median survival of 5 months and succumb to the disease (34). Disease development in these mice mimics human pancreatic adenocarcinoma progression with commonly found mutations in $Kras$ and $p53$ genes, and is thought to provide a representative model of the human disease in an immunocompetent host. The main downside of this model is that tumor progression cannot be monitored as easily as in xenograft model, so we opted for 6-week treatment at a fixed age of three months, during which controls will have progressed to PDAC but are less likely to die from the disease. Mice were treated with 1 mg/kg of PD173074 intraperitoneally, 10 mg/kg of TAE684 by oral gavage, a combination of both drugs PD173074 + TAE684, or with vehicle. H&E tumor sections were examined independently by three observers, blinded to the respective treatment groups (Bhaskar Kallakury, MD, Eveline Vietsch, MD and myself).

A range of PanIN lesions and of invasive cancer was diagnosed (Fig. 20).
Figure 20. Representative pancreatic intraepithelial neoplastic (PanIN) lesions and invasive cancer in LSL-Kras<sup>wt/mut</sup>LSL-p53<sup>wt/mut</sup>p48<sup>wt/Cre</sup> transgenic mice at five months of age. Pictures are representative for control mice and each stage of PanIN lesions or cancer. Pictures were taken at 20x magnification.

Moreover, metastases to liver and/or lungs were detected frequently (in 8 of 32 animals), and less commonly in lymph nodes adjacent to pancreas (4 of 32 mice), diaphragm (1), spleen (1), small intestines (1), perinephric adipose tissue (1), as well as perineural and lymphovascular invasion (1). In one animal, bile duct dysplasia was found. Furthermore, bloody ascites was present in 3 of 32 mice.

In Fig. 21, a distribution of pancreatic lesions across the different treatment groups is shown. PanIN1 and PanIN2 were grouped together because they are considered to be early lesions that can revert, while PanIN3 is a later lesion (“in situ cancer”) that eventually will progress to invasive cancer. In many pancreatic tissue sections different stages of PanIN lesions were detected and as is common practice the diagnosis was set by the highest grade of the lesion found even if that stage was not the most abundant one. It
is important to note that several mice in the initial cohorts died prior the treatment initiation (before the age of 3 months) and they were not included in further analyses.

![Figure 21](image)  
**Figure 21. Distribution of pancreatic lesions in LSL-Kras<sup>wt/mut</sup>LSL-p53<sup>wt/mut</sup>p48<sup>wt/Cre</sup> transgenic mice at five months of age across the treatment groups.** Groups consisted of at least 6 mice, with the number of mice per stage indicated. Statistical significance was determined by the chi-square test for trend.

Interestingly, the PD173074 treatment significantly prevented pancreatic cancer progression to the higher grade PanIN lesions and metastatic cancer in this model.

### 4.2 Circulating miRNA analysis in the LSL-Kras<sup>wt/mut</sup>LSL-p53<sup>wt/mut</sup>p48<sup>wt/Cre</sup> transgenic pancreatic cancer mouse model

Serum samples were collected on the last day of the 6-week treatment and pooled from the mice that belonged to the same experimental group. Pooled serum samples were analyzed for mature miRNA expression by q-PCR and the data were normalized for the
median Ct value of a panel of 155 mature miRNAs detectable in serum/plasma samples at Ct values lower than 30 cycles.

We compared: 1) vehicle treated transgenic mice with PanIN3 lesions (n=3); 2) vehicle treated transgenic mice with metastatic pancreatic cancer (n=3); 3) PD173074 treated transgenic mice of mixed stages as shown in Fig. 21 (n=7); and 4) TAE684 treated transgenic mice of mixed stages as shown in Fig. 21 (n=7). This set up allowed for detection of tumor progression and perhaps metastasis related circulating miRNAs, in comparison of vehicle treated transgenic mice with PanIN3 lesions to the ones with the metastatic PDAC (Fig. 22A). Moreover, we monitored PD173074 (Fig. 22B and C) and TAE684 (Fig. 22D and E) related serum miRNAs. Hundred serum miRNAs were more than 2 fold down-regulated in at least one of comparisons.
[ Fig. 22 A to E and legend are on the following pages ]
B

PD173074 treatment effect

PD173074 treatment - VEHICLE metastatic PDAC

$2^{-(\text{deltadeltaCt})}$

fold change

miRNA
Figure 22A to E. Distribution of serum miRNAs related to the disease progression and treatment of LSL-Kras$^{wt/mul}$LSL-p53$^{wt/mut}$p48$^{wt/Cre}$ transgenic mice with PD173074 and TAE684. The expression level of 155 mature miRNAs was analyzed by q-PCR and normalized for the median Ct value. A progression of PanIN3 lesions towards...
metastatic pancreatic cancer was defined by deregulated circulating miRNAs derived from a comparison of transgenic mice with metastatic pancreatic cancer to the mice with PanIN3 lesions (A). In defining treatment related serum miRNAs, two comparisons were done where drug treated group was compared to: (i) vehicle treated mice with metastatic PDAC (B and D); and (ii) vehicle treated mice with PanIN3 lesions (C and E). The miRNAs are arranged in descending order of fold changes, and differ between the panels.

With the tumor progression from PanIN3 lesions towards invasive pancreatic cancer, 63 miRNAs change more than 2 fold: 28 miRNAs get up-regulated, and 35 miRNAs down-regulated, while 37 miRNAs stay unchanged. This suggests a big impact of tumor progression on the serum miRNA pattern (Fig. 22A, Table 4 and Fig. 23).
Moreover, we compared drug treated groups of transgenic mice to the control groups of vehicle treated mice (one group that had PanIN3 lesions, and the other with metastatic pancreatic cancer). Since all panels of Fig. 22 are drawn to the same scale of the y-axis (but in a different rank order of miRNAs) it is fair to compare the shape of miRNA expression curves. It is interesting that treatment related circulating miRNA expression curves are more “flat” after normalization for vehicle treated group of transgenic mice that carried PanIN3 lesions versus those compared to vehicle treated group with metastatic cancer. This suggests that treatment drives miRNA expression towards levels observed in earlier lesions before invasive cancer occurred. The most extreme example is shown in Fig. 22E and also visible from the Table 4, in the comparison of TAE684 treated animals to the vehicle group with PanIN3 lesions. In this case, 64 miRNAs do not
change more than 2 fold, while only 6 miRNAs are ≥ 2 fold up-regulated and 30 miRNAs are ≥ 2 down-regulated (Fig. 23). For comparison, in the group of PD173074 related miRNAs normalized for vehicle group with PanIN3 lesions, 13 miRNAs are up-regulated and 35 miRNAs down-regulated, while 52 miRNAs stay unchanged, indicating bigger deviation from PanIN3 lesions relative to TAE684 treatment.

Figure 23. Distribution of up- and down-regulated miRNAs in the LSL-Kras\textsuperscript{wt/mut} LSL-p53\textsuperscript{wt/mut} p48\textsuperscript{wt/Cre} transgenic mouse model. Statistical significance was determined by using chi-square test for trend. * p<0.05; ** p<0.01.
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| Table 4. A list of all deregulated miRNAs with the indicated fold change in the LSL-
The serum miRNA profiles derived from the transgenic mouse model roughly and only partially resemble the profile derived from COLO357PL xenograft mouse model related to tumor presence/progression and PD173074 and TAE684 treatment, meaning that the particular miRNAs changed its expression in the same direction. However, several miRNAs changed in the opposite directions, which can be explained by the use of different mouse models, different duration of treatment and different comparisons of groups. More analysis is needed and will be performed in future.

4.3 Isolation of primary pancreatic cancer cells from the LSL-\(Kras^{wt/mut}\)\(\text{LSL-}p53^{wt/mut}\)\(p48^{wt/Cre}\) transgenic mice

In order to circumvent the difficulty of disease staging, we decided to take an alternative approach, i.e. an allograft mouse model. The allograft model has an advantage of growing cells in an immunocompetent host and at the same time it allows for an easy assessment of tumor growth if mouse pancreatic cancer cells are injected subcutaneously.

The first step was the isolation of primary pancreatic cancer cells from a pancreas of LSL-Kras\(^{wt/mut}\)\(\text{LSL-}p53^{wt/mut}\)\(p48^{wt/Cre}\) transgenic mouse with pancreatic ductal adenocarcinoma or PanIN lesions (Fig. 24). Primary cells were isolated from the pancreata of 6 transgenic mice. Four mice were in PanIN3 stage and two mice developed metastatic PDAC. Primary cells were grown as a heterogeneous population until subcloning of single cell populations. First, cells from the pancreas of a mouse with PanIN3 lesions were subcloned in passage 6. Single cells were plated in a 96-well plate,
and 37 out of 96 clonal cell lines grew out. The concern raised in this case, was that by
passage 6 some of the clonal lines took over the initially heterogeneous cell population.
Next, primary cells from metastatic PDAC were subcloned from the first passage, and
resulted in 16 clonal cell lines out of 96, which is roughly one half of the number of
clones derived from the cloning of passage 6. This might suggest that more aggressive
clones took over the initially heterogeneous population by the later passage and gave
more clonal cell lines in comparison to the subcloning of primary cells from passage 1.
Subcloning in earlier passages suggests more heterogeneous starting population of cells
where some clones are aggressive enough to grow in vitro, but many others still did not
acquire enough mutations to allow for survival in cell culture. Moreover, we can
conclude that a subpopulation of aggressive cells already exists in PanIN3 lesions.
Figure 24. A scheme of experimental process from isolation of primary pancreatic cells from a LSL-Kras^{wt/mut} LSL-p53^{wt/mut} p48^{wt/Cre} transgenic mouse, through subcloning them from heterogeneous population to unique clones, and finally allografting them into wild type littersmates intraperitoneally and subcutaneously.
Among the first observations after subcloning from passage 1, was the phenotypic difference between clonal lines as some of the cell lines were more cuboidal (clones #1 and #2 in Fig. 24), while others looked fibroblast-like (clones #3 and #4 in Fig. 24).

Initially, eight clonal cell lines subcloned from primary cells originated from metastatic PDAC were tested for tumor growth in vivo, after intraperitoneal and subcutaneous injection into wild type littermates and all of them formed tumors (Fig. 24). Interestingly, pancreatic cancer cells that were injected intraperitoneally homed to the pancreas and formed tumors very similar to the ones observed in the LSL-Kras\textsuperscript{wt/mut}LSL-p53\textsuperscript{wt/mut}p48\textsuperscript{wt/Cre} transgenic mouse model. Surprisingly, subcutaneously injected allograft tumors also retained phenotypic features as seen in transgenic mice that develop PDAC (Fig. 24).

Two clonal cell lines formed lung and liver metastases in both mice that they were injected into; two other cell lines formed metastases in either the lung or liver in one of the two injected mice; and lastly, four clonal lines did not form metastases; suggesting heterogeneity in the metastatic ability of different clones isolated from the same primary pancreatic ductal adenocarcinoma.

Subsequently, six clones subcloned from the transgenic mouse with metastatic PDAC were selected based on their phenotype, and further characterized by Eveline Vietsch, MD.

4.4 Discussion

The LSL-Kras\textsuperscript{wt/mut}LSL-p53\textsuperscript{wt/mut}p48\textsuperscript{wt/Cre} transgenic mouse model is an excellent model for studying pancreatic cancer because of its high resemblance to human disease.
progression on the histopathological and genetic level. However, the main pitfall of this model was inability of monitoring disease progression. Ideally, we would start the treatment when mice are all in the same disease stage. However, that assessment could not have been done reliably, thus we defined treatment start when mice reached 3 months of age. Certainly, that might have impacted results as some mice might had progressed to earlier PanIN lesions, while others might had already developed cancer. Nonetheless, we randomly assigned mice to the treatment groups hence no bias was introduced. Additionally, it would be useful to increase number of mice per group, to account for disease stage variability at the beginning of treatment.

Interestingly, it seems that TAE treatment of transgenic mice caused a change in serum miRNA expression towards PanIN3 related miRNA profile to the greater extent than PD173074 treatment. However, PD173074 treatment efficiently prevented occurrence of metastatic PDAC. Furthermore, both drug treatments and combination treatment (PD173074 + TAE684) showed a trend in increasing a fraction of mice that developed only early PanIN lesions compared to the vehicle treated mice.

Furthermore, isolation of primary pancreatic cells from LSL-Kras\textsuperscript{wt/mut}LSL-p53\textsuperscript{wt/mut}p48\textsuperscript{wt/Cre} mouse model provided us with an excellent opportunity to circumvent the inability of prediction and/or following disease stage at the treatment initiation. The capability of these cells to grow in wild type littermates and the striking resemblance of newly formed allografted tumors to the tumors developed in LSL-Kras\textsuperscript{wt/mut}LSL-p53\textsuperscript{wt/mut}p48\textsuperscript{wt/Cre} mice, offered opportunity of more controlled experiments in a very similar microenvironmental setting.
Moreover, clonal cell lines derived from the same primary PDAC represent heterogeneous cell population and among other possibilities, this could allow us to study intratumoral heterogeneity and drug resistance developed after an initially successful treatment course.
CHAPTER 5: TARGETING THE ALK RECEPTOR WITH MONOCLONAL ANTIBODIES
5.1 Binding of monoclonal anti-ALK antibodies to the extracellular domain of ALK receptor

Considering the success of targeting the ALK receptor signaling in the treatment of pancreatic cancer in a xenograft mouse model by a small molecule kinase inhibitor (TAE684) (see Fig. 15), we proceeded further with an idea of targeting the ALK extracellular domain by monoclonal anti-ALK antibodies. The custom antibody production service (Abmart, Shanghai, China) provided us with 12 different monoclonal antibodies in a form of lyophilized ascites fluid. Antibodies were raised against 5 different peptides, each 10 amino acid long, that are contained within a portion of the extracellular domain (ECD) of the ALK receptor which is approximately 50 amino acids up and downstream from the ligand binding domain (LBD) (Fig. 25) (15).
Figure 25. A schematic representation of the ALK receptor and location of peptides against which monoclonal antibodies were raised. A scheme of the ALK receptor with indicated domains and amino acid residue numbers (A). Human and mouse protein sequence of the extracellular domain (ECD) of the ALK receptor, with the indicated amino acid residue number, are aligned and amino acid residues that differ between human and mouse are indicated in bigger font size (B) or underlined (C). Twelve monoclonal antibodies were raised against 5 peptides (C) that are in the ECD surrounding the ligand–binding domain (LBD).

Dissolved powder from ascites fluid, containing monoclonal antibodies was tested in a direct ELISA assay against the MBP-ECD fusion protein (maltose binding protein, MBP, fused with the 90 amino acids long portion of the extracellular domain, ECD of ALK receptor, which also includes the ligand binding domain, LBD) (indicated in Fig. 26B). As a positive control a direct ELISA assay was run against specific peptide for each of the 12 antibodies (Fig. 26B).
Figure 26. A direct ELISA assay to determine the binding of twelve monoclonal anti-ALK antibodies to the MBP-ECD antigen (A) and to the specific peptide (B). Dissolved lyophilized ascites fluid containing antibody provided by Abmart was diluted 1:50 or 1:500 in PBS and tested for binding to the MBP-ECD fusion protein. MBP alone was used as a negative control and data in (A) are normalized to it. As a positive control, each antibody in 1:100 dilution was verified for binding to its specific peptide (B). As a negative control binding of each antibody to the other 4 peptides was used, and the data were normalized to it. Antibodies are color coded according to the color code of specific peptides presented in Fig. 25.

All antibodies did bind to the specific peptides they were raised against, with the exception of Ab#10 (Fig. 26B). However, we were limited in drawing a conclusion about each antibody titer from Fig. 26, since the antibody solution came from ascites fluid and antibodies were not purified. Thus their concentration and titer will vary. Nonetheless, we obtained a qualitative answer about the antibody binding to their specific peptides and to the MBP-ECD fusion protein. Unlike the binding of eleven antibodies to their specific peptides, there were only several antibodies that bound to MBP-ECD. Interestingly, the best signal was obtained for Ab#7 and Ab#3, considering that they were raised against the same peptide. The third antibody raised against the same peptide, Ab#1, showed about 1.6 fold binding to the MBP-ECD over MBP control.

Western blotting of the MBP-ECD fusion protein with the twelve monoclonal anti-ALK antibodies is in shown in Fig. 27.
Figure 27. Western blot detection of MBP-ECD fusion protein by twelve monoclonal anti-ALK antibodies. Ten µg of MBP-ECD fusion protein was loaded per well, and blotted with 12 anti-ALK antibodies at a 1:150 dilution. Antibodies were obtained from lyophilized ascites fluid provided by Abmart. The anti-MBP antibody was used as a positive control. M – marker or protein standard.

Similarly to the direct ELISA results, Ab#7 showed the strongest signal, followed closely by Ab#3 and Ab#1. Again, all three antibodies were raised against the same peptide that is a part of the extracellular domain of ALK receptor. Interestingly, we detected a lower intensity signal after blotting with Ab#10, which was raised against a different peptide that is also part of ECD domain in the proximity of LBD domain.

An interesting observation from Fig. 27 is the background that each antibody gave. Different background intensities can be explained by the fact that all of the antibodies came from ascites fluid, and preparations might contain different amounts of contaminants since no purification was performed. Of all the antibodies that gave a signal in the Western blot, Ab#10 had the lowest background, followed by very low background by Ab#3 and Ab#7, and moderate background by Ab#1.
5.2 Biological effect of monoclonal anti-ALK antibodies

After ELISA and Western blot experiments proved the binding of monoclonal anti-ALK antibodies to the specific peptide and/or extracellular domain of the ALK receptor, we tested the antibody effect in different biological assays.

To assess anti-ALK antibody effect on cell proliferation, we used cell lines that express the ALK receptor and depend on ALK/PTN signaling. The first cell line used in this set of experiments was the SK-N-SH neuroblastoma cell line that carries an activating F1174L mutation in the ALK kinase domain (106-108). Duijkers et al. suggested that neuroblastoma cell lines with mutated ALK receptor have a better response to anti-ALK treatment compared to the lines that carry wild type ALK (109).

Indeed, several anti-ALK antibodies inhibited SK-N-SH cell growth (Fig. 28A). Ab#1, Ab#4, Ab#5, Ab#6 and Ab#10 reduced cell proliferation by about 50% or more compared to vehicle. Additionally, Ab#3 and Ab#11 had a good effect in reducing cell proliferation by 25 and 50%.
Figure 28. Cell proliferation ECIS assay with the addition of monoclonal anti-ALK antibodies. SK-N-SH neuroblastoma cell line (A), MDA-MB-231 breast cancer cell line (B), and COLO357PL pancreatic cancer cell line (C) were tested for cell growth rate in the presence of anti-ALK antibodies at a dilution of 1:50. Antibody solution from ascites fluid provided by Abmart was filter sterilized before adding it to the cells. Graphs were normalized to vehicle control that did not contain antibody and its growth rate that was set to 100%. Mean +/- SEM of duplicate measurements is shown, and statistical significance was determined by using a t-test. n.s. – non significant.

We screened two more cell lines for the effects of anti-ALK antibody treatment. In the MDA-MB-231 breast cancer cell line, Ab#3 and Ab#4 moderately reduced cell proliferation between 25 and 50%, while surprisingly Ab#9 increased it (Fig. 28B). In the
COLO357PL pancreatic cancer cells, Ab#1 showed a trend of decreasing cell proliferation (Fig. 28C).

We also tested the ability of the anti-ALK antibody to prevent disruption of HUVEC endothelial cell monolayer by MDA-MB-231 breast cancer cells (Fig. 29).

Figure 29. Monoclonal anti-ALK antibodies affect the ability of MDA-MB-231 breast cancer cells to disrupt a HUVEC endothelial cell monolayer. HUVEC cells formed a stable monolayer prior to addition of MDA-MB-231 cells together with the antibody. Antibody solution from ascites fluid provided by Abmart was filter sterilized before adding it to the cells at a 1:50 dilution. Cell index was measured every 5 min and was followed for the first 14 h after addition of cancer cells. The experiment was done in duplicate wells. Mean +/- SEM, and results are represented as a percentage of cancer cell invasion through HUVEC monolayer. Data was statistically analyzed with t-test and p-values are indicated. n.s. – non significant.

Ab#3 showed the greatest effect in preventing the disruption of an endothelial monolayer by cancer cells. Similarly, there was a trend in reducing the effect of MDA-MB-231 cells after treatment with Ab#4, while Ab#9 had no effect.

In the final ECIS experiment done with MDA-MB-231 breast cancer cells, we used two anti-ALK antibodies, Ab#3 and Ab#4, that successfully reduced cell proliferation and invasion through a HUVEC monolayer. In this experimental set up we tried to reverse the anti-proliferative antibody effect by adding an excess of the specific peptide which
antibody recognized (Fig. 30A). A similar experimental setup was done with neuroblastoma cells and Ab#1, Ab#3 and Ab#10 (Fig. 30B).

Figure 30. Breast cancer MDA-MB-231 (A) and neuroblastoma SK-N-SH (B) cell proliferation assay and the reversal of an anti-ALK antibody effect by addition of the specific peptide the antibody recognized. Anti-ALK antibody derived from ascites fluid provided by Abmart at a 1:50 dilution was incubated overnight at 4°C together with 0.01 mg/mL of its specific peptide. The experiment was done in duplicate wells, with indicated mean +/- SEM. Data were normalized to the cell growth of vehicle control at the 120 h (A) and 96 h (B) time point after plating cells. Results form (A) were analyzed with t-test and p-values are indicated.

First, there is a partial inhibition of cell proliferation after addition of an anti-ALK antibody to the MDA-MB-231 cell culture media. Incubation of media containing an antibody with the respective specific peptide antigen reversed that effect in part. In the case of MDA-MB-231 cells, there is a significant reversal, while there is only a trend in the case of SK-N-SH cells due to a lack of an effect, or too small an effect of the antibodies alone.
The anti-ALK antibodies were also tested for their effect on proliferation of the mouse pancreatic cancer clonal cell lines isolated from the LSL-Kras<sup>WT/mut</sup>LSL-p53<sup>WT/mut</sup>p48<sup>WT/Cre</sup> transgenic mouse model that developed metastatic pancreatic cancer by the age of 5 months (Table 5).

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<th>PDAC clone F2</th>
<th>PDAC clone C5</th>
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Table 5. An effect of anti-ALK antibodies on the proliferation of mouse pancreatic cancer clonal cell lines. Six different clonal lines were treated with filter sterilized antibody solution derived from ascites fluid provided by Abmart at a 1:50 dilution. n.s. – non significant.

We tested a smaller panel of at least three antibodies per clonal line. Antibodies were selected based on their efficacy in the other in vitro assays.

The six pancreatic cancer clonal cell lines had a varied response to the antibody treatment. This result suggests intratumoral heterogeneity and will warrant follow-up with purified monoclonal antibodies (see below).
5.3 Production and purification of monoclonal anti-ALK antibodies

Based on the data on antibody binding to the extracellular domain of the ALK receptor in the ELISA and biological data in proliferation and invasion experiments, we selected 5 anti-ALK antibodies to further work with: Ab#1, Ab#3, Ab#4, Ab#7 and Ab#10. Hybridoma cells that produce monoclonal IgG antibody raised against a peptide antigen contained in the extracellular domain of ALK receptor were obtained and grown in RPMI + 10% FBS media. Supernatants were harvested for purification of the antibody when cells reached >90% confluency.

The isotype of each antibody was determined (IsoQuick, Sigma-Aldrich, St. Louis, MO) (Table 6).

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<th>Ab</th>
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<th>IgG2a</th>
<th>IgG2b</th>
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Table 6. Isotypes of monoclonal anti-ALK antibodies determined by IsoQuick Kit for mouse monoclonal antibodies. Isotyping was performed on the supernatants from hybridoma cells after cells reached >90% confluency, according to the manufacturer’s instructions.

Surprisingly, the five selected monoclonal anti-ALK antibodies cover the whole panel of IgG isotypes. Moreover, it is very interesting that three anti-ALK antibodies raised against the same peptide (Ab#1, Ab#3 and Ab#7), cover three different IgG isotypes. Consequently, the different IgG isotypes might partially explain the differences in the antibody efficacy.
Hybridoma cells that produce monoclonal anti-ALK antibodies were propagated and after approximately one month in cell culture, supernatants from the growth media were tested by ELISA for binding to the extracellular domain of ALK and to peptides the antibodies cross-react with (Fig. 31).

**Figure 31.** A direct ELISA assay for five monoclonal anti-ALK antibodies to MBP-ECD antigen (A) and to the respective specific peptide (B). Supernatant of hybridoma cells that produce antibody was added straight or 1:10 diluted in PBS and tested for binding to MBP-ECD fusion protein or to a specific peptide. MBP alone was used as a negative control and data in (A) is normalized to it. As a positive control, each antibody was verified for binding to its specific peptide (B). As a negative control only blocking solution was used in the wells, and the data was normalized to that. Mean +/- SEM of duplicate measurements from two experimental wells is shown.

Similarly to the results obtained with antibodies from the ascites fluid, Ab#7 and Ab#3 showed the highest binding to the MBP-ECD fusion protein, while Ab#4 showed no binding (Fig. 31A). In this case, Ab#10 from the hybridoma supernatant shows a higher signal for the MBP-ECD (Fig. 31A) compared to the antibody from ascites fluid (see Fig. 26A), which might be explained by a cleaner preparation generated in cell culture. The same antibody also gave a good signal in binding to the peptide it recognized (Fig. 31B), compared to no signal when ascites fluid was used (Fig. 26B). Ab#1 gave a lower signal...
for binding to the MBP-ECD than the other two antibodies raised against the same peptide, which is most likely due to a lower antibody concentration in that particular supernatant preparation.

Antibodies typically showed a reduction in binding to the antigen at the 10 times lower concentration (Fig. 31).

Unpurified antibodies obtained from the hybridoma cell supernatant were tested in ECIS proliferation assay on SW13-PTN cells (W2-8) that overexpress PTN, and on SK-N-SH neuroblastoma cells (Fig. 32). These two cell lines were chosen because of their dependence on the ALK pathway.

Figure 32. Cell proliferation assay with the addition of monoclonal anti-ALK antibodies from hybridoma cell supernatants. SW13 adrenal carcinoma cells that overexpress PTN, W2-8 (A), and SK-N-SH neuroblastoma cells (B) were tested for the cell growth rate in the presence of anti-ALK antibodies harvested from the supernatant of hybridoma cell culture media. Graphs were normalized to vehicle control that did not contain antibody and its growth rate was set to 100%. Mean +/- SEM of duplicate measurements from two experimental wells is shown, and statistical significance was determined by t-test. n.s. – non significant.
The greatest effect on cell proliferation in both cancer cell lines was seen with Ab#3. Interestingly, Ab#4 was the second best in its ability to reduce proliferation of W2-8 cells, while it was not effective in SK-N-SH cells.

Finally, based on the above-described experiments, Ab#3 was selected for purification. This monoclonal anti-ALK antibody was purified on FPLC by Protein G affinity chromatography, and its binding to the MBP-ECD fusion protein was monitored using Biacore (Fig. 33).

**Figure 33. Binding of purified monoclonal anti-ALK antibody Ab#3 to the MBP-ECD fusion protein determined by Biacore.** The anti-ALK Ab#3 was purified on Protein G column by FPLC from the supernatant of hybridoma cell growth media. The antibody solution was immobilized on the chip surface, and MBP-ECD antigen was passed to determine the binding. MBP was used as a negative control.

The purified antibody could be used in future *in vivo* studies, where it could be compared to the effect of the small molecule ALK kinase inhibitor, TAE684.
5.4 Discussion

Anti-ALK therapy could be of great interest in targeting tumor-stroma crosstalk in the treatment of pancreatic and other types of cancer that overexpress the ALK receptor or its ligand PTN. There are two approaches in targeting the ALK receptor, one of which is the use of a small molecule inhibitors of the intracellular kinase domain, i.e. drugs like TAE684. The other approach is the use of monoclonal antibodies that bind to the extracellular domain of the receptor, or antibodies that bind and sequester ALK ligands (23,110).

Treatment with the kinase inhibitor, TAE684, successfully delayed tumor growth in the pancreatic cancer xenograft mouse model (Fig. 15), and it also delayed tumor progression in transgenic LSL-Kras\(^{\text{wt/mut}}\)LSL-p53\(^{\text{wt/mut}}\)p48\(^{\text{wt/Cre}}\) mice (Fig. 21). Therefore, we anticipate that the use of a monoclonal anti-ALK antibody might impact tumor progression in a similar fashion.

First, \textit{in vitro} characterization of the anti-ALK antibody was performed. Antibodies Ab#1, #3, #4 and #7 are produced to recognize both, mouse and human extracellular domain in proximity of the ligand binding domain of the ALK receptor, while Ab#10 recognizes ligand binding domain of the mouse ALK receptor that differs in 2 amino acids from the human sequence (see Fig. 25). Binding experiments in a direct ELISA gave a clear signal to noise ratio for binding of Ab#1, #3, #7 and #10 to the recombinant extracellular domain of the ALK receptor. Moreover, similarly to the results of the ELISA, most of the selected antibodies, with the exception of Ab#4 gave a signal in Western blot with the MBP-ECD. However, Ab#4 showed a biological effect in reducing cancer cell proliferation and invasion. Moreover, antibodies #3 and #4 inhibited MDA-MB-231 cell
proliferation level in a peptide antigen reversible manner. Finally, Biacore confirmed binding of purified Ab#3 to the MBP-ECD recombinant protein in comparison to MBP. All of these experiments produced promising data and suggested that at least some of the five selected anti-ALK antibodies can be successful in in vivo experiments.

It is noteworthy that five selected monoclonal anti-ALK IgG antibodies cover the whole range of IgG isotypes, which might be of importance if these antibodies can additionally boost antibody dependent cellular cytotoxicity (ADCC) response, where isotype IgG2a (anti-ALK monoclonal antibodies #1 and #4) is predicted to give the best ADCC response (111).

Promising in vitro studies with monoclonal anti-ALK antibodies are going to be moved forward to in vivo studies, and possibly, antibody therapy could be coupled with a small molecule kinase inhibitor therapy against ALK to increase efficacy of anti-ALK treatment.
CHAPTER 6: CONCLUSIONS AND DISCUSSION
The stromal compartment is crucial for tumor cell growth, survival and metastasis. This is especially true in the case of a desmoplastic cancer such as pancreatic ductal adenocarcinoma. Also, the presence of stromal cells makes pancreatic cancer cells more aggressive and more treatment-resistant (112). Disruption of this axis that supports tumor growth appears to be important for the development of pancreatic cancer treatment, especially when the standard of care has not made a big impact on patient survival (113). We believe that targeting tumor-stroma crosstalk will make an impact on cancer cell survival, tumor growth and metastasis.

6.1 Tumor-stroma targeting in vitro

Small molecule kinase inhibitors that target the FGFR or ALK receptors prevented cancer cell invasion through an endothelial cell monolayer (see Fig. 1). The doses of PD173074 (100 nM) and TAE684 (32 nM) used in these assay were not toxic for the cancer cells and caused little if any apoptosis. The slight anti-proliferative drug effect after 48 h treatment could be caused by small fraction of cancer cells entering G1 phase arrest in combination with slight increase in percentage of cells undergoing early apoptosis. This result suggests only a small dependence of these cancer cells on autocrine signaling. Moreover, a reduction in cancer cell invasive ability seen in the endothelial disruption assay in vitro is not explained by a cancer cell autonomous drug effect, but rather the crosstalk with endothelial cells. Also, the 6 h time frame in the cancer cell/endothelial assay was too short to be due to cell death, and we conclude that it is due to the disruption of tumor-endothelium crosstalk. Hence, both drugs significantly reduced the disruption of
an endothelial monolayer by tumor cells, suggesting a predominantly paracrine signal that requires both compartments: cancer cells and stromal cells, to be present.

Similar results were obtained when cancer cells were treated with monoclonal anti-ALK antibodies. Several antibodies had a great effect on proliferation in cells that highly depend on ALK/PTN signaling. However, even in this case, the antibody effect was more prominent in preventing the cancer cell invasion through the endothelial monolayer, than in reducing cancer cell proliferation, with the same distinct time frame of the assay distinguishing this further.

6.2 Tumor-stroma targeting in vivo and treatment follow-up by serum miRNA expression changes

Encouraging in vitro studies that showed the ability of the PD173074, FGFR and TAE684, ALK kinase inhibitors to target tumor-stroma interactions, led towards the in vivo xenograft mouse study, and later to the LSL-Kras$^{wt/mut}$LSL-p53$^{wt/mut}$p48$^{wt/Cre}$ transgenic mouse model study.

First, in the pancreatic cancer xenograft study, we decided to treat mice for a short period of time and follow the changes in serum miRNA readout due to an early treatment effect, as opposed to deregulated miRNAs involved in excessive cell death potentially caused by prolonged treatment. The short 7-day treatment did not have an impact on gross tumor size measurements. However, both drugs induced histologically visible effects on angiogenesis (number of capillaries) and cancer cell growth (mitotic cancer cells) after the treatment (see Fig. 3). Interestingly, in spite of the same efficacy measured by histological analysis, we obtained two distinctive serum miRNA profiles for the two
inhibitors. Moreover, we measured miRNA expression level in tumor tissue from vehicle and drug treated mice, and compared the drug impact on miRNA expression in the circulation that reflects the host and in tumors. Another noteworthy goal of this study was deconvolution of the drug effect sites seen in the tumor versus affecting tissues in the host that are reflected in the deregulation of serum miRNAs. The analysis of treatment effect site of preference could be of interest while considering treatment benefits or comparing several different drugs to derive side effects.

Interestingly, after applying a provisional cutoff of sine (angle) = 0.3, which corresponds to the angle of 18°, in Fig. 14, the initial TAE684 treatment had better effects in targeting the tumor rather than the host, compared to PD173074. From this cutoff one can conclude that the two miRNAs that are outside of this cutoff after initial TAE684 treatment, miR-1 and miR-93, are the ones that are also very interesting for a distinction between PD173074 and TAE684 treatment. Perhaps the up-regulation of the tumor suppressive miR-1 in the circulation of TAE684 treated mice might help provide the tumor suppressive activity to the host and induce apoptosis.

Importantly, both drugs caused down-regulation of most of the selected tumor tissue miRNAs, with the exception of miR-1 after PD173074 treatment, while serum miRNAs were up and down-regulated after treatment. miR-1 could be of special interest for distinguishing between TAE684 and PD173074 treatment. Since potential clinical use would be in the assessment of circulating miRNA levels, it is interesting that serum miR-1 gets significantly up-regulated (> 2 fold) after initial TAE684 treatment, while it is not significantly deregulated in serum of mice with xenografts after PD173074 treatment. A
more than 2 fold up-regulation of miR-1 in serum after the initial treatment could suggest successful treatment.

However, it is still open if miR-1 is a unique marker of successful therapy or it should be used together with another serum miRNAs (e.g. miR-93 or others) that are part of the treatment signature derived after TAE684 treatment.

Unlike miR-1, miR-93 is a known oncogenic miR, that gets down-regulated in serum after TAE684 treatment, and has been shown to be involved in enhancing angiogenesis, cell survival, and tumor growth through targeting of integrin-β8 (114) in the context of ALK-signaling-dependent U87 glioblastoma cells (115,116). It was also found that miR-93 targets vascular endothelial growth factor, VEGF (117), and tumor suppressor LATS2, which belongs to YAP signaling pathway, and when present in cells, promotes cell invasion and tube formation (118). The same miRNA gets down-regulated in the circulation upon TAE684 treatment and this observation suggests a systemic inhibitory response targeting cancer cells.

Additionally, there was a striking difference between the two drugs in the overall miRNA shift after treatment: the selected miRNAs displayed a significantly larger deregulation in tumor tissue after the TAE684 treatment (see Fig. 13).

On the other hand, we have to be aware that some of the unchanged miRNA levels observed in serum and tumor might be a consequence of a drug affecting two different pathways that cause miRNA changes in opposite directions. For example, a drug could cause an effect in two pathways downstream of the receptor that has been targeted, and one of the affected pathways might cause up-regulation of the miR, while other pathway might cause down-regulation of the same miR. Finally if those two effects are
quantitatively similar, but in the opposite directions, they will cancel out and the final miRNA readout could be the same as in the vehicle control. This is also true of miRNAs from different organs shed into the circulation when a drug affects pathways in two different organs in a different manner causing the miRNA changes in opposite directions.

6.3 Predictive potential of circulating miRNAs for the response to treatment

Since both drugs had very similar effects on tumor histology, we were unable to predict which drug might cause a better effect in prolonged treatment or if they would act the same. That was addressed in a follow-up study: three-week treatment with TAE684 extended survival of mice twice longer than vehicle or PD173074 treatment. This distinct result was suggested by the hierarchical clustering based on serum miRNA expression after the initial treatment that grouped vehicle treated mice together with the PD173074 treated group, while there was still the histological effect of PD173074 (see Fig. 7). Furthermore, the fact that bigger changes in miRNA expression were caused by initial TAE684 treatment rather than PD173074, was more appreciated since TAE684 ultimately was the more effective drug in the 3-week treatment xenograft study. This finding shows that the extent of miRNA changes might be important and probably not only reflect but also contribute to the final drug effect.

Taken together, every piece of data suggests a superior effect of TAE684 treatment in the pancreatic cancer xenograft mouse model. This fact, if extrapolated to patients, might be very significant when the outcome cannot be easily predicted after initial treatment cycles. In conclusion, the ultimate exciting result of connecting a prolonged treatment effect with
the serum miRNA profile triggered already upon initial treatment, displays a high relevance of this research for eventual clinical use. In the clinic, an easily accessible serum miRNA readout could possibly be used as an early predictor of therapy success or failure.

6.4 Utilization of pancreatic cancer transgenic mouse model in studying treatment effects on serum miRNA profile

Finally, we also applied PD173074, TAE684 and a combination treatment to the LSL-Kras<sup>wt/mut</sup>LSL-p53<sup>wt/mut</sup>p48<sup>wt/Cre</sup> transgenic mouse model, and assessed miRNA expression in serum samples afterwards in addition to histological analysis of tumors. We anticipated a possibility of confirming deregulation of 4 circulating miRNAs after PD173074 treatment, as we have already detected their deregulation in two independent xenograft mouse models. However, we did not observe the same miRNA deregulation in the transgenic mouse model. It is very important to state that this study differs on several levels from the xenograft studies, and consequently it is difficult to compare results between the models. First of all, there is a difference in genetic background of tumors (human versus mouse), and there is also a difference in the mouse background (nude versus immunocompetent). Nude mice used in xenograft study are immunocompromised and this can likely affect baseline miRNA expression when compared to the baseline miRNA levels in immunocompetent mice. Moreover, in the transgenic study, mice were treated for 6 weeks followed by miRNA analysis, while in xenograft study mice were treated for only 7 days. Additionally, the tumor burden at the experimental endpoint was higher in the transgenic mice, and many of them had already developed metastasis to
lymph nodes, lung and liver by 5 months of age which is also the median survival of these mice (34). Finally and most importantly, the serum miRNA analysis in the transgenic study was fundamentally different. We did not use serum miRNA level from healthy mice as a baseline. Rather, we compared vehicle treated transgenic mice that developed PanIN3 lesions to those that developed metastatic PDAC by the age of 5 months. Those two groups compared to each other gave us an insight to miRNA changes during disease progression. However they are not necessarily miRNAs that discriminate between healthy and cancer bearing mice. Furthermore, serum miRNA levels in treated transgenic mice were compared to: 1) vehicle treated transgenic mice with PanIN3 lesions; and 2) vehicle treated transgenic mice with metastatic PDAC. The two comparisons gave us two serum miRNA profiles that do not overlap. However, after treatment, mice expressed circulating miRNA profile more similar to vehicle treated mice with PanIN3 lesions, rather than to mice with metastatic PDAC, suggesting that treatment moved the profile in the desired direction. Moreover, we obtained a serum miRNA signature that resembles more precancerous lesions (PanIN3) after TAE684 than after PD173074 treatment. It is interesting, that the number of deregulated circulating miRNAs is similar after both treatments in comparison to vehicle treated transgenic mice with metastatic PDAC. However, the difference between the effects of the drugs is visible in the comparison to vehicle treated transgenic mice with PanIN3 lesions. Interestingly enough, this result is in line with the prolonged treatment study in pancreatic cancer xenograft mouse model where TAE684 treatment had an impact on prolonged survival and it also caused hierarchical clustering of deregulated miRNAs after initial TAE684 treatment to separate from the PD173074 or vehicle treated group of cancer bearing mice.
6.5 Future directions

Here we developed and characterized the tools that can be used in future research to study the impact of tumor-stroma interaction on pancreatic cancer growth. There is a big potential in the use of pancreatic cancer cells derived from the LSL-Kras\textsuperscript{wt/mut}LSL-p53\textsuperscript{wt/mut}p48\textsuperscript{wt/Cre} transgenic mouse model in allograft studies, since they would allow for closer assessment of tumor size at the treatment initiation and during prolonged treatment. Importantly, this mouse model still retains phenotypic features of the pancreatic tumors developed in transgenic model, such as desmoplastic response and duct-like formation. Furthermore, a development of pancreatic cancer clonal cell lines derived from the same primary tumor offers an opportunity to study intratumoral heterogeneity and possibility to answer some of the burning questions about development of therapy resistance. Treatment of pancreatic cancer allografted mice with different drugs and drug combinations, and eventual development of therapy resistance will let us study the cells that survived a harsh selection in an immunocompetent host, and describe the mutations carried by resistant clones. The characteristics of therapy resistant cells will lead us towards better understanding of underlying mechanisms of resistance and towards new drug targets.

Furthermore, a successful targeting of tumor-stroma crosstalk in xenograft and transgenic studies, move us towards new therapeutic targets in the context of pancreatic cancer. Even though targeting of the FGF receptor with the PD173074 drug did not provide striking results in longer treatment of xenografted mice, initial results of successful inhibition of FGFR1 activation, reducing number of mitotic figures within the tumor and
reducing the number of capillaries are promising. Perhaps a dose adjustment and combination with another drug would result in the desired treatment effect and prolonged survival. Interestingly, PD173074 delayed tumor progression and occurrence of invasive pancreatic adenocarcinoma in the transgenic mouse model when mice were treated for 3 weeks.

A potentially larger deregulation of circulating miRNAs upon initial PD173074 treatment caused by higher drug dose could potentially impact tumor growth during prolonged treatment in a xenograft study. Furthermore, PD173074 might also be coupled with another drug for additive or synergistic effect.

Nevertheless, the study in the xenograft mouse model can serve as a proof of principle and provide a basis for a larger scale animal study with different treatments and / or drug combinations versus single drugs or vehicle treatment. The application could also be seen in a clinical trial where patients are randomized into different treatment arms, and followed during the course of time with blood collection after every cycle of therapy. Long term follow up of patients would tell us about tumor progression, remission or recurrence, that could later be correlated with the circulating miRNA profile, and a potential predictive serum miRNA biomarker could be associated with specific drug efficacy.

The promising antitumor activity of the ALK kinase inhibitor, TAE684, might suggest a similar effect of a monoclonal anti-ALK IgG antibody in reduction of tumor growth. We believe that we could potentially couple TAE684 drug effect with a monoclonal anti-
ALK antibody. This effect joined with the proven efficacy of TAE684, could possibly increase the therapy response.

The advantage of treatment with an anti-ALK antibody could be in an enhanced antitumor effect if the antibody is able to induce antibody dependent cellular cytotoxicity by engaging the immune response against cancer cells that carry the ALK receptor on their surface. Preliminary *in vitro* studies with the anti-ALK antibodies generated promising results by reducing *in vitro* proliferation of several different cancer cell lines that depend on ALK/PTN signaling, as well as in reducing cancer cell ability to invade through an endothelial monolayer. In the future, we would like to perform a study in an allograft mouse model with the primary and clonal pancreatic cancer cell lines isolated from the LSL-Kras\(^{wt/mut}\)LSL-p53\(^{wt/mut}\)p48\(^{wt/Cre}\) mouse, and follow the anti-ALK antibody effect on tumor growth. A potential experimental approach is to choose the most effective antibody from *in vitro* experiments, and screen it against different allografted pancreatic cancer clones. Another approach would be screening of all five selected antibodies against a pooled mixture of the 6 different clonal pancreatic cancer lines. In this case, each clonal cell line should be characterized by a unique tag – most likely a unique mutation that is present in only one of six clonal lines, so we could trace the resistant and sensitive clones.

Along the lines of searching for new anticancer treatments, circulating miRNA profiles can be used to monitor therapy. Our studies presented evidence that miRNA deregulation already upon initial treatment can distinguish between successful and failing therapy in
the prolonged treatment context and this principle can hopefully be translated to clinical studies.

With the new directions in pancreatic cancer therapy and monitoring treatment response based on easily accessible circulating miRNA profile, we hope that this study will bring us a step closer to the development of an effective treatment of pancreatic cancer.
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