

Review

Pancreatic Ductal Adenocarcinoma: MicroRNAs Affecting Tumor Growth and Metastasis in Preclinical *In Vivo* Models

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Abstract. Patients with pancreatic ductal adenocarcinoma have a dismal prognosis because at the time of diagnosis, in the vast majority of patients the tumor has already disseminated to distant organs and the therapeutic benefit of approved agents such as gemcitabine is limited. Therefore, the identification and preclinical and clinical validation of therapeutic agents covering new targets is of paramount importance. In this review we have summarized microRNAs and corresponding targets which affect growth and metastasis of pancreatic tumors in preclinical mouse *in vivo* models. We identified four up-regulated and 16 down-regulated miRs in PDAC in comparison to corresponding normal tissues. Three sub-categories of miRs have emerged: miRs affecting tumor growth and miRs with an impact on both, tumor growth and metastasis or metastasis only. Finally, we discuss technical and therapeutic aspects of miR-related therapeutic agents for the treatment of pancreatic ductal adenocarcinoma.

Pancreatic ductal adenocarcinoma (PDAC) comprises 85% of pancreatic tumors, is one of the most lethal solid human tumors with a yearly prevalence of 50,000 patients in the USA and a slightly lower death rate (1). The incidence of PDAC is increasing and in 2020, PDAC is expected to cover the second most common cancer-related death rate after lung carcinoma (2). Only 10-20% of PDACs can be resected and it is an exception to find a resected carcinoma that is still

limited to the primary tumor (3). The median survival time after diagnosis is 3-10 months in patients with unresectable tumors and 10-20 months after resection (4). Gemcitabine together with 5-Fluoro-Uracil (5-FU) and subsequently FOLFIRINOX (5-FU, irinotecan, oxaliplatin and leucovorin) as well as a nanobody-based formulation of paclitaxel (nab paclitaxel), give rise to slightly improved survival rates in comparison to gemcitabine, depending on the clinical scenario (5).

Activating mutations of Kirsten rat sarcoma viral homolog (*K-RAS*) and inactivating mutations of tumor suppressor genes (TS) such as cyclin-dependent kinase inhibitor 2A (*CDKN2A*), *TP53* and SMAD family member 4 (*SMAD4*) have been identified as the most prominent drivers of pathogenesis and metastasis of PDAC (6, 7). Mutated *K-RAS* leads to activation of oncogenic and pro-metastatic mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K) and ral guanine nucleotide dissociation stimulator (RALGDS) pathways (6, 7). Inactivating mutations in *CDKN2A* prevent inhibition of cell-cycle progression from the G₁ phase to the S phase of the cell cycle and proteasomal degradation of p53 inhibitor mouse double minute 2 homolog (MDM2) (6, 7). *TP53* mutations interfere with G₁/S and G₂/M arrest and with inhibition of apoptosis (6, 7). Inactivating mutations in *SMAD4* impair the protein's tumor suppressor anti-metastatic function (6, 7). Further treatment-obstructive features of PDAC are: intratumoral heterogeneity, pronounced desmoplastic tumor micro-environment (TME), intrinsic resistance to chemotherapy, hypovascularity and resistance to immuno-therapy (5, 8, 9). Another issue complicating treatment of PDAC is its dependence on a super-enhancer based regenerative program with cellular myelocytomatosis (c-MYC) as a switch engaging and maintaining the program (10). Although, the pathogenesis of PDAC starting with pancreatic intraepithelial neoplasia can be mimicked faithfully in transgenic mice, the impact of these models on the discovery of new drugs against PDAC remains to be validated (11, 12). Therefore, the identification of new targets and

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modalities for the treatment of PDAC is of paramount importance (13, 14). In this review we focus on the role of microRNAs (miRs) on the pathogenesis and metastasis of PDACs. We restricted the selection to PDAC-related miRs with documented efficacy in preclinical *in vivo* models.

Metastasis of PDAC

From a preclinical point of view, PDAC-derived exosomes have been shown to be involved in metastasis of PDAC to the liver. These exosomes contain macrophage inhibitory factor (MIF), which can induce liver metastasis by mediating pre-metastatic niche formation in the liver (15). Uptake of PDAC-derived exosomes by Kupffer cells causes secretion of TGF β 1 and enhancement of fibronectin secretion by hepatic stellate cells, which are responsible for the generation of a fibrotic TME and recruitment of bone-marrow-derived macrophages (15). Blockade of MIF prevents liver pre-metastatic niche formation and metastasis (15). MIF primes the liver for metastasis and is markedly higher expressed in exosomes from PDAC patients who later develop liver metastasis (15).

From a clinical point of view, the liver was found to be the most common site of metastasis, followed by the peritoneum and the lungs. Colonization of adrenal glands and bones comprise approximately 10% of metastatic disease derived from pancreatic cancer (16-18).

microRNAs: General Issues and Involvement in Cancer

microRNAs (miRs) are non-coding RNAs in the range of 22 to 25 nucleotides (nts) which regulate gene expression at the post-transcriptional level by inducing degradation or blocking translation of corresponding mRNAs (19, 20). miRs are transcribed by RNA Pol II as about 700 nts comprising pri-miRNAs precursors in the nucleus and then cleaved by RNase of type III DROSHA, from both ends to pre-miRs of 70-100 nts in length (21, 22). The pre-miRs are subsequently transferred to the cytoplasm by carrier-protein exportin-5 (21, 22). Finally, RNase type III member DICER, which acts as an endoribonuclease, cleaves the stem-loop of the pre-miRs to yield shorter, double-stranded RNAs comprising 22 to 25 nts with two unpaired 3' nts at each end (21, 22). One of the strands is finally bound to the RNA-induced silencing complex (RISC) (21, 22). The RISC-miR complex matches the target mRNA in its 3'-untranslated region (3'-UTR) and induces its degradation or inhibition of its translation (21, 22). Approximately 1,000 miR-genes have been identified in humans (23). The transcripts are capped at their 5'-ends, polyadenylated at their 3'-ends and sometimes they are spliced. The corresponding genes can be localized in exons, introns or intergenic regions (23). It has emerged as an important

principle that one type of miR can target several distinct mRNAs and a single mRNA can be cleaved by several different types of miRs, thus enabling interference with several cancer-related cellular networks with the potential of rewiring the transformed and metastatic state (24).

The involvement of miRs in the pathogenesis of cancer has been demonstrated by the involvement of miR-15a and miR-16-1 in the pathogenesis of B-cell chronic lymphatic leukemia (B-CLL) (25-27). It was observed that tumor suppressors on chromosome 13q14, a locus containing miR-15a and miR-16-1, are frequently deleted in patients with B-CLL. In a proof-of-concept experiment, deletion of miR-15a and miR-16-1 in mice recapitulated the human disease phenotype due to circumventing cleavage of the anti-apoptotic protein B-cell lymphoma 2 (BCL2) (25-27).

We have recently reviewed the role of miRs in metastasis of breast-, ovarian- and prostate cancer (28-30). In this review, we outline the role of miRs in the pathogenesis and metastasis of PDAC. We focus on miRs with documented *in vivo* efficacy in pathogenesis- or metastasis-related preclinical models of PDAC.

Growth-promoting miRs Up-regulated in PDAC

miRs discussed in this chapter enhance tumor growth. Since they have not been tested in metastasis-related *in vivo* models, some of them might also function as mediators of metastasis.

miR-10b steady-state levels are increased in PDAC patients in comparison to normal pancreatic tissues and its up-regulation correlates with a poor prognosis (31, 32). miR-10 enhances epidermal growth factor (EGF)-dependent invasion and proliferation of PANC-1 and COLO-357 pancreatic carcinoma cells and targets TAT-interacting protein 30 (TIP30) directly (31). TIP30 regulates endocytosis of EGFR (31, 33). miR-10b enhances the ability of EGF to activate MAPK and loss of TIP30 up-regulates epidermal growth factor receptor (EGFR) (Figure 1) (31, 33). However, the mechanistic interactions underlying these phenomena have to be resolved in more detail. Pancreatic carcinoma derived T3M4 cells overexpressing miR-10b exhibit enhanced proliferation and tumor growth in an orthotopic xenograft model (31). Data derived from The Cancer Genome Atlas (TCGA) did not reveal tendency of increased steady-state levels of miR-101 in PDAC samples in comparison to normal pancreatic tissues (Figure 2).

miR-194 is up-regulated in PDAC, mediates migration, proliferation and colony formation of PANC-1 cells and suppresses apoptosis of these cells (34). miR-194 transfected PANC-1 cells exhibit reduced growth in an orthotopic pancreatic mouse model in comparison to the untransfected cell line (34). miR-194 targets dachshund 1 (DACH1), a cell fate determination factor, which functions as a TS in several

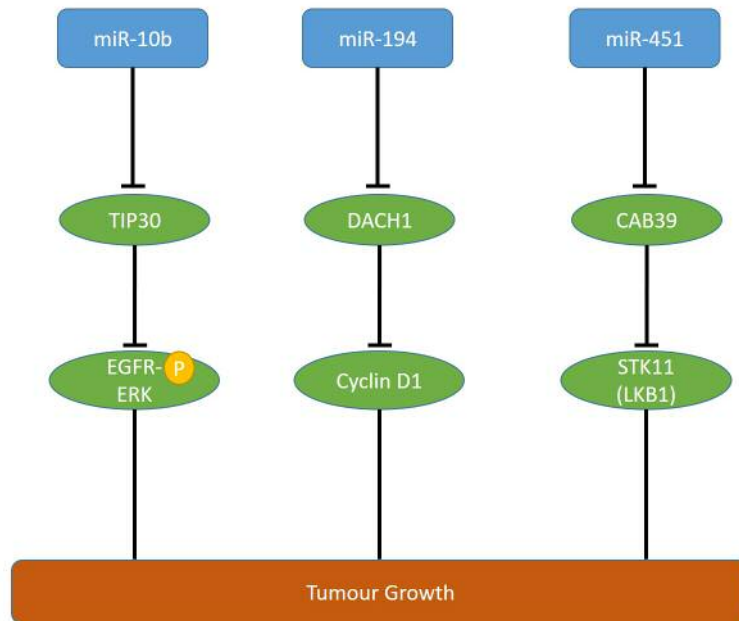


Figure 1. Up-regulated miRs affecting growth of pancreatic ductal adenocarcinoma targeting tumor suppressors in preclinical *in vivo* models. miRs-10b, -194 and -451 affect tumor growth via the outlined targets. CAB39: Calcium binding protein 39; DACH1: dachshund homolog 1; EGFR-P: phosphorylated epidermal growth factor receptor; ERK: extracellular signal regulated kinase 1; LKB1: liver kinase B1; STK11: serine-threonine kinase 11.

types of cancer (34-36) (Figure 1). Steady-state RNA levels for miR-194 derived from TCGA did reveal a tendency of increased expression of miR-194 in PDAC samples in comparison to normal pancreatic tissues (Figure 2).

Overexpression of miR-451 is related to poor prognosis in patients with PDAC (37). miR-451 promotes *in vivo* growth of subcutaneously implanted PANC-1 and AsPC-1 pancreatic carcinoma cells (37). Calcium binding protein 39 (CAB39) was identified as a direct target of miR-451 (37). CAB39 stimulates tumor-suppressive serine threonine kinase 11 (STK11), also referred to as liver kinase B1 (LKB1) (38, 39) (Figure 1). miR-451 steady-state levels in PDAC samples in comparison to normal pancreatic tissues did not reveal a tendency of increased levels in PDAC samples (Figure 2).

Growth-inhibiting miRs Down-regulated in PDAC

miRs discussed in this chapter inhibit tumor growth. Since they have not been tested in metastasis-related *in vivo* models, some of them might have an impact on metastasis. miR-96 is down-regulated in PDAC in comparison to corresponding normal pancreatic tissues (40). miR-96 inhibits invasion, migration and proliferation of MIA PaCa-2 and PANC-1 pancreatic carcinoma cells *in vitro* and growth of both cell lines subcutaneously injected into the flanks of nude mice (40). KRAS has been identified as a direct target of miR-96 (40) (Figure 3). KRAS is one of the

three members of the RAS oncogene family which encode small GTPases involved in signaling transduction mediating cell growth, differentiation and survival (41). Oncogenic KRAS promotes pancreatic tumorigenesis through multiple pathways including PI3K/AKT, extracellular signal-regulated kinase (ERK) and nuclear factor κ B (NF κ B) (41, 42).

miR-130b is down-regulated in PDAC in comparison to normal pancreatic tissues and is associated with worse prognosis (43). miR-130b suppresses proliferation and invasiveness of PANC-1 and AsPC-1 pancreatic carcinoma cells *in vitro* as well as growth of PANC-1 cells transfected with miR-130b in nude mice in comparison to untransfected cells (43). Signal transducer and activator of transcription (STAT3) has been identified as a direct target of miR-130b (43). STAT3 is a transcription factor which is frequently overexpressed in human cancers including PDAC, and inhibits apoptosis and promotes angiogenesis, invasion and metastasis (44, 45) (Figure 3).

Decreased expression of miR-141 is associated with poor prognosis in patients with PDAC (46). In PANC-1 and MIA PaCa-2 transfectants, miR-141 inhibits cell proliferation, colony formation, invasion, G1-phase arrest and apoptosis (46). PANC-1 cells transfected with miR-141 exhibit reduced tumor growth as xenografts in nude mice in comparison to untransfected PANC-1 cells (46). Mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) has been identified as a direct target of miR-141 (46) (Figure 3). MAP4K4, a

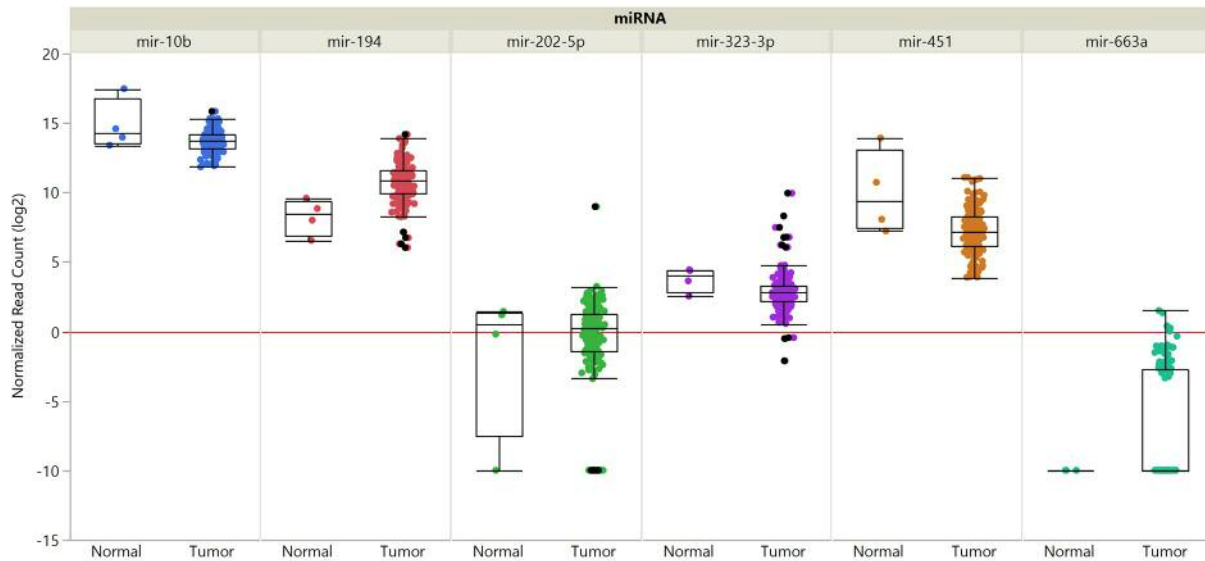


Figure 2. Expression of selected microRNAs in pancreatic adenocarcinoma compared to matching normal tissues. Expression data are shown for miRs-10b, -194, -202-5p, -323-3p, -367, -451 and -663a. Data from 179 pancreatic cancer samples and 4 matching normal samples derived from the PAAD cohort of The Cancer Genome Atlas (TCGA) are shown. miRNA expression was quantified by RNA sequencing and is shown as log₂ of normalized read counts. The red lines indicate low versus higher expression. Expression data are shown as box plots. The line in the middle of the box represents the median values, the rectangles show the upper and lower 25% quartiles and 50% of all data points are included in the greater rectangle. All other data points, except for outliers are located within the upper and lower whiskers.

serine-threonine kinase, is involved in migration, proliferation and regulation of focal adhesion dynamics (47, 48). Expression levels of MAP4K4 are associated with worse prognosis in patients with stage II PDAC (49).

miR-148 is down-regulated in PDAC and correlates with lymphatic invasion, distant metastases and worse prognosis (50). In PANC-1 and BxPC-3 cells, miR-148 inhibits invasion and proliferation and induces apoptosis and cell-cycle arrest (50). miR-148 inhibits growth of PANC-1 cells subcutaneously injected into nude mice (50). 5'-AMP activated protein kinase catalytic subunit $\alpha 1$ (AMPK $\alpha 1$) was identified as a direct target of miR-148 (50) (Figure 3). AMPK $\alpha 1$ interferes with growth of human cancer cells by inhibition of mechanistic target of rapamycin (mTOR) signaling (51, 52). AMPK $\alpha 1$ acts as a cellular energy sensor conserved in all eukaryotic cells and regulates the activity of key metabolic enzymes through phosphorylation (53).

miR-663a is down-regulated in PDAC in comparison to normal pancreatic tissues and its down-regulation is associated with decreased survival (54). In PANC-1 and AsPC-1 cells, miR-663a attenuates cell proliferation and invasiveness and induces apoptosis (54). PANC-1 cells transfected with miR-663 exhibit decreased tumor growth in comparison to untransfected cells after subcutaneous implantation into the flanks of immuno-deficient mice (54).

Elongation factor 1A2 (eEF1A2) has been identified as a direct target of miR-663 (54). eEF1A2 is critical for protein synthesis because it negatively regulates protein biosynthesis by binding to amino-acylated t-RNAs and facilitating their recruitment to the ribosome (55) (Figure 3).

Up-regulated miRs Mediating Metastasis in PDAC Xenografts

High levels of miR-367 correlate with poor prognosis in PDAC patients (56). miR-367 promotes migration and invasion of PANC-1 and BxPC-3 pancreatic carcinoma cells (56). miR-367 induces epithelial- mesenchymal transition (EMT) by increasing TGF β -induced transcriptional activity (56). *In vivo*, PANC-1 cells transfected with miR-367 and injected into the spleen of nude mice give rise to increased numbers of liver metastatic colonies in comparison to non-transfected cells, whereas no effect on tumor growth was observed (56). SMAD family protein 7 (SMAD7) was identified as a direct target of miR-367 (56) (Figure 4). SMAD7 acts as an inhibitor of TGF β signaling and as a metastasis suppressor in human cancer (57-59). In PDAC, TGF β signaling contributes to cell invasion and metastasis by inducing EMT (60). Low levels of SMAD7 correlates with poor prognosis and lymph node metastasis in patients

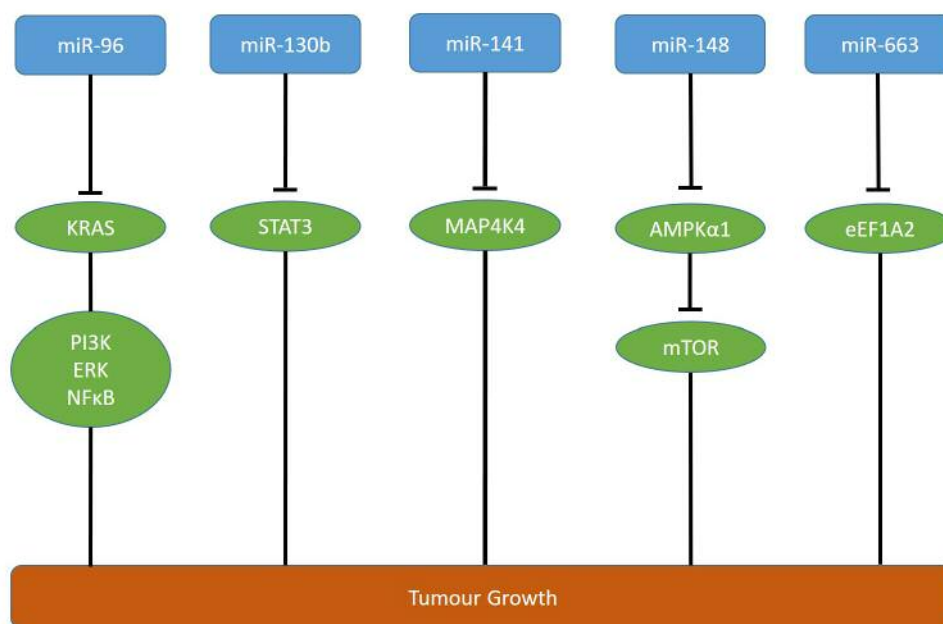


Figure 3. Down-regulated miRs affecting growth of pancreatic ductal adenocarcinoma-related xenografts. miRs -96, -10b, -141, -148 and -663 are down-regulated and interfere with the targets as outlined. AMPK α 1: AMP-activated protein kinase; eEF1A2: eukaryotic initiation factor A2; ERK: extracellular signal regulated kinase; KRAS: Kirsten rat sarcoma; mTOR: mammalian target of rapamycin; MAP4K4: mitogen-activated protein kinase kinase kinase 4; NF κ B: nuclear factor κ B; PI3K: phosphoinositide 3-kinase; STAT 3: signal transducer and activator of transcription 3.

with pancreatic cancer (61). Nevertheless, one should be aware that TGF β can have opposite effects on tumor progression and metastasis depending on the stage of carcinogenesis and the responsiveness of tumor cells (62, 63). TCGA-derived steady state levels of miR-663a revealed very low expression in PDAC and normal pancreatic tissues (Figure 2).

Down-regulated miRs Mediating Metastasis in PDAC Xenografts

Down-regulated expression of miR-29c and matrix metalloprotease 2 (MMP2) correlate with progression of PDAC (64). miR-29c suppresses migration and invasion of miR-29c transfected PANC-1 and BxPC-3 pancreatic carcinoma cells and targets MMP2 directly (64) (Figure 4). *In vivo*, miR-29c transfected Hs-766 T pancreatic carcinoma cells give rise to reduced liver metastasis in comparison to non-transfected cells after injection into the pancreas (64). MMP2 can act as a promoter of metastasis, but its functional role is context-dependent and clinical trials failed to validate MMP2 as an anti-metastatic target in several types of cancer (65-68).

miR-96 decreases proliferation, migration and invasion of PANC-1 and CFPAC-1 pancreatic carcinoma cells *in vitro* (69). CFPAC-1 cells transfected with miR-96 exhibit reduced growth after subcutaneous injection into the flanks of nude

mice and no metastatic nodules after injection into the pancreatic capsule in contrast to the untransfected cell line (69). Human ether-a-go-go 1 (HERG1) potassium channel was identified as a direct target of miR-96 (69, 70) (Figure 5). HERG1 is often up-regulated in PDAC and functions as an oncogene (71). HERG1 is overexpressed in many types of cancers and has been shown to be involved in functions such as invasion, proliferation and apoptosis (72, 73).

miR-98-5p inhibits migration, invasion and proliferation of PDAC cell lines CFPAC-1 and MIA-PaCa-2 (74). Low levels of miR-98-5p correlate with tumor size and lymph node metastasis (74). *In vivo*, tumor volume and weight of miR-98-5p overexpressing pancreatic carcinoma cells was decreased, whereas silencing of miR-98-5p increased tumor growth *in vivo* (74). In an orthotopic pancreatic cancer *in vivo* model, liver nodules were elevated in MIA PaCa-2 cells with silenced expression of miR-98-5p in comparison to the control cell line (74). MAP4K4 was identified as a direct target of miR-98-5p (74) (Figure 5). It was shown that overexpression of MAP4K4 increases growth of hepatocellular carcinoma cell lines *in vitro* and *in vivo* (75). In gastric cancer cells, silencing of MAP4K4 by short hairpin RNA was shown to suppress proliferation and to induce G₁ cell cycle arrest and apoptosis (76).

miR-143 inhibits migration and invasion of PANC-1 pancreatic carcinoma cells *in vitro* and liver metastasis of

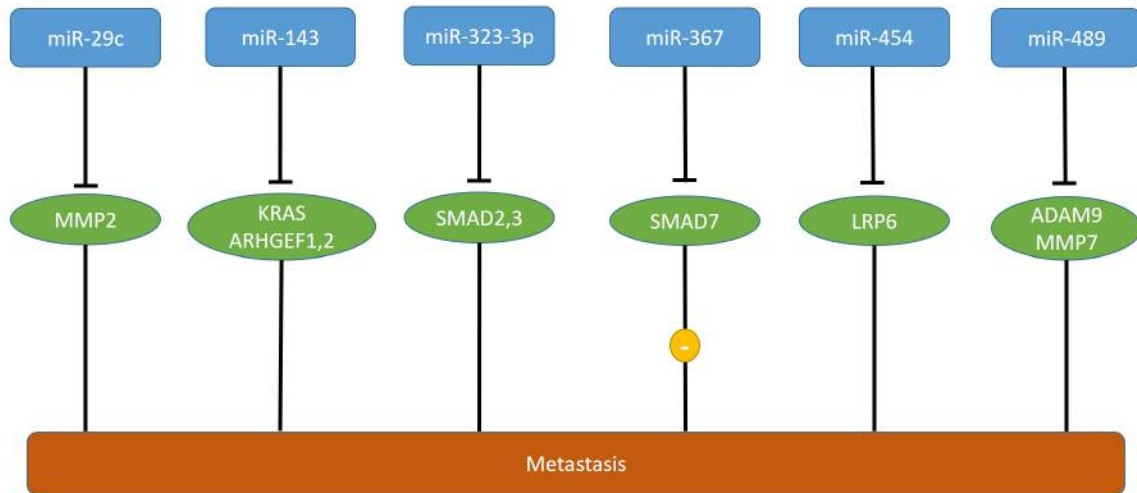


Figure 4. miRNAs affecting metastasis of pancreatic ductal adenocarcinoma xenografts. miR-367 is up-regulated, whereas miRNAs -29c, -143, -323-3p, -367, -454-5p, and miR-489, are down-regulated in pancreatic tumors in comparison to corresponding normal pancreatic tissues models. The corresponding targets are shown. ADAM 9: A disintegrin and metalloproteinase 9; ARHGEF1,2: rho guanine nucleotide exchange factor 1,2; KRAS: kirsten rat sarcoma; LRP6: low-density lipoprotein-related protein C; MMP2,7: matrix metalloproteinase 2,7.

these cells in an orthotopic xenograft model (77). miR-143 targets directly KRAS and RHO guanine nucleotide exchange factors ARHGEF1 and ARHGEF2 (77) (Figure 4). miR-143 decreases RHO GTPase activity of ras-related C3 botulinum toxin substrate 1 (RAC1), cell division control protein 42 (CDC42) and RHO GTPase A (RHO A), inhibits MMP2 and MMP9 and elevates the protein level of E-cadherin (77). Guanine nucleotide exchange factors commonly activate RHO GTPases through catalysis of the exchange of GDP for GTP and are crucial for cell migration and invasion (78-80).

miR-195 inhibits proliferation, migration and invasion of PANC-1 and AsPC-1 pancreatic carcinoma cells *in vitro* and *in vivo* and low miR-195 expression indicates a poor clinical outcome (81). Overexpression of miR-195 in PANC-1 cells inhibits subcutaneous tumor growth and alleviates metastasis of PANC-1 cells transfected with miR-195 to the liver after injection into the spleen of nude mice (81). Doublecortin-like kinase 1 (DCLK1) was identified as a direct target of miR-195 (81) (Figure 5). miR-195 regulates pERK1/2, p38, p21, vimentin, E-cadherin, RHOA and MMP9 expression in pancreatic carcinoma cells by targeting DCLK1 (81). DCLK1 is a microtubule-binding member of the calmodulin-dependent kinase family, is overexpressed in intraepithelial lesions as well as in surgical resection specimen of PDAC and is a stem cell marker, inhibitor of proliferation and mediator of EMT in PDAC (82-85).

Expression of miR-202-5p in PANC-1 cells reduces tumor growth and liver metastasis in athymic mice after orthotopic implantation in comparison to the untransfected cell line (86). In an immuno-competent mouse model, making use of

PAN 02 cells, derived from a carcinogen-induced mouse model of pancreatic carcinoma, miR-202-5p reduced tumor burden and liver metastasis after orthotopic implantation (86). miR-202-5p inhibits TGFβ1-induced EMT of PDAC cells (86). Also, miR-202-5p interferes with TGFβ-mediated activation of pancreatic stellate cells, accumulation of extracellular matrix (ECM) proteins and fibrotic stromal deposition (86). Transforming growth factor β receptor 1 and 2 (TGFRβ1, TGFRβ2) have been identified as direct targets of miR-202-5p (86) (Figure 5). TGFβ signaling is a mediator of EMT of pancreatic carcinoma cells (87-91). The TGFβ-TGFβR complex recruits downstream effectors such as SMAD2 and SMAD3 (R-SMADs), which form complexes with SMAD4 and these complexes translocate into the nucleus, resulting in activation of genes involved in EMT by recruitment of transcriptional coactivators or corepressors (87-91). TCGA derived steady-state levels of miR-202-5p did not reveal a tendency of deregulation of its expression in PDAC samples in comparison to normal pancreatic tissues (Figure 2).

Down-regulation of miR-323-3p correlates with PDAC progression (92). miR-323-3p inhibits invasion and migration of PANC-1 and BxPC-3 pancreatic carcinoma cells (92). Metastatic lung foci were inhibited in PANC-1 cells transfected with miR-323-3p after tail vein injection in nude mice in comparison to control cells (92). miR-323 inactivates the TGFβ signaling pathway by directly targeting SMAD2 and SMAD3 which are functional effectors of the TGFβ pathway in PDAC cells (92-95) (Figure 4). Down-regulation of miR-323-3p, with increased expression of SMAD2 and SMAD3,

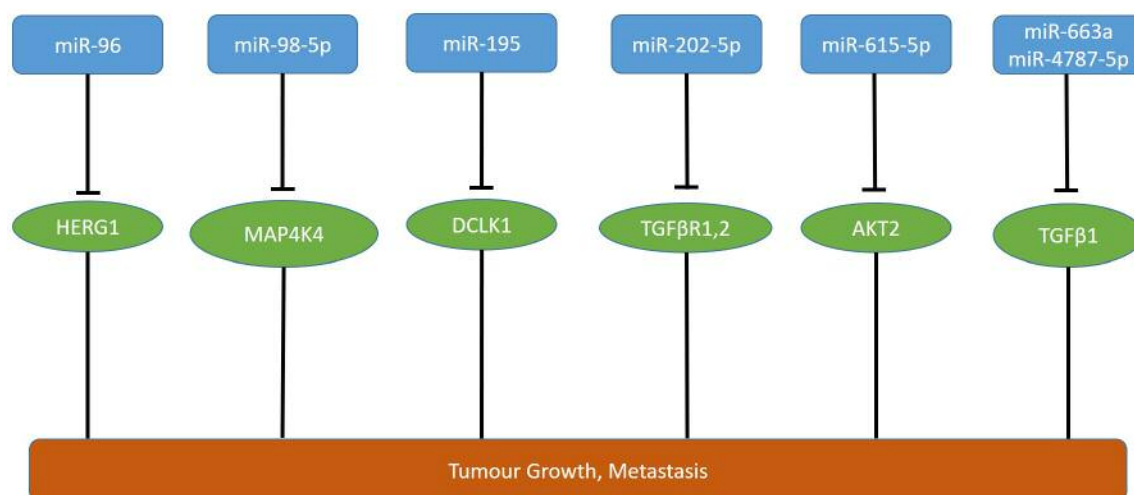


Figure 5. miRNAs affecting tumor growth and metastasis of pancreatic ductal adenocarcinoma xenografts. miR-96, -98-5p, -195, -202-5p, -615-5p, -663a and 4787-5p are down-regulated in pancreatic tumors in comparison to corresponding normal tissues. AKT2: Protein kinase β ; DCLK1: doublecortin-like kinase 1; HERG1: human ether-a-go related gene product; MAP4K4: mitogen-activated protein kinase kinase kinase 4; TGF β 1: transforming growth factor β 1; TGF β R1,2: transforming growth factor β receptor 1 and 2.

contributes to a more aggressive phenotype of PDAC (92). Steady state levels for miR-323-3p did not show a tendency of deregulation of its expression in PDAC in comparison to normal pancreatic tissues (Figure 2).

miR-454 inhibits invasion, proliferation and arrests PANC-1 and MIA PaCa-2 pancreatic carcinoma cells in the G2/M phase (96). Furthermore, miR-454 inhibits tube formation of endothelial cells (96). Tail vein injection of miR-454 transduced PANC-1 cells revealed reduced lung metastasis in comparison to the corresponding control cell line (96). As a direct target, low density lipoprotein receptor-related protein 6 (LRP6) has been identified (96) (Figure 4). LRP6 together with LRP5 and the family of frizzled receptors are activating components of the wingless/integrated (WNT) signaling pathway (97-99). Calcipotriol has been identified as a WNT-pathway inhibitor targeting LRP6 (100). Targeting of WNT signaling in cancer is pursued by many ongoing drug discovery programmes (101-103).

miR-489 inhibits migration of PANC1 and BxPC3 pancreatic carcinoma cells (104). PANC-1 cells transfected with miR-489 exhibit reduced lung metastasis after tail vein injection in comparison to the untransfected cell line. Substantially reduced liver metastasis was observed after injection of these cells into the mouse spleen (104). Conversely, inhibition of miR-489 in BxPC-3 cells leads to increased metastasis to the lungs after tail vein injection and enhanced liver metastasis after injection into the spleen of immuno-deficient mice in comparison to the control cell line (104). miR-489 is down-regulated by the KRAS-NF κ B-YY1 axis in PDAC cells (104). KRAS and NF κ B are constitutively

activated in the vast majority of PDACs and yin-yang (YY1) is a ubiquitously distributed transcription factor that binds to the promoter region of its target genes (105-107). A disintegrin and metalloproteinase 9 (ADAM9) and matrix metalloproteinase 7 (MMP7) were identified as direct targets of miR-489 (104) (Figure 4). ADAM9 and MMP7 are metalloproteinases that remodel the ECM, facilitating cancer cells to form local or distant metastases (108-112). Expression of miR-489, YY1, ADAM9 and MMP7 correlated with progression of human PDAC (104).

High expression of miR-615-5p correlates with significantly higher survival time in PDAC patients (113). Overexpression of miR-615-5p inhibits MIA-PaCa-2 and PANC-1 cell migration and invasion *in vitro* (113). Tumor growth of MIA-PaCa-2 cell transfected with miR-615-5p was inhibited after subcutaneous implantation and fewer and smaller colonies in the liver were observed after tail vein injection in comparison to the control cell line (113). Serine-threonine kinase AKT2 was identified as a direct target of miR-615-5p (113) (Figure 5). AKT2 together with AKT1 are isoforms of AKT (114). Activation and amplification of AKT2 has been observed in human PDACs (115-117).

miR-663a and -4787-5p

miR-663a and -4787-5p suppress TGF β 1-induced EMT in PDAC cells MIA PaCa-2 and PANC-1 (118). Expression of both miRNAs in PANC-1 cells reduces tumor weights and volumes and the number of metastatic lesions in liver, spleen, lungs and kidneys after orthotopic implantation into

the pancreas in comparison to the non-transfected cells (118). Both miRs target TGF β 1 directly (118) (Figure 5). In PDAC, TGF β 1 can act both as a tumor suppressor and promoter depending on tumor stage and cellular context (119, 120).

Synopsis of Identified PDAC-related miRs

miRs modulating growth of PDAC xenografts. The identified miRs of this sub-category modulate tumor growth of PDAC-related preclinical *in vivo* models. They might also be involved in metastasis, but corresponding preclinical *in vivo* experiments are pending. Up-regulated tumor growth promoting miRs such as miR-10b (31), miR-194 (34) and miR-451 (37) interfere with mRNAs of TS genes such as *TIP30*, *DACH1* and *CAB39*. Down-regulated tumor growth inhibiting miRs, miR-96 (40), miR-130b (43), miR-141 (46), miR-148 (50) inhibit signal transducers such as KRAS and HERG1, STAT3, MAP4K4 and AMPK α 1. miR-663a (54) interferes with translation by targeting elongation factor eEF1A2.

miRs modulating metastasis of PDAC xenografts. In the category metastasis-related miRs, examples of tumor growth and metastasis affecting miRs as well as miRs affecting metastasis only have been summarized. miR-367 (56), the only up-regulated miR in this category, targets SMAD7, a negative regulator of TGF β signaling (57-59).

Down-regulation of miRs-29c (64), -143 (75), -323-3p (92), -367 (56), -454 (96), -489 (104) promote metastasis, but not tumor growth in preclinical *in vivo* models. miRs-96 (69), -98-5p (74), -195 (81), -202-5p (86), -615-5p (113), -663a (118) and -4787 (118) inhibit tumor growth as well as metastasis in corresponding preclinical *in vivo* models.

As outlined, miR-29c (64) and miR-489 (104) interfere with metalloproteinases MMP2, MMP7 and ADAM9. However, these targets have pro- and tumoral activities and clinical studies with small-molecule inhibitors did not reach the projected endpoints (66-68). Other metastasis-related miRs such as miR-96 (96), -98-5p (74), miR-454 (96) and miR-615p (113) affect signal transduction (113) through targets such as HERG1, MAP4K4, LRP6 and AKT2. miR-195 (81) inhibits DCLK1 which modifies microtubules and therefore has an impact on migration and metastasis. None of the latter targets has reached the status of a clinically validated target.

Noteworthy, 4 miRs such as miR-202-5p (86), -323-3p (92), -663a (118) and -4187 (118) are down-regulated and activate TGF β signaling through up-regulation of targets such as TGFR β 1 and TGFR β 2, TGF β 1, SMAD2, 3, emphasizing the role of activation of this pathway in the progression of PDAC. However, miR-367 is up-regulated and targets SMAD7 which is a negative regulator of pro-metastatic TGF β signaling as outlined above (56-59) and therefore it is a

candidate for functional inhibition. Galunisertib (LY2157299), a small molecule kinase inhibitor of TGFR β 1, is presently evaluated together with gemcitabine for first-line treatment in patients with unresectable PDAC (121). Furthermore, combination studies of galunisertib and checkpoint inhibitory antibody durvalumab (MEDI 4736) are underway (122). Further candidates for functional reconstitution are miR-96 (KRAS and HERG1), miR-130b (STAT3), miR-141 (MAP4K4), miR-148 (AMPK α 1) and miR-663 (eEF1A2). In addition to miR-367 (SMAD7), further candidates for functional inhibition are miR-10b (TIP30), miR-194 (DACH1) and miR-451 (CAB39) which target TS genes.

Therapeutic Aspects

As outlined, 4 up-regulated and 16 down-regulated miRs have been identified as possible therapeutic candidates for treatment of PDAC. However, for miRs -96, -143, -195, -202-5p, -454, -663 and -4787-5p, data correlating expression status and clinical outcome are pending. Of note, miR-96 and -663 can be found in both categories: down-regulated miRs with growth inhibitory function and down-regulated miRs inhibiting metastasis. This is not a contradiction, because miR-96 and miR-663 modulate different targets in these categories. From a therapeutic point of view, up-regulated miRs are candidates for inhibition of function and down-regulated miRs are candidates for reconstitution of function. miRs-10b (31), -194 (34), -451 (37) and -367 (56) are candidates for inhibition of function. Among the miRs to be functionally reconstituted are two miRs targeting KRAS (miR-96 and -143) and four down-regulated miRs with an inhibitory function of TGF β signaling (miRs-202-5p, -323-3p -663a and -4187). The latter are candidates for reconstitution therapy. An exception in this context is miR-367 which is up-regulated and inhibits SMAD7, an inhibitory component of TGF β signaling and therefore is a candidate for functional inhibition. Analysis of the expression status of miRs and components of the TGF β signaling pathway in clinical specimens will guide the selection of the appropriate therapeutic miR candidates. Inhibition of KRAS by reconstitution of miRs-96 and -143 is critical, because these miRs do not discriminate between wild-type and mutated KRAS due to targeting of the conserved 3'-UTRs of their corresponding mRNAs.

miR inhibitors are referred to as antagomirs and are single-stranded RNA molecules in the range of 22-25 nts complementary to a site of the mRNA which is the target of the miR under consideration, preventing binding of the corresponding miR to its target mRNA (21, 22, 123, 124). Usually, chemical modifications such as 2'-methoxy groups and phosphothioates are incorporated into antagomirs to make them resistant to degradation (21, 22). Another version of miR-inhibitors are miR sponges. They are artificial constructs with multiple reiterated miR-binding sites that

compete with the natural mRNA target for binding of the corresponding miR and are usually delivered into mammalian cells by expression vectors (125, 126). The alternative therapeutic option, functional reconstitution of miRs, can be performed making use of miR mimetics, small double-stranded synthetic RNA molecules designed to mimic endogenous mature miRs leading to cleavage, deadenylation or inhibition of translation of the corresponding mRNAs (126). They mimic the transient duplex product of DICER complex processing. The two strands of the synthetic mimic separate and the single-strand mature miR is incorporated into the RISC to down-regulate mRNA transcripts. Reconstitution of miR function by delivery of expression vectors for the corresponding miR is another option for therapeutic intervention (126).

After identification of the target, the miR-related agent has to be designed, its chemistry and delivery efficiency have to be optimized, therapeutic efficacy of the candidate miR-related agent has to be worked out in preclinical *in vitro* and *in vivo* studies and toxicity in rodents and non-human primates has to be assessed. Finally, issues such as dose-finding, toxicity and therapeutic efficacy have to be addressed in clinical studies (22, 124).

There are many issues which are potential bottlenecks and have to be optimized for the therapeutic performance of miR-related therapeutic agents. One of the issues is the improvement of the delivery systems. Optimization of liposomal carriers, conjugation of nucleic-acid related agents to poly-ethylene glycole (PEG), use of biodegradable polymers, cyclodextrin and dendrimers, have resulted in significant progress (127-131). Conjugation of miR-related agents to N-acetyl-D-galactosamine has significantly improved delivery to the liver based on its high affinity to the asialo glycoprotein receptor expressed in liver (132). The size of the unconjugated RNA is another critical issue for delivery of miR-related agents. They have a size between 7 and 20 kD, are filtered by the kidneys and secreted which holds true for all complexes smaller than 50 kD (133). After internalisation into target cells, escaping the endosomal vesicles for release into the cytoplasm is a further bottleneck, because the delivery complexes are often trapped as phagolysosomes (134). Further critical issues are immunogenicity of the miR-related agents, removal from extracellular spaces by macrophages and monocytes and carrier toxicity such as hemolysis, thrombogenicity and complement activation (135-138). Toxicity due to hybridisation of the miR-related agents to non-target nucleic acids is another alerting issue.

Since PDAC has already metastasized to distant organs in the vast majority of patients after diagnosis, miR-related therapeutic agents should be able to eradicate metastasis, which is a significant hurdle. This scenario is reflected in none of the preclinical *in vivo* models. miR-related

therapeutic agents should exhibit anti-proliferative together with anti-invasive properties as a desired profile. In case the miR-related agent has only anti-invasive capacity, the desired profile can be probably achieved by combination with other agents.

In the near past, miR-based therapeutic agents have witnessed serious drawbacks in clinical studies (139). In 2018, Regulus Therapeutics has halted clinical studies of treatment of polycystic kidney disease with anti-miR agent RGLS4326 due to toxicity issues and paused enrollment for a phase II study of miR-21 inhibitor RG-012 in patients with Alpert syndrome, a rare genetic kidney disease (139). In 2016, Regulus has halted a phase I study of RG-101 for treatment of infection with hepatitis C virus with anti-miR-122 due to jaundice in two patients, despite excellent efficacy data (139). In the oncology area, Mirna Therapeutics has shut down a phase I study of MRX-34 for treatment of multiple types of cancers in 2016 (139). Presently, miRagen is clinically evaluating Cobomersen (MRG106) in several types of hematological cancers in phase I studies without reporting serious side effects (139). The clinical evaluation of second-generation miRs without the side-effects as outlined above will be soon underway and tell us more about the therapeutic utility of miRs in cancer therapy.

Conflicts of Interest

FB and AN are employees of Roche, UHW has been a former employee of Roche.

Authors' Contributions

UHW and AN were jointly designing and writing the paper, FB has performed the bioinformatics analysis and was preparing the figures.

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