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Desease models in vertebrates : from hypoxia to cancer

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CHAPTER 4

RETINOIC ACID RECEPTOR (RAR) ANTAGONISTS INHIBIT MIR-10A EXPRESSION AND BLOCK METASTATIC BEHAVIOR OF PANCREATIC CANCER

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Abstract

The infiltrating ductal adenocarcinoma of the pancreas is among the most lethal of all solid malignancies, largely due to a high frequency of early metastasis. We identify microRNA-10a (miR-10a) as an important mediator of metastasis formation in pancreatic tumor cells and investigate the upstream and downstream regulatory mechanisms of miR-10a. Northern blot analysis revealed elevated expression levels of miR-10a in metastatic pancreatic adenocarcinoma. The role of miR-10a was analyzed by Morpholino and siRNA transfection of pancreatic carcinoma cell lines and resected specimen of human pancreatic carcinoma. Metastatic behavior of primary pancreatic tumors and cancer cell lines was tested in xenotransplantation experiments in zebrafish embryos. We show that miR-10a expression promotes metastatic behavior of pancreatic tumor cells and that repression of miR-10a is sufficient to inhibit invasion and metastasis formation. We further demonstrate that miR-10a is a retinoid acid target and that retinoic acid receptor (RAR) antagonists effectively repress miR-10a expression and completely block metastasis. This anti-metastatic activity can be prevented by specific knock down of HOX genes, HOXB1 and HOXB3. Interestingly, suppression of HOXB1 and HOXB3 in pancreatic cancer cells is sufficient to promote metastasis formation. These findings suggest that miR-10a is a key mediator of metastatic behavior in pancreatic cancer which regulates metastasis via suppression of HOXB1 and HOXB3. Inhibition of miR-10a expression (with RAR antagonists) or function (with specific inhibitors) is a promising starting point for anti-metastatic therapies.

Introduction

Pancreatic cancer is a highly invasive malignancy which is characterized by early metastasis formation. Although metastases spawned by malignant tumors are

responsible for over 90% of all cancer death (Jemal, et al., 2007), our understanding of the mechanisms involved in metastasis formation is still rather limited.

There is increasing evidence that microRNAs can function as oncogenes or as tumor suppressors and that differential expression of specific microRNAs is critically involved in the progression of many cancers (Johnson, et al., 2005; Esquela-Kerscher, et al., 2006; He, et al., 2005; Tavazoie, et al., 2008). Large scale transcriptome analysis data are available for microRNA signatures of different human tumors (Calin, et al., 2006; Lu, et al., 2005) including pancreatic cancer (Roldo, et al., 2006; Bloomston, et al., 2007). These studies used microarray technology to reveal specific microRNA expression profiles in specimen from pancreatic adenocarcinoma, which could be differentiated from normal pancreatic tissue and chronic pancreatitis.

In a recent publication, it was shown that miR-10b is highly expressed in human breast tumors and that expression correlates with metastatic potential (Ma, et al., 2007). In pancreatic cancer miR-10b expression is not elevated, but instead an upregulated expression of the related miR-10a has been described (Roldo, et al., 2006; Bloomston, et al., 2007) Both microRNAs belong to the same microRNA family, but miR-10b is located in the HOXD cluster on chromosome 2, whereas miR-10a is present in the HOXB cluster on chromosome 17 (Tanzer, et al., 2005). Since related microRNAs are expected to share similar target genes, we hypothesized that elevated miR-10a levels in pancreatic tumors could be involved in their metastatic behavior.

It is known that many of the 39 mammalian Homeobox genes are regulated by retinoids through retinoic acid response elements (RARE) and miR-10a is located in the 3'genomic region of the well-known RA target HOXB4, in the vicinity of a

RARE (reviewed in Mainguy, et al., 2003). Retinoids, that is, retinoic acid (RA) and its derivatives, regulate a wide variety of different biological processes during development. Experimentally applied retinoid agonists and antagonists promote differentiation, inhibit proliferation and accordingly show promise for the prevention and treatment of cancer (Hong, et al., 1997). Retinoid derivatives have been reported to inhibit invasion of breast and hepatocellular carcinoma cells *in vitro* (Wu, et al., 2006; Simeone, et al., 2006). Interestingly, RAR antagonists also seem to have anti-invasive potential, possibly by their inhibitory activity to matrix metalloproteinases (MMPs) (Schoenermark, et al., 1999).

In this study, we investigated the importance of miR-10a in pancreatic cancer metastasis and the possible link with retinoic acid. We demonstrate that miR-10a is involved in the causal mechanism of tissue invasion and metastasis formation in pancreatic cancer. We find that miR-10a is under transcriptional control of RA in pancreatic cancer cells and that two target genes, HOXB1 and HOXB3 are involved in promoting metastatic behavior. Our findings show that both, miR-10a inhibitors and RAR antagonists are effective compounds for targeting metastatic tumor behavior through regulating miR-10a function and expression.

Materials and methods

Animal care and handling

Zebrafish (*Danio rerio*) (Tuebingen line, Alb strain (Albinos) and Tg (fli1:eGFP) were handled in compliance with local animal care regulations and standard protocols of the Netherlands and Germany. Fish were kept at 28°C in aquaria with day/night light cycles (10h dark versus 14h light periods). The developing embryos were kept in an incubator at constant temperatures of 28°C.

Retinoic acid and RA Inhibitors

The selective RAR α antagonist Ro-41-5253 was purchased from Biomol International, dissolved in DMSO and cells were treated overnight prior to injections at a final concentration of 200nM (50% inhibition at 60nM) (Keidel, et al., 1994; Bertram, et al., 2005) Retinoic acid (ATRA – all trans retinoic acid, Sigma) was added to cells ON at a final concentration of 1 μ M.

Cell Culture

Cell lines PaTu8988T, PaTu8898S, AsPC1, Capan1, Capan2, MiaPaCa2, PANC1 and PaTu8902 cells were cultured in DMEM high glucose, with 10% FCS and 1:100 Pen/Strep.

Cell Staining, Injections, Incubations and Antibodies

Cells were stained with CM-Dil (red fluorescence) at a 1:1000 dilution according to the manufacturers' protocol (Vybrant, Invitrogen). Cells were suspended in 67% DPBS for injection into embryos. 2dpf zebrafish embryos were dechorionated and tranquilized with tricaine (Sigma). An injection needle (2.8 μ m diameter) with a manual injector (Eppendorf; Celltram Vario) was used to inject cells into the yolk sac of 2dpf zebrafish embryos. Between 100 and 200 cells were injected per embryo. On average 80 embryos were injected in the experiments for each treatment. Presence of cells was checked after 2h incubation at 31 $^{\circ}$ C and embryos were subsequently kept at 35 $^{\circ}$ C. We defined three categories based on the number of migrated cells (<5; between 5-20; and >20). We defined here migration for the two latter categories and percentages given refer to this. All data are shown in Table 2 and Supplemental Table 1. A micrometastasis is

defined as a cluster of at least 4 cells in close proximity indicating origin from cell division.

PaTu-S cells were transfected ON with indicated concentrations of miR-10a siRNA in the presence of ICA-Fectin TM442 (Eurogentec, Belgium). Whole cell lysates were prepared 36h post-transfection and 40µg of total lysates were separated on 7.5% PAA-gels and blotted with anti E-cadherin, α-catenin, β-catenin (all mAbs, Transduction Labs) and actin antibodies (mAb Sigma-Aldrich, Germany).

Tissue preparation for transplantation into zebrafish embryos

Human resection specimens were obtained at the Universitätsklinikum Greifswald according to local ethical guidelines and after obtaining informed patient consent. Tumor tissue was cut into small pieces using a scalpel blade. Tissue fragments were then washed with 67% DPBS and stained with 1:500 CM-Dil. Stained tissue fragments of approximately $\frac{1}{5}$ to $\frac{1}{2}$ the size of the yolk sac were transferred with a glass transplantation needle into the yolk. After 2h at 31°C the embryos were checked that the yolk had sealed itself and the embryos were incubated at 35°C for the remainder of the experiment.

Northern Blot, RT-PCR and quantitative RT-PCR (RT-qPCR)

Northern Blot analysis was performed essentially according to Kloosterman *et al.* (2006) using a miR-10 LNA probe (CACAAATTCGGATCTACAGGGTA) (Exiqon, Denmark). We had previously described protocols for RT-PCR (Ott, et al., 2007) and RT-qPCR (Marques, et al., 2008). Primers for the RT-PCR were: HOXB1: sc-38686 and HOXB3: sc-38690-PR and RARα: sc-29465-PR all from Santa Cruz Biotechnology. For quantitative RT-PCR we used 100 ng of total RNA and the

Roche Master SYBR Green kit. Single-band amplification was verified by melting curve dissociation protocols. A standard curve for β -actin using 1, 5, 10, 100, and 500 ng of total RNA was used for normalization. RT-qPCR primers for HOXB1 were: forward 5'-ctccgaggacaaggaaacac-3' and reverse 5'-ccttcatccagtcgaaggtc-3' and for HOXB3: forward 5'-actccaccctcaccaaacag-3' and reverse 5'-ttgcctcgactctttcatcc-3'.

Morpholino knockdown, siRNA expression and Sensor Construct

Morpholino antisense oligonucleotides were obtained from Gene Tools, LLC (OR, USA) and used as previously described (Woltering, 2008). The miR-10a antisense Morpholino sequence is: CACAAATTCGGATCTACAGGGTA. Standard control Morpholino was CCTCTTACCTCAGTTACAATTTATA. Both miR-10a and control morpholinos were fluorescein labeled. The siRNAs for human HOXB1, human HOXB3 and human RAR α were purchased from Santa Cruz Biotechnology (HOXB1: sc-38686, HOXB3: sc-38690 and RAR α : sc-29465). Endoportor (Gene Tools, LLC) was used to deliver Morpholinos and siRNAs into cells and efficiencies for Morpholinos were checked by fluorescence microscopy to be >95%. For the miR-10a siRNA, the sense strand corresponds to UACCCUGUAGAUCGAAUUUGUGUG, The sequence of control siRNA (miR-196a) was UAGGUAGUUUCAUGUUGUUGG and a corresponding antisense sequence). 30 μ l of each siRNA oligonucleotide (50 μ M) were annealed by gradually cooling from 98°C to 20°C in a volume of 75 μ l annealing buffer (50mM Tris, pH 7.8, 100 mM NaCl RNase free). The effect of miR-10a Morpholinos on miR-10a function was tested with a sensor construct: pCS2+ EGFP 4x miR-10 AS containing 4 copies of a miR-10a antisense sequence.

Statistical Analysis

Significance between groups was assessed by Student's t-test.

Imaging

Confocal pictures were taken with the Biorad Confocal microscope 1024ES (Zeiss microscope) combined with Krypton/Argon laser, a dual laser scanning confocal microscope Leica DM IRBE (Leica) or with the Nikon TE300 confocal microscope and a coherent Innova 70C laser (Chromaphor, Duisburg, Germany). Pictures were further taken in a differential interference contrast (DIC) microscope (Axioplan 2) with an AxioCam MR5 camera (Carl Zeiss). For fluorescent stereomicroscope images we used a Leica MZ16FA microscope and a Leica DFC 420C camera.

Results

miR-10a expression is elevated in pancreatic tumor cell lines and specimen of chronic pancreatitis and pancreatic tumors.

To evaluate the role of miR-10a in pancreatic tumor cells, we performed northern blot analysis in a series of pancreatic tumor cell lines including PaTu8988-S and PaTu8988-T (Elsässer HP, 1992). (PaTu-S and PaTu-T), and observed expression of this specific microRNA in all but two (PaTu-S and AsPC1) cell lines (Figure 1 A). Expression differences in PaTu-S and PaTu-T cells are interesting as both sister cell lines originate from liver metastases of the same human pancreatic adenocarcinoma (Elsässer HP, 1992) and we and others have previously shown that E-Cadherin expression in PaTu-S cells correlates with the maintenance of functional cell-cell contacts and a reduced tendency of cells to migrate (Menke, et al., 2001; Mayerle, et al., 2005; Marques, et al., 2009) In

PaTu-T cells, loss of E-Cadherin is associated with little cell aggregation and enhanced migratory capabilities, which we confirmed in a zebrafish xenotransplantation model (Marques, et al., 2009). These different migratory capacities seem to correlate with an elevated miR-10a expression in PaTu-T cells and a very low expression in PaTu-S cells (Figure 1 A).

We also assessed the expression of miR-10a in resection specimen of 4 pancreatic adenocarcinoma, 4 chronic pancreatitis patients and 2 normal pancreatic tissues and found a ~2.8 fold upregulation of miR-10a expression in all 4 pancreatic tumors in comparison to control pancreatic tissue. In contrast to the previous microarray study (Bloomston, et al., 2007) we also observed increased miR-10a expression in chronic pancreatitis. (Figure 1 B).

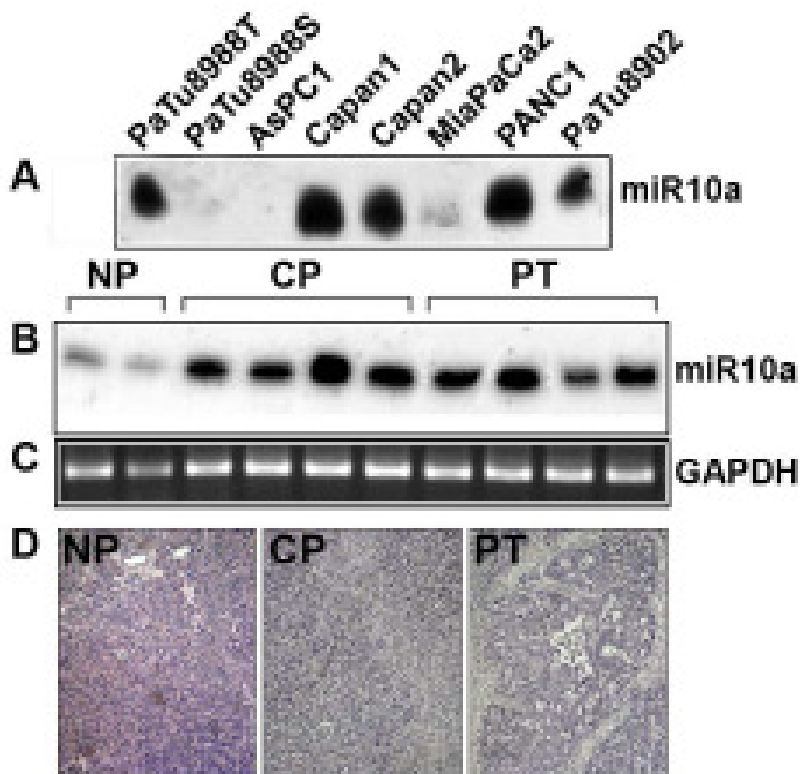


Figure 1 MicroRNA-10a expression in pancreatic tumor cell lines and human specimen of pancreatic tumors or chronic pancreatitis. By Northern blot analysis we evaluated miR-10a expression in human pancreatic cancer cell lines (A) and in resection specimen (B) of normal human pancreas (NP), chronic pancreatitis (CP) and pancreatic tumors (PT). Densitometric analysis (Biorad GS800) showed average intensities of 4.2 (+/-0.5) for NP, 11.9 (+/-1.1) for PT and 12.7 (+/-0.6) for CP, suggesting a ~3-fold elevated miR-10a expression in chronic pancreatitis and pancreatic tumor compared to normal pancreatic tissue. Control GAPDH expression, analyzed by RT-PCR of the same RNA samples used in the northern blot, is shown in (C). HE staining of representative histological sections of the utilized human tissue specimen are shown in (D) (NP: normal pancreas, CP: chronic pancreatitis, PT: pancreatic tumor).

miR-10a is required and sufficient for tissue invasion and metastasis formation in

pancreatic tumor cells

The zebrafish and its transparent embryos have recently been established as a model system to investigate tumor development (Langenau, et al., 2003; Stoletov, et al., 2007; Park, et al., 2008; Nicoli, et al., 2007). Xenotransplantation in fish is utilized to study invasiveness and metastatic behavior of established cancer cells (Marques, et al., 2009; Stoletov, et al., 2007; Nicoli, et al., 2007; Topczewska, et al., 2006; Nicoli, et al., 2007; Lee, et al., 2005; Haldi, et al., 2006) and of fragments of resected gastrointestinal tumor tissue (Marques, et al., 2009)

To study the role of miR-10a in metastatic behavior, we labeled the pancreatic tumor cell lines with a carbocyanine dye (CM-Dil) and ectopically transplanted them in the yolk sac of 2 dpf zebrafish embryos. As expected, PaTu-S cells remained in the yolk, whereas PaTu-T cells invaded the embryo and showed metastatic behavior (Table 1 and Supplemental Table 1). The expression of miR-10a in these cell lines therefore correlates with their metastatic potential in the zebrafish xenotransplantation model. We further tested 6 additional pancreatic tumor cell lines and find a correlation of metastatic behavior with the presence of miR-10a but not with its absolute levels of expression (Figure 1A and Supplemental table 1).

To investigate the involvement of miR-10a in the metastatic behavior *in vivo*, we performed a loss of function analysis by silencing miR-10a with specific Morpholino antisense oligonucleotides which we previously have demonstrated to work well in silencing miR-10a in the developing zebrafish embryo (Woltering, 2008). More than 95% of cancer cells incorporated the miR-10a Morpholino (Figure 2 B) and also resection specimen of human tumor tissues showed significant uptake of morpholino (Figure 2 C and D).

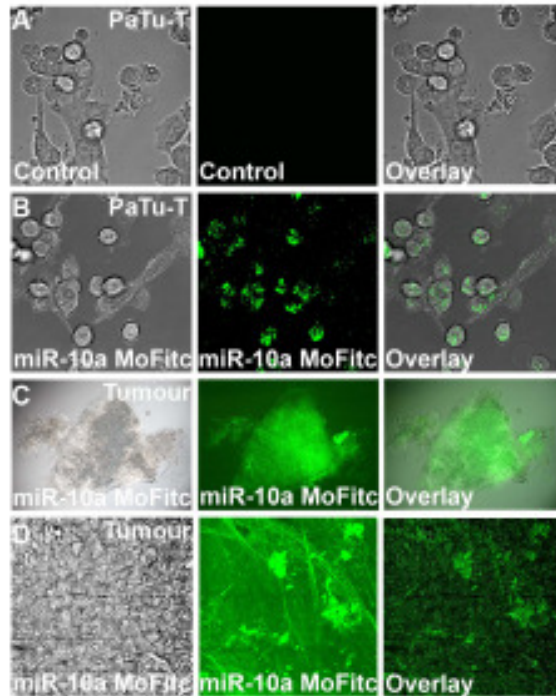


Figure 2 Uptake of miR-10a Morpholino into pancreatic cancer cells and tumor tissue

PaTu-T cells (A-F) and an ~1 mm long tissue fragment of a human resection specimen of pancreatic tumor (G-L) were incubated for 24h in the presence of Endoportor with control Morpholino (A-C) or miR-10a-FITC Morpholino (D-L). Uptake of FITC-labeled Morpholino was analyzed by confocal microscopy (B, E, H, K). Analysis of bright field (A, D, G, J) and overlay images (C, F, I, L) demonstrates uptake efficiencies of >95% in PaTu-T cells. Notably significant uptake of Morpholino into cells within the resection specimen was also detected even though exact transfection efficiency could not be determined. No auto fluorescence of tumor tissue was observed (Supplemental Fig. 5).

Expression of an EGFP miR-10a sensor construct, containing 4 miR-10a target sequences in the 3'UTR is repressed by the presence of the endogenous miR-10a in PaTu-T cells (the cell line highly expressing miR-10a) (Figure 3, left panel).

Delivery of anti-miR-10a Morpholino to the cells hinders this repression, indicating that miR-10a Morpholinos work well in silencing miR-10a function (Figure 3, right panel).

Targeting endogenous miR-10a with Morpholinos rendered PaTu-T-cells non-invasive and prevented metastatic behavior in zebrafish embryos, whereas a control Morpholino had no such effect (Figure 4 B, Table1 and Supplemental Table 1). Over a three day time course we observed no adverse effects of morpholino treatment on general cell survival (analyzed by propidium iodine (PI) staining - data not shown).

Complementary to this loss of 'metastatic' function, we also performed a gain of function analysis using miR-10a siRNA which can be effectively used to study miR-10a over expression (Woltering, 2008). We transfected PaTu-S cells with miR-10a siRNAs and subsequently transplanted the cells into the yolk sac of 2 dpf embryos. The delivery of miR-10a siRNAs actually conferred invasiveness and metastasis formation to PaTu-S cells (39.2% (SD 7.4)) that were previously non-invasive *in vivo* (Figure 4 C). As a control we used miR-196a siRNAs, which showed no effect on cellular invasion and migration (0.57% (SD 1)) (Figure 4 D, table 1 and supplemental table 1).

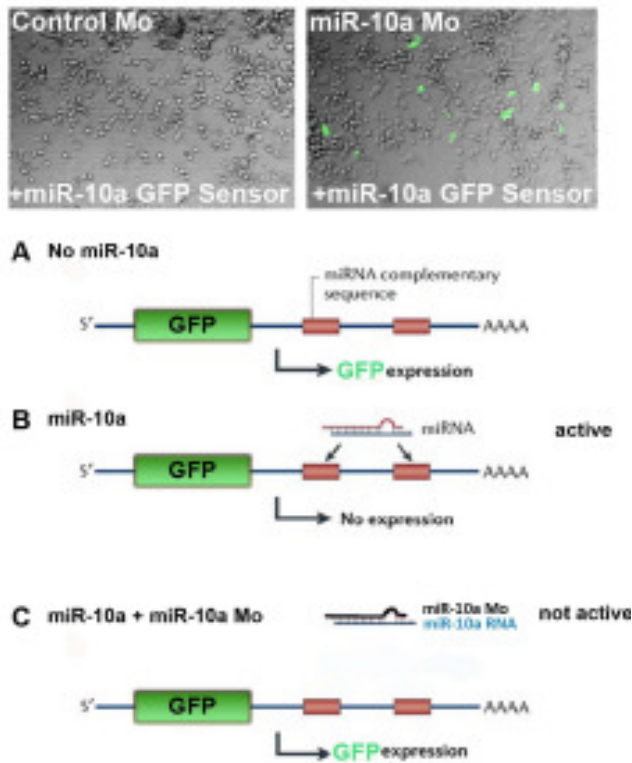


Figure 3: Inhibition of miR-10a function by miR-10a specific Morpholinos in pancreatic cancer cells. PaTu-T cells were transfected with a sensor construct containing a green fluorescence protein (GFP) coding sequence followed by multiple miR-10a target sites. As demonstrated in the schematic representation, GFP is expressed from this sensor construct only in the absence of miR-10a (A). Due to expression of miR-10a in PaTu-T cells, miR-10a can bind to the 3' target sites and therefore no GFP signal is detected (B). This suppressive effect of miR-10a can be released by addition of a specific miR-10a Morpholino which itself targets miR-10a and prevents its binding to the sensor construct (C). Following transfection, PaTu-T cells were incubated for 24 h with control or miR-10a Morpholinos and investigated by fluorescence microscopy (upper panel). In contrast to the control cells, fluorescent cells were only detected in miR-10a Morpholino treated PaTu-T cells. A low transfection efficiency of PaTu-T cells is responsible for only limited numbers of fluorescent cells (tested with a GFP-expression construct; data not shown).

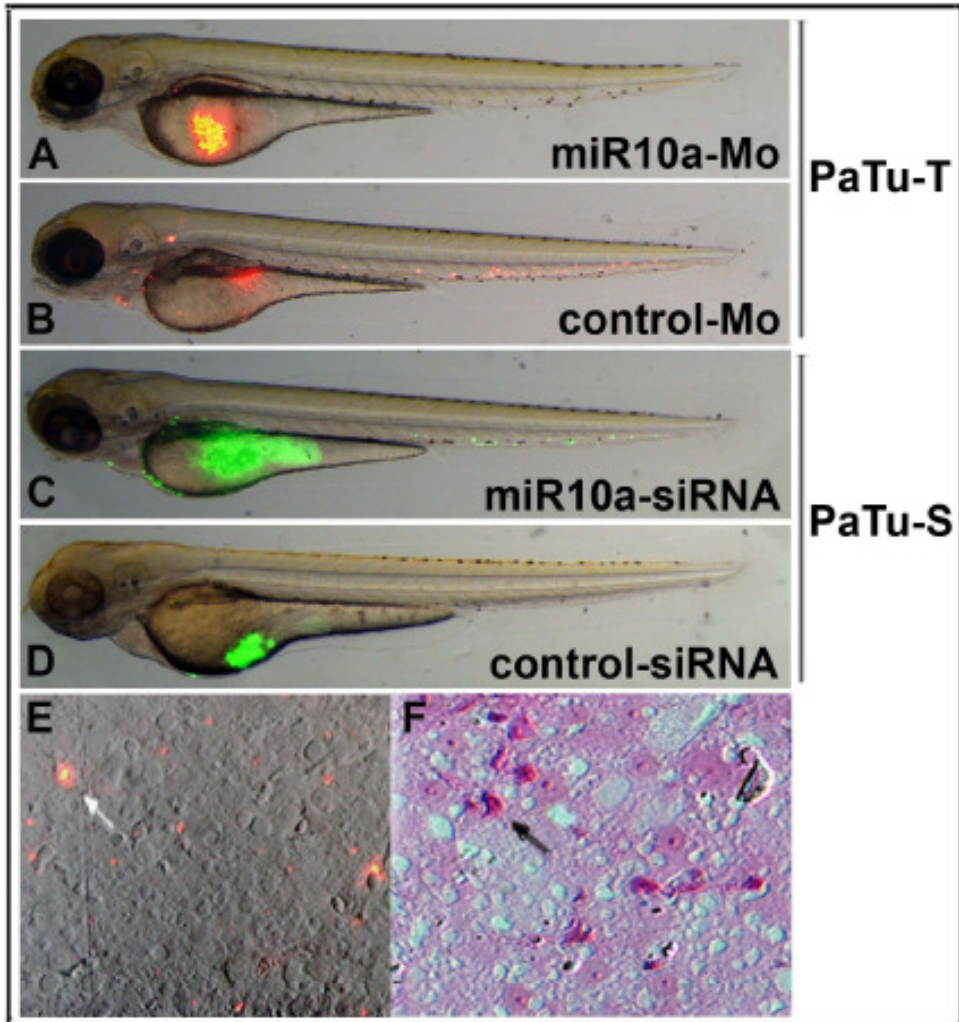


Figure 4 MicroRNA-10a is required and sufficient for tissue invasion and metastasis formation of pancreatic tumor cells PaTu-T cells were incubated with control and miR-10a

Morpholinos, stained with fluorescent CM-Dil and transplanted into the yolk of zebrafish embryos. Blockage of miR-10a by specific Morpholino was found to inhibit migration of PaTu-T cells in xenotransplanted zebrafish (A) whereas PaTu-T cells treated with a control Morpholino still invaded the embryo and formed metastases (B). To determine whether miR-10a is able to induce tissue invasion and migration of non-invasive PaTu-S cells, we incubated the cells with synthetic miR-10a siRNA and, as a control, synthetic miR-196a siRNA (control-siRNA). We observed tissue invasion and migration in miR-10a siRNA (C), but not in control-treated cells (D), which remained in the yolk. Images (A-D) were taken by high resolution fluorescence stereomicroscopy at 3 days post fertilization and 1 day post injection (dpi). The presence of representative tumor cells and micrometastasis at 3 dpi is shown in contrast microscopy pictures overlaid with a fluorescence image (E, F), HE staining (G, I, J) and fluorescence images (H) of transversal sections of zebrafish liver. Arrows point to micrometastasis in the liver.

miR-10a is required for invasion and metastatic behavior of xenotransplanted primary human tumors

We investigated whether miR-10a gene-specific morpholinos could also block metastatic behavior of xenotransplanted primary human tumors. Small fragments of resected tumor tissue were treated with fluorescein labeled miR-10a Morpholinos (miR-10a Mo-FITC) and cellular uptake of miR-10a Mo-FITC was observed (Figure 2 C-D). Treatment of small tumor fragments from three different patients with miR-10a Mo prior to xenotransplantation led to over 90% reduction of invasion and metastatic behavior, when compared to control Mo treated tissue of the same tumor specimen (Table 2 and Supplemental Table 1). Our results indicate a regulatory function of miR-10a in the metastatic behavior of human pancreatic tumor cells.

miR-10a expression is increased by retinoids and decreased by RAR antagonists in pancreatic tumor cells

To analyze if miR-10a expression is under control of RA signaling in pancreatic cancer, we analyzed miR-10a expression in cells treated either with retinoids or a selective RAR α antagonist. Our northern blot data show that miR-10a expression in non-metastatic PaTu-S cells is positively regulated by stimulation with all trans retinoic acid (ATRA) (Figure 5 A and Supplemental Figure 1), whereas a significant decrease in miR-10a expression was observed in the metastatic PaTu-T cells treated with either a selective RAR α antagonist (Ro-41-5253; Biomol) or RAR α -specific siRNAs (Figure 5 A and Supplemental Figure 1). Using chromatin immune precipitation (CHIP analysis), we identified the DR5 type 10 retinoic acid response element (RARE) as a functional target sequence for RAR α binding in PaTu-T cells (but not in PaTu-S cells). This RARE has been previously identified as a mediator of *hoxb4* neural expression (Gould, et al., 1998) and thus is likely to regulate miR-10a (Supplemental Figure 2).

RAR α inhibition prevents metastatic behavior of pancreatic cancer cells and xenotransplanted tumors

We used the selective RAR α antagonist Ro-41-5253; to analyze its anti-migratory activity in an *in vitro* migration assay (“scratch assay”) and in the zebrafish xenotransplantation model, respectively. The RAR α antagonist significantly decreased PaTuT-T cell migration in the *in vitro* assay (Figure 5 B) and inhibited invasion and metastatic behavior in zebrafish embryos (Table 1 and Supplemental Table 1). Similar results were obtained when PaTu-T cells were treated with RAR α -specific siRNAs (Supplemental Table 1 and Supplemental Fig. 1). We observed no adverse effects of RAR α antagonist treatment on general cell

survival (analyzed by propidium iodine (PI) staining or cell cycle distribution (Supplemental Figure 3).

Moreover, we treated small fragments of resected pancreatic tumors specimen with the selective RAR α antagonist prior to xenotransplantation into zebrafish embryos. Compared to the DMSO treated control tissue of the same tumor specimen, we observed a strong reduction (> 95%) of invasiveness and metastatic behavior (Table 2 and Supplemental Table 1).

miR-10-a regulates Cadherin/catenin expression

One of the known prerequisites for metastasis is the down regulation of cell-adhesion proteins e.g the proteins that constitute functional cadherin-catenin complexes (Jeanes, et al., 2008). We therefore analyzed if induction of migration in PaTu-S cells by miR-10a specific siRNAs affects expression of E-cadherin, α -catenin and β -catenin. We observed that miR-10a specific siRNAs clearly affected expression of all three proteins, however, to various extents (Figure 5 C). Our results suggest that regulation of metastasis by miR-10a in pancreatic tumor cells involves down regulation of cadherin/catenin proteins which may enable the tumor cell to start migration.

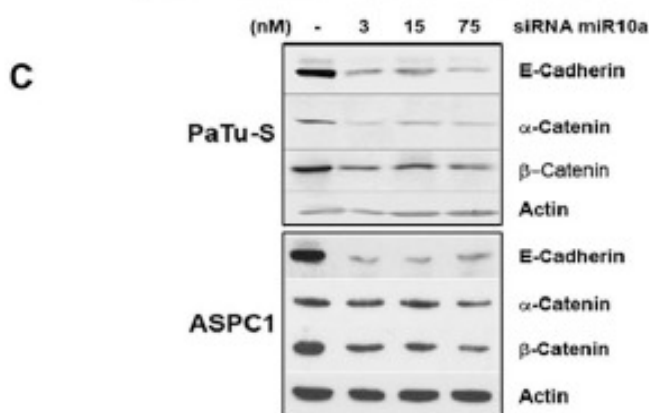
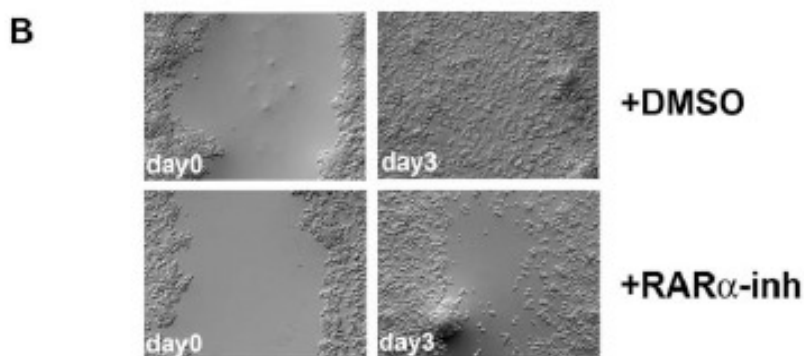
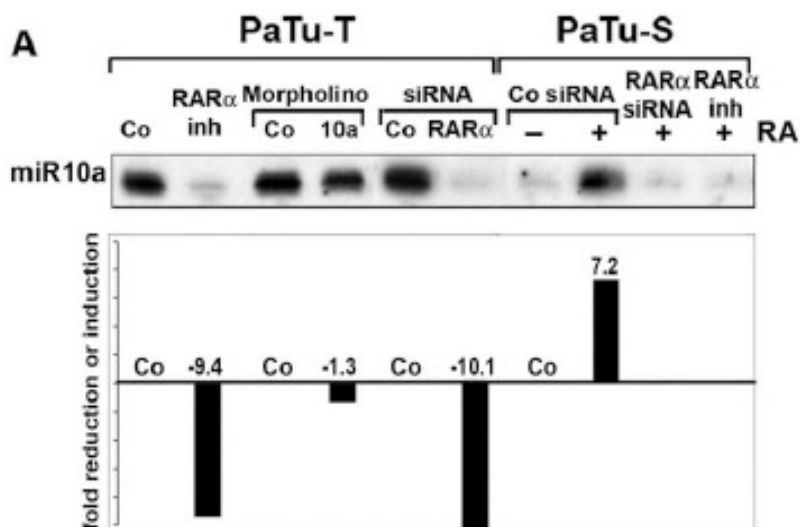


Figure 5: RAR antagonists inhibit miR-10a expression and migration of pancreatic cancer cells. PaTu-T cells were incubated, as indicated, with DMSO (Co), 200 nM Ro-415253 (RAR α inh), control-Morpholino, miR-10a Morpholino, miR-196 siRNA, or RAR α siRNA and PaTu-S cells were co-incubated with 1 μ M retinoic acid (RA) and control siRNA, RAR α siRNA or Ro-415253 and miR-10a expression was evaluated by Northern blot analysis **(A)**. Densitometric analysis (Biorad GS800) revealed in PaTu-T cells a 9.4 fold reduction of miR-10a expression following Ro-415253 treatment, a 10.1 fold reduction by RAR α siRNA treatment and a 1.3 fold reduction following miR-10a Morpholino treatment. In contrast miR-10a expression was upregulated by 7.2 fold in PaTu-S cells following treatment with retinoic acid (1 μ M final concentration). This upregulation was completely blocked by RAR α siRNA or RAR α inhibitor. RT-PCR control of GAPDH was used as a loading control (not shown). Dose response curves for RA and the RAR α antagonist and the RT-PCR control for RAR α siRNA treatment are shown in Supplemental Figure 1. The effect of Ro-415253 on cell migration was analyzed in an in vitro migration assay in PaTu-T cells. Representative images demonstrate significant inhibition of cell migration following Ro-415253 treated in comparison to control (DMSO) cells **(B)**. The regulatory function of miR-10a on expression of proteins of the cadherin catenin complex was demonstrated in PaTu-S cells. Western blot analysis of total lysates of PaTu-S cells (PaTu-T cells as control) showed reduced expression of E-Cadherin, α -Catenin and β -Catenin following treatment with indicated concentrations of miR-10a siRNA **(C)**. An actin re-blot confirmed equal loading.

miR-10-a promotes invasiveness and metastatic behavior through suppression of HOXB1 and HOXB3

In the zebrafish we have identified hoxB1 and hoxB3 as conserved downstream targets of the miR-10 family (Woltering, 2008). To investigate whether suppression of HOXB1 and HOXB3 is involved in the observed role of miR-10a in metastasis formation in human pancreatic cancer we used siRNAs to specifically

knock down HOXB1 and HOXB3. Suppression of either HOXB1 or HOXB3 by siRNAs conferred invasiveness and metastasis formation to otherwise non-metastatic PaTu-S cells (Table 1 and Supplemental Table 1). Simultaneous suppression of HOXB1 and HOXB3 resulted in an additive effect (Table 1 and Supplemental Table 1).

To examine whether HOXB1 and HOXB3 expression is required for the anti-metastatic effects of the RAR α antagonist, we pre-treated PaTu-T cells with both the RAR α inhibitor and HOXB1 and HOXB3 specific siRNAs. Both siRNAs were functional and no transcripts were detectable by RT-PCR (Figure 6 A). The anti-metastatic activity of RAR α antagonist was repressed by HOXB1 or HOXB3 suppression in PaTu-T cells (Table 1 and Supplemental Table 1). HOXB1 and HOXB3 suppression had an additive effect and by simultaneous suppression annulled the effects of the RAR α antagonist (Table 1 and Supplemental Table 1). PaTu-T cells transfected with HOX siRNA or control siRNA were equally viable as tested by PI staining (data not shown).

By quantitative PCR analysis we could show that RAR α antagonist treatment increased HOXB1 and HOXB3 expression in PaTu-T cells and that in contrast RA treatment decreased the expression of both genes in PaTu-S cells (Figure 6 B). These findings correlate with an inverse regulation of miR-10a expression, which is decreased by RAR α antagonist and increased by RA stimulation (Figure 5 A). Taken together, these results strongly suggest that miR-10a exerts its role in metastasis formation through suppression of HOXB1 and HOXB3.

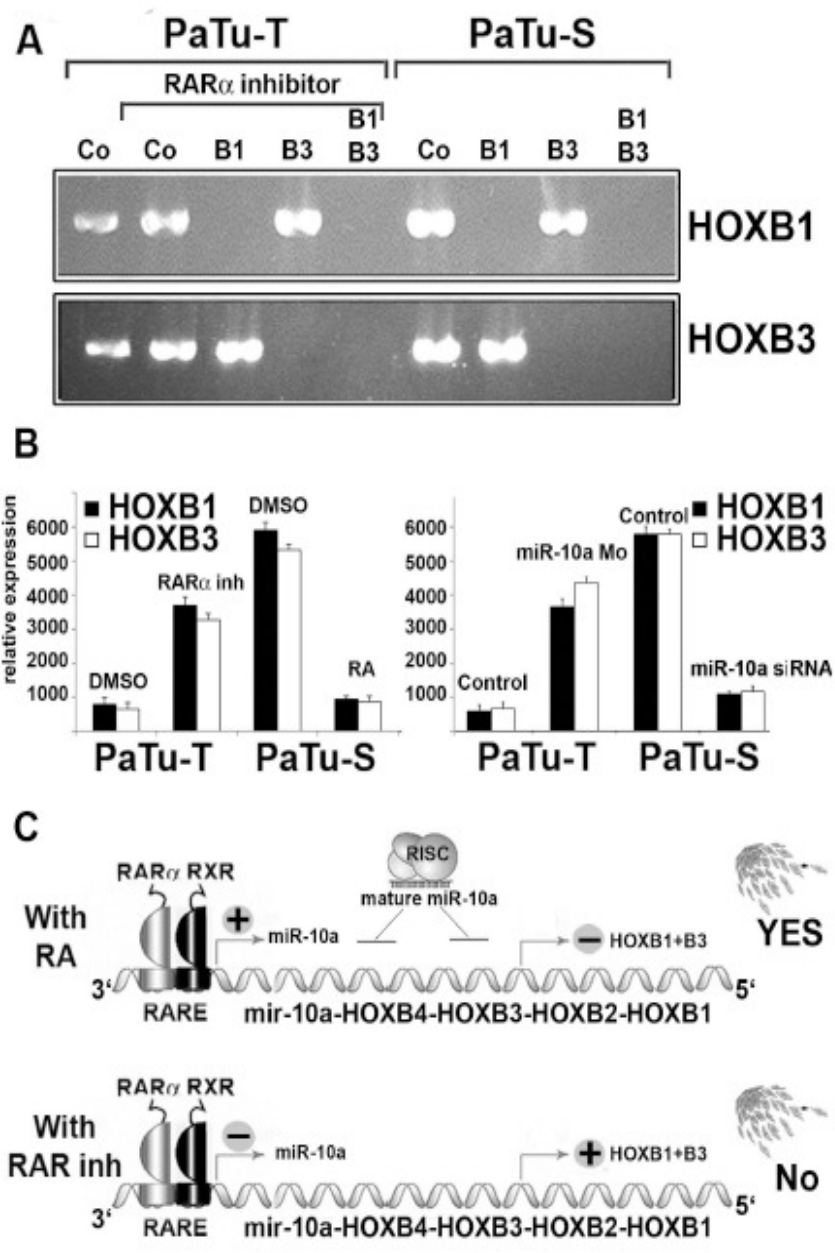


Figure 6 Suppression of HOXB1 and HOXB3 confers invasiveness and metastatic

behavior PaTu-S and PaTu-T cells were treated as indicated with specific siRNAs against HOXB1 (B1) and HOXB2 (B2) (Co: DMSO control) in presence or absence of Ro-415253 (RAR α inh). Migration of these cells was analyzed after transplantation into zebrafish embryos (see Supplemental table 1) and down regulation of HOXB1 and HOXB3 expression in these cells was confirmed by PCR analysis (A). In addition PaTu-S cells were treated with DMSO (control) or with 1 μ M ATRA (+RA) and inverse regulation of HOXB1 and HOXB3 expression in response to RAR α inh or RA was observed in quantitative RT-PCR (B). Results are normalized to β -actin and similar results were obtained in three independent experiments. The schematic model in (C) summarizes our findings: RA stimulates dimerization of RAR/RXR retinoid receptors which bind to retinoic acid response elements (RAREs) in the regulatory region of miR-10a and triggers increased miR-10a expression. Suppression of HOXB1 and HOXB3 by miR-10a then promotes invasion and metastatic behavior (upper panel). RA stimulation, miR-10a overexpression or suppression of HOXB1 and HOXB3 (by siRNAs) are all sufficient to confer metastatic behavior to these cells. Transcriptional repression of miR-10a by a selective RAR α inhibitor (Ro-415253) releases the suppression of HOXB1 and HOXB3 and prevents invasiveness and metastatic behavior (lower panel). The inhibitory function of Ro-415253 can be overruled by a suppression of HOXB1 and HOXB3 with siRNAs.

Discussion

Here, we identified and validated miR-10a as a novel gene with an important function in tissue invasion and metastasis formation. Over expression of miR-10a siRNAs alone suffices to acquire invasiveness and metastatic behavior in otherwise non-metastatic pancreatic cancer cells, whereas silencing miR-10a with gene specific Morpholinos blocks metastatic behavior of metastatic pancreatic cancer cells and primary human tumors. We demonstrate that specific miR-10a inhibitors can be used to prevent invasion and metastatic

behavior even of xenotransplanted resection specimen of human pancreatic tumors in zebrafish embryos. Morpholinos can remain functional for several days (Nasevicius, et al., 2000) and recently “*in vivo* Morpholinos”, which are covalently linked to a delivery component, have become available (Gene Tools, LLC).

The early embryos used here (2dpf) do not reject these primary tumor xenografts, due to the fact that their immune system is not fully developed. The major maturation events leading to immune competence occur in the zebrafish between 2 and 4 weeks post fertilization (wpf), coinciding with the larval to juvenile transitory phase (Lam, et al., 2004).

The identification of upstream regulators of miR-10a in pancreatic cancer cells is of importance. We show that miR-10a is regulated by RA signaling in pancreatic cancer cells. Retinoids have been used as chemo preventive and anticancer agents because of their pleiotropic regulator function in cell differentiation, growth, proliferation and apoptosis, however to date limited amount of information on the role of retinoids in metastasis formation is available and nothing is known on the role of RAR antagonists in this process. For pancreatic cancer we clearly demonstrate anti-metastatic properties of RARA α antagonists, which down regulate miR-10a, and therefore may have potential as anti-metastatic drugs. Whether this role of miR-10a and RARs is specific for pancreatic cancer or a common mechanism in other cancer types will need to be determined.

Notably we found upregulated miR-10a expression already in conditions of chronic pancreatitis. It is well recognized that chronic conditions of pancreatitis may represent a risk factor for the development of pancreatic cancer (Lowenfels, et al., 1993). Nevertheless, the route from inflammation to cancer must involve

many steps and general observations concerning the risk of pancreatic adenocarcinoma in patients with CP may suggest an accelerated process of mutagenesis and mutation accumulation rather than a unique process. However, the role that miR-10a plays in the progression from chronic pancreatitis to cancer cannot be answered at this time. Upregulation of miR-10a expression could be involved in a more complex regulation mechanism that controls or parallels the malignant transformation of a cell to cancer. miR-10a might regulate additional early steps in tumor progression which are not related to cell migration and additional triggers are necessary for the initiation of the metastatic phenotype that are absent in chronic pancreatitis, but are active in transformed carcinoma cells. It is known that the development of pancreatic cancer from chronic pancreatitis is a slow process and Lowenfels *et al.* ⁽¹⁹⁹³⁾ reported a cumulative risk of pancreatic cancer in subjects with CP of 1.8% after 10 years and 4.0%, after 20 years. Future experiments will have to address the specific role of miR-10a in these processes and identification of miR-10a targets in chronic pancreatitis and pancreatic carcinoma might give further insights in these early regulatory mechanisms.

We identified here downstream targets of miR-10a, namely HOXB1 and HOXB3 and the cadherin/catenin complex proteins E-cadherin, α -catenin and β -catenin. Recently, Ma *et al.*, (2007) showed inhibition of metastatic behavior by another microRNA, miR-10b, which is located in the HOXD cluster and targets HOXD10. This study described suppression of metastatic behavior via a regulatory module consisting of Twist-miR-10b-HOXD10-RHOC ⁽²⁰⁰⁷⁾. Upregulated miR-10b expression suppresses translation of HOXD10 and thereby leads to increased expression of the pro-metastatic gene *RHOC* ⁽²⁰⁰⁷⁾. Our results indicate that both, HOXB1 and HOXB3 also function as metastasis suppressor genes. Whether down

regulation of cadherin/catenin proteins is controlled via HOXB1 and HOXB3 or occurs through a parallel signaling pathway of miR-10a will have to be further analyzed. However, our results show that HOX genes can function as metastasis suppressor genes and HOXB1 and HOXB3 and HOXD10 might not be exceptional among the 39 existing mammalian HOX genes.

In summary, we identified miR-10a as a RA responsive mediator of invasiveness and metastatic behavior in pancreatic cancer which promotes its effects through HOXB1 and HOXB3 suppression. Inhibition of miR-10a expression or function represents a promising strategy for anti-metastatic therapy and both RAR antagonists and miR-10a inhibitors could be powerful anti-metastatic compounds.

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