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Clinical implications of miRNAs in the pathogenesis, diagnosis and therapy of pancreatic cancer

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Abstract

Despite considerable progress being made in understanding pancreatic cancer (PC) pathogenesis, it still remains the 10th most often diagnosed malignancy in the world and 4th leading cause of cancer related deaths in the United States with a five year survival rate of only 6%. The aggressive nature, lack of early diagnostic and prognostic markers, late clinical presentation, and limited efficacy of existing treatment regimens makes PC a lethal cancer with high mortality and poor prognosis. Therefore, novel reliable biomarkers and molecular targets are urgently needed to combat this deadly disease. MicroRNAs (miRNAs) are short (19–24 nucleotides) non-coding RNA molecules implicated in the regulation of gene expression at post-transcriptional level and play significant roles in various physiological and pathological conditions. Aberrant expression of miRNAs has been reported in several cancers including PC and is implicated in PC pathogenesis and progression, suggesting their utility in diagnosis, prognosis and therapy. In this review, we summarize the role of several miRNAs that regulate various oncogenes (KRAS) and tumor suppressor genes (p53, p16, SMAD4 etc) involved in PC development, their prospective roles as diagnostic and prognostic markers and their therapeutic targets.

1. Introduction

Pancreatic cancer (PC) is a lethal malignancy and remains a major clinical challenge. Due to its early metastatic nature, more than 80% of PC patients have invasive disease at the time of diagnosis, which makes surgical and medical intrusions mostly unsuccessful, resulting in high mortality and poor prognosis [1, 2]. With equal incidence to mortality ratio and a five

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The authors have no conflict of interest with any company or financial organization.

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year survival rate of less than 6%, PC is considered to be the most deadliest and aggressive cancer compared to other malignancies [3]. Limitations of the current multimodality therapeutic regimens for PC highlights the urgent need to understand the molecular mechanism/pathway(s) governing initiation, progression and metastasis for discovering novel diagnostic, prognostic and therapeutic targets for better management of this lethal disease. Although several research efforts are directed to discover specific bio-molecules with their utility in early diagnosis, prognosis and therapy for PC, these currently used biomolecules do not have adequate sensitivity and specificity to detect PC in its early stages, nor can they be used to target PC.

The advent of microRNAs (miRNAs) research has opened new avenues to understand the gene regulatory mechanisms at the post-transcriptional level. miRNAs are double stranded small non-coding RNA molecules of 19–24 nucleotides (nt) in length, regulating gene expression at the post-transcriptional level either by degradation or translational inhibition of their target mRNA [4, 5]. Details about the miRNA biogenesis, role in physiology, development and miRNA mediated pathological conditions have already been discussed elsewhere [5–13]. Association of miRNAs role in cancer was first described in Chronic Lymphocytic Leukemia (CLL) [14] and subsequently several miRNAs were reported to be aberrantly expressed in several other cancers including PC [12, 15–22]. They play significant roles in initiation, progression, metastasis, and therapeutic resistance [23]. Although miRNAs represent only 3% of the human genome, they regulate 20–30% of the protein coding genes [24, 25]. Recent studies have shown that 50% of miRNA genes are located in genomic instability regions that are generally associated with neoplastic transformation [26]. Therefore, miRNA signatures can provide better patho-genomic information about tumors than their transcriptome profiles [27]. Moreover, expression patterns of miRNAs are tissue specific [28, 29] and unique to tumor type and tissue of origin [12, 27, 30], thus making miRNAs valuable tools for diagnosis, prognosis and therapy. Pancreatic cancer pathogenesis is a multistep process that involves a compendium of sequential genetic alterations in oncogenes like *KRAS* and several tumor suppressors including *p53*, *p16*, *SMAD4* etc [31]. In this review article, we have summarized the role of miRNAs in PC pathogenesis with specific emphasis on their role in regulating *KRAS*, *p53*, *p16*, and *SMAD4* expression, along with their utility as early detection/prognostic markers and therapeutic targets.

1.1 Deregulated miRNAs in Pancreatic Cancer

Several research studies have proposed an association between altered miRNA expression and PC. Identification of miRNA(s) at various stages of PC progression is critical to understand as their biological functions will help us develop unique diagnostic/prognostic markers and therapeutic targets. In this context, miRNA profiling of tumor samples, pancreatic juice, serum and cyst fluid engenders a potential avenue for miRNA-based biomarker development. The first miRNA expression study was done by Poy *et al*, where the expression of miR-375 and miR-376 was observed in mouse pancreas (pancreas specific miRNAs) but not in the brain, heart, and liver tissues [32]. Later, high throughput analysis identified several miRNA signatures that were able to precisely classify tumors and differentiate PC from normal pancreas and pancreatitis [33]. In addition, profiling of PC

tissue or desmoplasia revealed several precursor miRNAs that were aberrantly expressed and localized to tumor cells including miR-221, -376a, and -301 [34]. Further, extensive miRNA expression profiling in healthy individuals, pancreatitis, precursor pancreatic intraepithelial neoplasm (PanIN) lesions, and pancreatic ductal adenocarcinoma (PDAC) patients have revealed their diagnostic and prognostic utility [11, 35–37]. Subsequently, over expression of miR-376 was reported in human Panc-1 PC cells compared to lung, breast, head and neck, colorectal, prostate, and hematopoietic cancer cells [38], although no difference in expression of miR-375 was observed [12, 38]. While expression of miR-216 and 217 (pancreas specific miRNAs), and the lack of miR-133a are characteristic of normal pancreatic tissues [33], over expression of miR-103 and miR-107, and downregulation of miR-155 are characteristic of PC specimens [39]. Furthermore, up regulation of miR-21 and downregulation of miR-150, miR-30d in PC tissues compared to normal pancreas was reported [40]. The miR-221, miR-376a and miR-301 are present specifically only in the tumor cells of PC and are among the top differentially expressed miRNAs [34]. Recently, miRNA microarray analyses in a panel of 15 PC cell lines revealed overexpression of miR-10a, miR-92, and miR-17-5p [41]. In addition to cell lines, several studies have reported differential expression of miRNAs that can distinguish between PDAC from chronic pancreatitis and normal pancreas [33, 42–44] and a cluster of miR30a-3p, miR-105, miR-127, miR-187, miR-452, and miR-518a-2 was also associated with better prognosis of lymph node positive patients [33]. The miR-203 was over expressed in PC and associated with poor prognosis of patients with no residual tumors [45]. Yu *et al* profiled 700 miRNAs in PanIN lesions and observed an overexpression of let-7f/g, miR-18a, -15b, -21, -29a/b/c, -31, -93, -95, -101, -103, -106b, 146a, -155, -182, -190, -193b, -194, -196b, -200a/b, -203, -222, 338-3p, -429, and 486-3p, and no or weak expression of miR-107, -139-3p/5p, -216a/b, -217, -218 and -483-5p in PanIN-3 lesions [46]. A similar miRNA expression profile using 10 PC cell lines and 17 pairs of PC/normal tissues has revealed significant up-regulation of miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95 [47]. In addition, miR-376a, miR-301, miR-132 and miR-212 were reported to be over expressed specifically in PC compared to normal or benign adjacent pancreas [34, 48]. On the contrary, miR-132 was also found to be down regulated in PC tissues compared to normal and benign tissues [49].

The miRNA deregulation is not only observed in human PC cell lines and tissues but is also evident in the circulation of PC patients. Many studies have identified differential up-regulation of miRNAs including miR-642b, miR-885-5p, miR-22, miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, miR-191, miR-155, miR-196a, and miR-18a in blood of PC patients and these miRNAs could differentiate PC patients from healthy individuals [50]. In addition, miR-2 was over expressed in serum [51], whereas, over expression of miR-196a, miR-217, miR-451, and miR-486-5p [52] and down regulation of let-7c, let-7d, let-7f, and miR-200c was observed in the fine needle aspiration samples (FNA) of PC patients [52].

2. miRNA and KRAS in pancreatic cancer

KRAS is a membrane bound Guanosine Triphosphate (GTP) binding protein from the RAS family of proteins that regulates several signal transduction pathways like RAF, MAP2K,

MAPK Tiam1, Ral-GEF and PI3K AKT upon receiving extracellular stimuli. Usually, RAS associated signaling activation is short lived due to its intrinsic GTPase activity, switching off the RAS signaling cascades. However, KRAS is mostly mutated (Kras^{G12D}) in 90% of PC [53] that results in gain of function and hyper activation of downstream signaling cascades involved in the change of transcriptional dynamics, cell survival and proliferation facilitating tumor progression and metastasis [54]. In spite of establishing KRAS^{G12D} as a potent oncogene, attempts to inhibit the hyper activity or identifying various factors that induce this mutation have failed. Recent discoveries have identified several miRNAs that target KRAS^{G12D} by binding to the 3'UTR of the *KRAS* mRNA transcript and lead to its degradation or translational inhibition. In this section we have attempted to elucidate the role of miR-96, -126, -143/145, -217 and Let-7 mediated regulation of oncogenic *KRAS* in PC.

Recently, Yu *et al.* has observed that miR-96, which is usually lost in PC tissues compared to normal pancreas targets *KRAS* by perfect base-pairing with its 3'UTR [55]. Overexpression of miR-96 in PC cell lines resulted in decreased cell migration, invasion, and proliferation, indicating its tumor suppressive potential [55]. Similarly, miR-126 was also found to be down regulated in PDAC compared to benign cystic tumors and was identified to inhibit translation of *KRAS* by binding to its two putative seed regions on the 3' UTR suggesting that re-expression of miR-126 can be a powerful therapeutic strategy for *KRAS* mediated oncogenesis [56]. Not only do miRNAs target wild type/mutated *KRAS* gene expression, KRAS^{G12D} also modulates miRNA(s) expression during cellular transformation. RAS-responsive element-binding protein (RREB1) binds to the promoter of miR-143/145 cluster and negatively regulates their expression. Surprisingly, oncogenic KRAS^{G12D} induces RREB1 expression in PC as means to check expression of the miR-143/145 cluster for enhanced tumorigenesis [57]. While constitutive miR-143/145 cluster expression targets RREB1 protein to inhibit a feed forward circuit of *KRAS* signals *via* RREB1, the KRAS^{G12D} mediated over expression of RREB1 in turn represses the miR-143/145 cluster expression and therefore facilitates further *KRAS* mediated signaling [57]. Besides the miR-143/145 cluster, another study using *in situ* hybridization technique revealed down regulation of miR-217 in 76% of PC tissues [58]. The same study also reported that over expression of this miR-217 in PC cells decreased *KRAS* expression and phosphorylated AKT levels, suggesting that miR-217 not only downregulates *KRAS* expression but also affects the downstream signaling molecules involved in cell survival and proliferation [58]. Let-7 family miRNAs have multiple target sites on the 3' UTR of *KRAS* and downregulate its expression [59]. Several studies have shown significant down-regulation of let-7 expression in PC samples compared to cancer adjacent normal tissues, and PC patients with loss of let-7 expression were ineligible for surgery due to aggressiveness of the disease [60]. They also observed that ectopic over-expression of let-7 in PC cells significantly downregulates *KRAS* expression and inhibits MAPK with concomitant decrease in cell proliferation. However, let-7 over-expression fails to inhibit tumor growth and progression [60]. In addition to affecting *KRAS* expression in PC, miRNAs also modulate *KRAS* expression in breast and lung cancer. Kopp *et al.*, observed an inverse correlation between *KRAS* expression and miR-200c expression in a panel of breast cancer cell lines [61]. Furthermore, they also observed that miR-200c directly regulates *KRAS* activity and additionally affects other tumorigenic pathways inhibiting

tumor progression and resistance to therapy. Therefore, miRNAs definitely play a key role in modulating PC as they regulate the expression of key oncogenes such as KRAS which has been shown to be the initiating event for PC and important for tumor maintenance (Figure 1).

3. miRNA(s) and the p53 pathway

Inactivation of tumor suppressor genes is commonly observed in the multistep progression of various cancers. The Trp53 (p53), referred to as the guardian of the genome is located on chromosome 17p and plays an important role in controlling cellular responses involving cell cycle arrest and programmed cell death due to various physiological stress [62]. Wild type p53 binds to DNA and induces p21 expression to negatively regulate cell division-stimulating proteins including cyclin D1 and cyclin-dependent kinase-2 resulting in growth arrest [63]. However, more than 50% of adult human tumors have inactivating mutations or deletions in the p53 gene leading to loss of its function without affecting its translation [64]. Inactivation of p53 due to somatic mutations is observed in 50–75% of PDAC [65]. Gain of function mutations (R175, G245, R248, R249, R273 and R282) in the DNA binding domain of p53 leads to loss of its function associated with uncontrolled cell growth, cell survival, and genetic instability [64]. More precisely, R175/R248/R273 mutations of p53 are mainly observed at late PanINs with dysplasia and occurs mostly in single allelic loss coupled with an intragenic mutation of the second allele, leading to uncontrolled cell proliferation and inhibition of apoptosis [66]. These mutations may be structural affecting folding of the protein or they can affect p53-DNA interactions, thereby altering transcriptional regulation of its target genes [67–69]. However, mutant p53 recognizes and binds to transcription factors/cofactors/p63/p73 proteins that leads to transcriptional upregulation of genes involved in uncontrolled growth [70–73]. Besides *in vitro* studies, recently transforming capabilities of mutant p53 were successfully demonstrated using genetically engineered mouse models (GEMs) for various mutant p53 [65, 74, 75]. Recently, a co-operative association of mutant p53 (p53^{R172H}) and other genes was observed in cellular signaling pathways and cell fate decisions by regulating cell proliferation, motility and invasion, apoptosis, inflammation and angiogenesis either in the cancer cell itself or in the surrounding microenvironment during the tumorigenesis and metastasis [76]. In addition, the co-operative role of mutant p53^{R172H} and Kras^{G12D} has also been investigated during PC pathogenesis using GEM models [77].

3.1 p53 mediated regulation of miRNAs

Besides regulating proteins involved in cell cycle and apoptosis, wild type p53 has recently been shown to modulate expression of several miRNAs in cancer cells, while siRNA mediated knockdown of p53 abolished effects on miRNA expression [78]. Surprisingly, global sequence analysis has revealed that >46% of miRNA promoters have p53 binding sites suggesting that p53 can be a master regulator of miRNA expression [78] and subsequently other studies also showed transactivation of several miRNAs by p53 [79]. miR-34 family members are evolutionary conserved between species and are transcribed from two different gene loci [80]. While miR-34a is located at chromosome 1p36 and is expressed in wide variety of tissues, miR-34b and miR-34c are localized to chromosome

11q23 and mainly expressed in lung tissues [81]. Recently p53 has been shown to bind to p53 responsive elements on the promoters of miR-34 family (miR-34a/b/c) of miRNAs, thereby inducing cell cycle arrest and apoptosis [82–87]. Ectopic overexpression of miR-34 leads to G₁ phase cell-cycle arrest [82, 84, 87], inhibition of proliferation, colony formation [83], increased apoptosis [86–88], and induced cellular senescence in human diploid fibroblasts by targeting several of its putative target genes like *CDK4*, *BCL-2*, *Cyclin E*, *CDK6*, *c-MET*, *SIRT1*, *c-MYC*, *N-MYC*, *Axl*, *SNAIL* and *LDH* etc [84]. However, several cancers including PC harbor CpG methylation on promoters of miR-34a/b/c and this significantly inhibits their transcription by p53 [89, 90]. PC cells have a low or undetectable level of miR-34a [91], however their low/loss of expression was neither associated with loss of heterozygosity (LOH), p53 mutation, nor with promoter CpG methylation [82].

Several reports have shown that mutant p53 also regulates transcription of miR-130b, miR-155, and miR-205 and thus alter the expression of their target genes like ZEB1 and ZNF652 either by inhibiting/degrading translation of their mRNA transcripts, thereby influencing invasive and metastatic potential of cells [92–94]. The mutant p53 regulates these miRNAs in either p53 dependent (miR-155 and miR-205) or p53-independent manner (miR-130b) [92–94]. The miR-15a/16-1 family is another transcriptional target of p53 that is significantly down regulated in PC [15]. Ectopic expression of miR-15a was shown to down-regulate WNT3A and FGF7 and results in reduced survival and proliferation of PC cells [95]. Furthermore, the tumor suppressor functions of this cluster was confirmed both *in vitro* and *in vivo*, whereby it downregulated *ALDOA* and *TPI1* expression, affecting glycolysis and cell metabolism [15]. By inducing miR-145 expression, p53 downregulates c-MYC expression and impedes tumor growth both *in vitro* and *in vivo* [96]. However, miR-145 expression was significantly down regulated in PC tissues as compared to healthy control groups [43]. The p53 regulated miR-107 expression inhibits hypoxia inducible factor-1 β (HIF-1 β) expression, that on its interaction with HIF-1 α forms HIF-1 complex and induces genes that are involved in the pathogenesis of cancer [97]. However, miR-107 is up regulated in many solid tumors including PC [12, 97]. Besides PC, overexpressed miR-107 in breast cancer negatively regulates expression of tumor suppressor let-7 miRNA [97]. Ectopic expression of miR-107 destabilizes let-7, and thereby upregulates let-7 target genes that are involved in progression of breast cancer [97]. The miR-200 family members (miR-200c/141, miR-200a/b/429) inhibit expression of Zeb1 and Zeb2 transcription factors favoring mesenchymal to epithelial transition and reduced tumor growth [98, 99]. Transcription factor Zeb1, by upregulating Jag1, mastermind-like co-activators Maml2 and Maml3 promotes pancreatic cancer progression through notch signaling. p53 has been shown to induce expression of miR-200 family members accompanied by downregulation of Zeb1 and Zeb2 expression that resulted in decreased tumor progression. Furthermore, knockdown of p53 expression resulted in abrogation of miR-200 family members associated with up regulation of Zeb1 and Zeb2. However, Soubani *et al.* has recently shown that chemo-resistant PC cell lines have downregulated expression of miR-200 family members including miR-200a/b/c suggesting deregulated p53 signaling in these cells [100]. In addition, upregulated expression of Zeb1 and Jag1 was also associated with decreased miR-200 family members in PC [101]. Similarly Kim *et al.* has shown that p53 also induces the expression of miR-192 family members (miR-192/194/215) that target Zeb1 expression

and inhibit epithelial to mesenchymal transition and induces cell cycle arrest by targeting MAD2L1, CUL5, and CDC7 genes [98, 99, 102]. Contrary, expression of miR-192 was high in PDAC tissues as compared to normal pancreas [103]. Besides transcriptional regulation, p53 also regulates miRNA maturation post-transcriptionally by modulating the function of drosha that is involved in the processing of pri-miRNA to pre-miRNA in the nucleus. To achieve this process, it forms a complex with DEAD box RNA helicases p68 and p72 and interacts with drosha to process pri-miRNA to pre-miRNA (miR-16-1, miR-143 and miR-145) [104]. Significant downregulation of miR-145 and miR-16-1 as seen in PC [43, 95] speculates that point mutations like R175H/R273H in p53 may decrease the maturation of these miRNAs. Further, Dnmt3a directly interacts with p53 and inhibits p53 mediated transcription of p21 and other tumor suppressor genes in the genome [105].

3.2 miRNAs regulating p53 expression

Several *in silico* analysis tools have predicted potential binding sites on the 3'UTR of p53 mRNA for multiple miRNAs. Recently, A study revealed that ectopic expression of miR-491-5p in SW-1990 PC cells down regulates p53 expression suggesting that p53 not only has the potential to modulate miRNA expression, but in turn miRNAs can also regulate p53 expression for cooperative normal functioning of cells [106]. Besides effecting p53 expression, miR-491-5p overexpression also abrogated several signaling pathways like Jak/Stat3, PI3K/Akt accompanied by decreased cell proliferation and induction of apoptosis [106]. Tumor suppressor miR-34 cluster is also involved in inhibiting cell proliferation, migration, invasion, and epithelial to mesenchymal transition [89, 107]. It has been shown that p53, by up regulating miR-34a expression, represses NAD-dependent deacetylases that in turn increases acetylated p53 (lysine 382) and transcriptionally upregulates p21 and PUMA expression [108, 109]. Furthermore, miR-34a also represses HDM4, a negative regulator of p53 and therefore creates a positive feedback loop on p53 expression [110]. Therefore, any imbalance in this positive feedback mechanism decreases p53 activity accompanied by enhanced cell proliferation and cell survival. Unfortunately, due to CpG island promoter hypermethylation, miR-34s are frequently silenced in a variety of cancers including PC [107]. But ectopic expression of miR-34a in PC cells has been shown to induce cellular senescence and cell cycle arrest by targeting CDK6. Another miRNA, miR-214 is over expressed in PC [12] and is involved in cancer progression, metastasis and chemo-resistance [111, 112]. This overexpressed miR-214 targets p53 and relieves negative inhibition on Nanog expression thereby enriching the stem cell population in ovarian cancer cells [113]. While miR-125b is over expressed in PC [33], it negatively regulates p53 expression during stress, its ectopic expression also downregulates p53 expression and inhibits apoptosis in lung fibroblast and human neuroblastoma cells [114].

The p85 α is a regulatory subunit of the PI3K complex that negatively regulates p53 expression and plays a critical role in cell survival and apoptosis [115]. However, miR-29 mediated p85 α downregulation has been shown to activate p53 signaling pathway and induces apoptosis in Hela cells [116]. Interestingly, overexpression of miR-29a/b/c was observed in PC tissue and the PC cell lines MiaPaCa-2, PANC-1, and BxPC-3 as compared to normal tissues and HPDE cells [47]. In contrast, miR-504 downregulates p53 expression and inhibits p53 mediated apoptosis, cell cycle arrest that leads to tumor promotion *in vivo*

[117]. miR-122 by modulating cyclin G₁/PP2A and MDM2 expression activates and stabilizes p53 protein expression in hepatocellular carcinoma cells [118] but it is significantly down regulated in PC [43]. Whereas other miRNAs like miR-33, miR-1285, miR-380-5p, miR-25, miR-133 and miR-30d directly bind to the 3'UTR of p53 and repress its expression [119], out of which miR-133b is down regulated, whereas miR-25 is overexpressed in mouse PC [120]. P53 also modulates MDM2 by negatively regulating miRNAs such as miR-192/194/215 and miR-605 as a feedback loop mechanism to check p53 activity physiologically [121, 122], however, miR-192 is significantly upregulated in the PC patients [103, 123]. The overall role of p53 mediated miRNA transcription and its regulation by miRNAs is shown in Figure 2.

4. p16 and miRNA(s) in pancreatic cancer

The INK4A/p16, also known as cyclin dependent kinase inhibitor 2A (CDKN2A) is a tumor suppressor gene involved in cell cycle regulation and cellular senescence [124, 125]. However, promoter methylation, missense mutation and deletions of the p16 gene leads to its inactivation in most of the cancers including PC where its function is lost in around 95% of cases [126]. In addition, miRNA mediated regulation of p16 has been documented in many cancers including PC. Recent studies have shown that miR-24 was up regulated in PC [11, 127], and other study have shown that it binds to both the coding and 3'UTR regions of p16 mRNA that results in its translation inhibition associated with decreased cell proliferation [128]. Similarly, miR-10b, also overexpressed in PC [129], post-transcriptionally regulates p16 expression by binding to its 3'UTR region, whereas inhibition of this miRNA in glioblastoma resulted in inhibition of tumor growth through activation of cell cycle arrest and apoptotic pathway [130]. In addition, several other miRNAs like miR-128a upregulate p16 expression by targeting its negative regulator Bmi-1, resulting in decreased growth of medullablastoma cells [131]. Similarly, miR-20a also upregulates p16 expression and inhibits tumorigenesis [132]. DNA methyltransferase (DNMT1) has been shown to negatively regulates p16 expression, and silencing of its regulators like miR-148a and miR-152 leads to decreased p16 expression and enhanced cholangiocarcinoma growth [133]. Not only do miRNAs target p16 expression, indeed p16 signaling has been shown to regulate expression of miRNA(s). In this context, ectopic overexpression of p16 in breast and glioma cells increased miR-410 and miR-650 expression and reduced CDK1 protein levels [134]. In addition, UV induced DNA damage initiates association of p16 with sp1 transcription factor and CDK4 (cyclin dependent kinase) causing subsequent upregulation of miR-141 and miR-146b-5p [135].

5. TGF- β /SMAD signaling regulates miRNAs in PC

SMAD proteins are the backbone of the transforming growth factor beta (TGF- β) family of cytokines [136]. Binding of ligand to TGF- β Receptor II (TGF β RII) results in its heterodimerisation with TGF- β Receptor I (TGF β RI) with their subsequent trans-phosphorylation and activation of their substrate SMADs. The human genome encodes eight different SMAD proteins among which SMAD-1, SMAD-2, SMAD-3, SMAD-5 and SMAD-8 are regulated by the TGF- β family of receptors commonly referred as R-SMADs. A common SMAD protein, SMAD-4 is a co-mediator that translates extracellular signals

into response. Activated SMAD-2 and/or SMAD-3 heterodimerises with SMAD-4 protein and translocates into the nucleus, along with other co-regulators to engage in transcriptional regulation of various target genes. Whereas, SMAD-6 and SMAD-7 are inhibitory SMADs (I-SMADs), which act as a negative regulator of R-SMADs [137]. Previous studies have shown the existence of signaling cross talks between SMAD proteins and other non-SMAD pathways such as extracellular signal related kinase (ERK), MAPK, protein kinase C (PKC). These intracellular kinases can phosphorylate R-SMADs at potential serine residues preventing accumulation of R-SMADs in the nucleus [138]. In a normal epithelial cells, TGF- β acts as a potent tumor suppressor eliciting growth inhibition through downregulating transcription factor c-Myc and up regulating cell cycle inhibitor proteins such as p15 and p21 [139, 140]. In cancer cells, the TGF- β /SMAD signaling is modulated and loss of some components of the TGF- β /SMAD pathway leads to impairment of TGF- β /SMAD mediated growth arrest [141].

Several *in vitro* and *in vivo* findings have revealed that a variety of miRNAs are controlled and modulated under the influence of R-SMAD proteins [142–145] and the TGF- β signaling pathway. For example, TGF- β signaling induces maturation of miR-21 through various post transcriptional modifications of primary miR-21 into mature miR-21 through a microprocessor complex consisting of DROSHA and RNA helicase p68. Thus ligand specific activation of SMAD protein may be involved in the biosynthesis of miRNAs such as miR-21, which in turn downregulate the tumor suppressor genes PDCD4 and PTEN, enhancing tumor invasion and metastasis [144]. Kong *et al.* have demonstrated that TGF- β /SMAD-4 signaling can induce miR-155 expression and its restoration inhibits cell migration and invasion in normal murine mammary epithelial cells [145]. Naito *et al.* have identified the presence of increased miR-143 expression in scirrhus type gastric cancer but not in non scirrhus type gastric cancer specimens. Further, several studies have revealed that miR-143 is expressed mainly by stromal fibroblast cells, not by gastric cancer cells and this expression is under the influence of TGF- β /SMAD signaling activation [146]. Recently, a functional screening approach was done to identify miRNAs that can regulate SMAD-4 expression in gastric cancer cells. Using a luciferase reporter assay supported by bioinformatics analysis they identified that miR-199a directly targets SMAD-4 expression, suggesting the implication of miR-199a as a negative regulator of TGF- β /SMAD signaling [147]. In addition, Liu *et al.* have shown that the 130a/301a/454 of family of miRNAs regulates TGF- β signaling by repressing the SMAD-4 expression by directly binding to its 3'-UTR sequence [148], further, this cluster is upregulated in PC [149, 150]. Among the SMAD family of tumor suppressor genes, SMAD-4 (DPC-4) is frequently inactivated in pancreatic, bile duct adenocarcinoma, and colorectal cancer. Studies have explored that SMAD-4 gene is inactivated in 55% of PC patients either by deletion in both the alleles (35%) or by intra-genic mutation in one allele coupled with LOH (20%) [151]. In the classical PC progression model, SMAD-4 gene inactivation occurs at the later stages of PanINs, thus loss of SMAD tumor suppressor was correlated with disease advancement or metastasis [152]. A recent study demonstrated that co-expression of SMAD-6 and/or SMAD-7 along with SMAD-4 in patients with PDAC could be one possible mechanism of SMAD-4 inactivation [153].

Traditional small molecular inhibitors like SD-208, SB431542 and LY-2157299, specific for TGF- β receptor abrogated SMAD mediated responses like its anti-invasive effect in PC cells [154] and inhibited cancer advancement and metastasis in xenograft mouse models [155]. Treatment of mouse preantral granulosa cells with recombinant TGF- β leads to upregulation of miR-224. Further this upregulated miR-224 was diminished upon treatment of above cells with SB-431542 (a TGF- β type I receptor inhibitor), indicating that anti-TGF- β therapies could block the canonical TGF- β /SMAD signaling pathway and their respective miRNA biogenesis and regulation [156]. miRNAs play a critical role in cell mediated immune response by controlling the dendritic cells function in cancer patients. Recently, Du *et al.* identified that miRNA-146a is aberrantly expressed in human CD14+ monocyte-derived dendritic cells from PC patients *in vitro*. Further, miR-146a expression is correlated with impairment of differentiation and inhibition of the antigen presentation function of dendritic cells through the mechanism of SMAD-4 repression. Thus, miRNAs are one of the factors involved in the regulation of immune response indirectly by modulating dendritic cells [157]. Interestingly, overexpression of other miRNAs can also directly influence SMAD-4. The miR-483-3p and miR-421 are involved in PC progression by directly regulating the tumor suppressor DPC4/SMAD4. Specifically, the expression level of miR-421 is inversely correlated with DPC4/SMAD-4 expression in PC specimens. Through various *in vitro* studies, it has been identified that miR-421 can also play a dual role by targeting DPC4/SMAD4 along with its downstream effectors p21 and p15 in PC [158, 159]. More recently, Li et al had identified the negative regulatory role of miR-494 on transcriptional activator FOXM1 on pancreatic cancer cells. Clinically, miR 494 expression levels were demonstrated to be higher in adjacent normal pancreatic tissues compared to PDAC specimens, suggesting that reduced expression of miR 494 as a critical factor in pancreatic cancer progression and development. By restoration of miR-494, the authors have showed decreased pancreatic cancer cell growth and metastasis through various *in vitro* and *in vivo* assays. Finally, they correlated the reduced expression of miR-494 with increased expression of FOXM1 and enhanced nuclear translocation of betacatenin. They also identified the molecular mechanism and association of SMAD4/miR-494/FOXM1/Betacatenin signaling cascade and its biological impact on pancreatic cancer pathogenesis. In particular, miR-494 is the novel microRNA identified with tumorsuppressive function in PDAC [160]. Along similar lines, miR-182-5p was reported to be overexpressed in bladder cancer, which could potentially target SMAD-4 expression. The repressed SMAD-4 can influence nuclear transport of β -catenin thereby resulting in activation of WNT signaling cascade in cancer cells [19]. Also, another study by Geraldo *et al.* has shown that ectopic overexpression of miR-146b-5p in rat follicular thyroid carcinoma cells resulted in significant increase in cell proliferation and development of resistance to TGF- β mediated cell cycle arrest. Thus, miR-146b-5p regulates the TGF- β mediated signaling pathway by repressing SMAD4 in thyroid cancer cells [161]. Overall these studies provide evidence that SMAD proteins are (i) involved in miRNA biogenesis, (ii) lost in expression in various cancers, and (iii) downregulated by miRNAs (Figure 3).

6. Sonic Hedgehog signaling and miRNA activation in pancreatic cancer

The Sonic Hedgehog (SHH) signal transduction pathway is critically important during embryonic development and is activated by binding of Shh to its cognate Patched-1 (PTCH1) receptor. Following PTCH1 activation, transmembrane G-protein coupled receptor (GPCR)-like protein Smoothened (SMO) is freed and activated resulting in nuclear translocation of the GLI1 family of transcription factors for target gene activation [162]. In addition, through GLI1, SHH mediated cellular responses are also coordinated through MAPK and the phosphatidylinositol-3 kinase (PI3K) signaling cascades for enhanced proliferation and cell survival [163]. Besides being re-activated in injured adult tissues, SHH signaling is also aberrantly activated in several malignancies promoting resistance to chemotherapeutics. While there is no expression in normal pancreas, Thayer *et al.* have reported aberrant expression of SHH in 70% of PC specimens and in precursor PanIN lesions (PanIN1 to PanIN3), suggesting its importance in the initiation and maintenance of PC pathogenesis [164]. SHH also contributes to the acceleration of desmoplastic response in PC [165]. Earlier studies have shown that miRNAs regulate SHH signaling either by targeting proteins upstream of SHH or by directly targeting the components of SHH pathway members. For example, miR-196 and miR-452 by regulating Shh signaling effects limb development [8] and epithelial to mesenchymal transition in enriched neural crest cells respectively [166]. In addition, the miR-17~92 cluster (miR-19a, miR-20 and miR-92) was overexpressed and coordinated with the Shh signaling activation in murine and human brain cancer (medulloblastoma) [167]. Specific to PC, synthetic miR-3548 that targets GLI1 transcription factor resulted in effective inhibition of cell division and cell proliferation [168]. Not only do miRNA regulate SHH signaling, SHH signaling also effects miRNA expression, as evidenced by the fact that use of pharmacological hedgehog inhibitor vismodegib (GDC-0449) upregulates the expression of miR-200b and let-7c that results in reversal of EMT phenotype and attenuation of drug resistance in NSCLC cells [169]; whereas cyclopamine, a SHH inhibitor in PC cells significantly inhibited expression of let7, miR-34, miR-107, miR-125, miR-128, miR-130, miR-132, and miR-141 [170]. Therefore, identification of a complete miRNA profile that exist to regulate various components of SHH signaling, will be helpful in identifying the central mechanism of sustained SHH activation in cancer cells. The miRNAs identified through this mechanistic approach would be suitable candidates for therapeutic targets in pancreatic and other cancers.

7. The impact of miRNAs on cell cycle and proliferation of pancreatic cancer cells

Deregulated cell proliferation due to aberrant regulation of cyclins and cyclin dependent kinases (CDKs) is a major hallmark of cancer [171]. An efficient way through which cancer cells manipulate cell cycle regulatory proteins is by altering the miRNA(s) expression. Usually, miRNAs that downregulate cyclins, CDKs and other cell cycle progression proteins are silenced, whereas miRNAs targeting inhibitors of cell cycle progression are over expressed in lung cancers and PC [172–174]. Sequence homology analysis of seed regions of several differentially regulated miRNAs and 3' UTRs of mRNA indicate that the above strategy is employed by many cancers [175]. In PC, proliferation and cell cycle regulatory

proteins like CCND1, CDKN1A (p21 WAF1), CDKN1B (p27 Kip1), E2F1, Rb1, CDK4, CDK6, CDKN1C (p57 Kip2), ABL-1, EZH2, c-Myc, KRAS, CDC25B, PTEN, LATS2, Spry2, RREB1, AKT and STAT3 are all affected by aberrantly regulated miRNA [48, 55, 60, 90, 172–191]. Deregulation of these proteins leads to more rapid progression through the cell cycle and intern accelerated cell proliferation through various signaling pathways. For example miR-34a that downregulates CCND1, CDK4 and CDK6 is frequently lost in PC resulting in increased cell proliferation [173, 174]. CDK6 is also targeted by miR-107, which like several other miRNAs is epigenetically silenced in PC cells [172]. The miR-148a is also frequently downregulated in PC and targets the CDC25B phosphatase that otherwise activates CDK1/cyclinB complex for increased cell proliferation [185]. Conversely PC cells aberrantly upregulate miRNAs that target inhibitors of cyclins and CDKs [175]. Over expression of miR-106 targets p21 thereby preventing the G1 cell cycle block for uninterrupted cell cycle progression [183]. Similarly miR-221/222 and miR-106b family miRNAs allows cancer cells to progress through the cell cycle by down regulating CDKN1B (p27 Kip1) and CDKN1C (p57 Kip2) circumventing the restriction point [179, 181, 183, 189]. Inhibition of TGF- β /SMAD-4, p16, p53, and others tumor suppressors expression by miRNAs is a tactic frequently employed by PC to deregulate cell proliferation [48, 175, 188]. Overexpression of the miR-106b-25 cluster was reported in various cancers including PC and this cluster impairs the TGF- β signaling by targeting its downstream effectors like cell cycle inhibitor CDKN1A (p21) and the proapoptotic gene BCL2L11 (BIM) [192] leading to increased cell proliferation. Further, this cluster also activates TGF- β signaling by targeting SMAD7, which result in a tumor promoting role of TGF- β [193]. However, p53 can also have gain of function mutations that can increase cell proliferation and other tumorigenic traits; in this case miRNA against p53 can have beneficial effects [65, 194, 195]. Aberrantly expressed miR-132 and miR-212 suppress Rb1 expression in PC, allowing E2F transcription factor to activate the transcription of several cyclins for enhanced cell cycle progression [48]. In addition, overexpressed miR-21 significantly downregulates PTEN expression allowing uncontrolled PC cell proliferation. This downregulation of PTEN was reversed upon antisense inhibition of miR-21, suggesting a useful therapeutic approach to combat PC [48, 175, 188]. Overactive oncogenes such as KRAS commonly drive cell proliferation in PC, and the miRNAs (miR-217, miR-96, miR-126, and miR-143/145 cluster) capable of downregulating KRAS, are commonly downregulated in PC [55–58]. Key players from aberrantly overexpressed oncogenic pathways alter miRNA expression. Overexpressed Notch-1 in PC induces EMT by upregulating miR-21 and downregulating expression of Let-7a, Let-7b, Let7c, miR-200b and miR-200c [176].

8. Potential role of miRNAs in pancreatic cancer diagnosis

Traditionally, PC diagnosis and staging is performed by using tissues biopsies and ultrasound guided FNAs. Serum CA19.9 (carbohydrate antigen 19.9) is the only gold standard biomarker approved by Food and Drug administration for PC, but it lacks specificity and sensitivity. Although widespread research has been focused to identify early detection markers, none has yielded a biomarker specific to PC. Because of high stability, tissue specificity and ease of availability of miRNAs, recent studies are focused on performing miRNA expression profiling of PC tissues, serum/plasma, and other body fluids

to identify suitable biomarker(s) for early diagnosis of this deadly disease. Several studies have revealed that altered miRNA expression profiles in various cancers, and their association with various clinical parameters including disease progression, response to therapy, survival, lymph node metastasis etc. and their utility as prognostic and diagnostic markers [33, 46, 47, 47, 103]. Therefore, it is very important to accurately determine the miRNA expression profile with high specificity and sensitivity in a given tissue or specific cell type. But accurate profiling of individual miRNAs with high specificity and sensitivity is technically challenging as mature miRNAs are very short (20–25 bp), differ in their GC content, lack mutual sequence features in mature miRNAs like poly-A tails and single nucleotide difference within miRNA families. However, recent rapid technological advancements like deep sequencing/parallel sequencing [196], oligonucleotide microarrays [197–200], northern blotting with radiolabelled probes [201], qPCR-based detection of mature miRNAs (TaqMan assays) [202–204], single molecule detection in liquid phase [205] and *in situ* hybridization [206, 207] have made it feasible to profile miRNAs in various tissue/cell types with high accuracy. miRNA microarray platform is a high-throughput method to compare differential miRNA expression profiles between the disease and the control states, whereas, deep sequencing methods involves generation of cDNA libraries from small RNA population followed by deep sequencing using different methods [208, 209]. The advantages of deep sequencing includes (i) its inexpensive and helps in the discovery of novel miRNAs, (ii) detects mature miRNAs with variation in length as well as modifications [210] and (iii) helps to identify low-abundance miRNAs with modest expression differences [208, 211–213]. Differentially expressed miRNAs as identified by either microarray or deep sequencing and are further validated by real time Q-PCR using TaqMan probed assays with high sensitivity and specificity. In addition, *in situ* hybridization is used to view and localize miRNAs within cells and also help us to pinpoint their distribution in tumor/stromal cell compartment/cell type-specific expression [204]. The advantage of using northern blot techniques is that it does not require technical knowledge and special equipment but the disadvantage of this technique is poor sensitivity and more time consumption. But less time consumption and high specificity of miRNA detection was reported with the use of LNA-modified oligonucleotides [214].

Several studies have shown differential expression of miRNAs in body fluids of PC patients compared to normal healthy controls [50, 215, 216]. Using miRNA microarray analysis, a cohort of three miRNAs (miR-642b, miR-885-5p, and miR-22) were identified from blood that can identify early stage PC patients from healthy control group with a sensitivity and specificity of 91% [AUC= 0.97 ($p < 0.001$)] [217]. Similarly, Frampton *et al.* also identified a signature of seven upregulated (miR-21, -23a, -31, -100, -143, -155, and miR-221) and three downregulated (miR-148a, -217 and miR-375) miRNAs that can identify PC patients from healthy controls [218]. Furthermore, overexpression of miR-21 and miR-31, and downregulation of miR-375 in tumor tissues were associated with poor PC patient survival [218]. Recently, Danish BIOPAC (Biomarkers in patients with Pancreatic Adenocarcinoma) identified 2 diagnostic panels of circulating miRNAs (panel I: miR-145, miR-150, miR-223, miR-636 with sensitivity of 85% and specificity of 64% with AUC of 0.86; while panel II includes miR-636, miR-26b, miR-223, miR-122, miR-150, miR-145, miR-505 miR-34a, miR-885.5p, miR-126* having sensitivity and specificity of 85% with AUC of 0.93. Both

these panels can significantly distinguish PC patients from healthy individuals [219]. Li *et al.* analyzed expression profile of 735 miRNAs in the serum and identified miR-1290 that distinguished PC patients from healthy controls with sensitivity 81% and specificity of 80% with AUC of 0.96 [220]. Furthermore, they observed that increased serum miR-1290 was better than CA19.9 in distinguishing early stage PC patients from controls [220]. Additionally, combination of CA19.9 with miR-27a-3p [221] and miR-16 and miR-196a [222] expression can also precisely discriminate PC patients from healthy controls. In a similar study, a panel of seven miRNAs (miR-24, miR-20a, miR-25, miR-21, miR-185, miR-99a, and miR-191) in serum was used to accurately diagnose PC patients from the healthy individuals, and can differentiate different stages of PDAC patients (stage I patients with 96.2% and Stage II with 91.7%) with high sensitivity and specificity [223]. Similarly, increased levels of serum miR-192 can distinguish PC from healthy controls with a sensitivity of 76% and specificity of 55% [103]. In addition, overexpression of miR-192 in PANC-1 PC cells results in an increase in cell proliferation, migration, inhibition of apoptosis along with induced the progression of cell cycle from the G₀/G₁ to the S phase [103]. Plasma miR-221 level was significantly higher in PC patients and was used as indicative of non-resectable status of the PC patients and distant metastasis [225]. In a similar study, a panel of seven miRNAs including miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191 in serum can accurately (83.6%) diagnose PC patients [223]. In addition to body fluids, Bauer *et al.* reported that deregulated miRNAs from tissues can discriminate between PC and pancreatitis [226]. Analysis of fine needle aspirations (FNAs) revealed that increased expression of miR-196a and miR-217 can accurately discriminate PC from benign pancreatic lesions with a sensitivity of 90% and specificity of 100% [227]. Similarly, down regulation of let-7 was observed in the FNA from PC patients [60]. In addition, many other studies have reported significantly higher serum concentration of miR-210, miR-18a, and miR-155 in PC patients compared to healthy controls [50, 215, 216] and concentration of blood circulating miR-155, miR-21 and miR-210 was significantly higher in rats with PC [228]. Although, differentially expressed miR-27a-3p in PBMCs can sufficiently discriminate PC from benign pancreatic/peri-pancreatic diseases (BPD) with a sensitivity of 82.2% and specificity of 76.7% (AUC= 0.840), combination of miR-27a-3p expression in PBMC and CA19.9 serum levels had significantly higher diagnostic accuracy than either of them alone with a sensitivity of 85.3% and specificity of 81.6% (AUC= 0.886) [221]. However, it has been shown that blood based miRNA expression profiles could not distinguish pancreatitis from PC [226].

Recently, a global miRNA expression study in pancreatic juice (exocrine pancreatic secretions) from 6 PC and 6 non-pancreatic, non-healthy (NPNH) patients was performed using miRNA microarray. The top differentially expressed miRNAs (miR-205, miR-210, miR-492, and miR-1247) were further confirmed in pancreatic juice from 50PC, 19 CP patients and 19 non-pancreatic, non-healthy (NPNH) controls, which showed marked difference in their expression from PDAC patients compared to those without pancreatic disease. These four miRNAs can diagnose PDAC with a sensitivity and specificity of 87% and 88% respectively, but inclusion of CA19.9 enhanced the sensitivity and the specificity to 91% and 100% respectively. Over expression of the above mentioned four miRNAs in pancreatic juice was further associated with reduced overall survival (OS) and lymph node

metastasis [229]. Not only in humans, PC bearing rats also showed increased blood levels of miR-155, miR-21 and miR-210 compared to normal control [228]. Similarly, high levels of circulating miR-196a and -196b was observed in KPC (Kras^{G12D};Trp53^{R172H};Pdx1-Cre) mice with PanIN2/3 lesions as well as PDAC as compared to contemporary littermate control or KPC mice with PanIN1 lesions. Further, expression of these miRNAs were significantly higher in the serum of PC patients with sporadic/hereditary or individuals at risk (IAR) with multifocal PanIN2/3 lesions compared to patients with neuroendocrine pancreatic tumors or chronic pancreatitis, IAR with PanIN1 or no PanIN lesions and healthy controls with a sensitivity of 100% and specificity of 90% (AUC = 0.99). However, it was observed only in few samples, therefore, further validation of miR-196a and -196b should be carried out to test their utility in PC diagnosis [230]. A meta-analysis study was carried out on 18 articles with a total of 2,036 patients and 1,444 controls to determine the role miRNAs in early diagnosis and reported that pooled sensitivity of 82 % (95 % CI, 78–86 %); and specificity of 77 % (95 % CI, 73–81 %) with AUC of 0.86 (95 % CI, 0.83–0.89), suggesting the potential diagnostic value of miRNAs (inclusion of multiple miRNAs for diagnosis) to discriminate PC patients from healthy controls with high sensitivity and specificity [231].

9. miRNAs as therapeutic agents in pancreatic cancer

Contribution of miRNAs in the initiation and progression of PC suggest that miRNAs can be exploited for the development of novel therapeutic strategies. The strategies followed to develop miRNA based therapies are similar to that of other gene targeted therapies. The main rationale for developing miRNA based therapies over targeting a single oncogene is due to the ability of a single miRNA to target many genes and signaling pathways at once. Therefore, targeting of a single miRNA can produce dramatic results due to its ability to affect numerous cellular processes. Primarily, miRNA targeted therapeutics can be classified into miRNA mimics and miRNA antagonists. The miRNA mimics double-stranded RNAs that are chemically modified *in vitro* and used to restore the expression of miRNAs that are lost during the disease process and functionally mimic the endogenous miRNAs. On the other hand, miRNA antagonists are developed to target endogenous miRNAs that have shown a gain-of-function in diseased tissues. Previously, we have extensively discussed various methods that are used to deliver miRNA based therapeutics [233], however here we will briefly describe some currently used methods to deliver miRNA therapeutics.

The miRNA antagonists are chemically synthesized single stranded oligonucleotides complementary to specific a miRNA, with specific chemical modification (2'-O-methoxyethyl (2'-MOE) and 2'-fluoro (2'-F) and locked nucleic acid (LNA) chemistries) [234–239]. These modifications provide extreme stability, high binding affinity to target miRNAs, resistance to nuclease degradation, leading to the sequestration of target miRNA within the RISC, inhibiting it from being processed or degraded. These modified antisense oligonucleotides (2'-MOE, LNA, and morpholinos) are utilized to antagonize miRNA activity and its processing. These oligos are frequently complementary to the miRNA guide strand, blocking its activity. They can often produce similar effects by sterically blocking only the seed region due to increased binding strength. These oligos can also antagonize miRNAs by interfering with miRNA processing by binding to the Drosha and Dicer guide sequences.

A previous study has shown that successful targeting of miR-122 in liver by intravenous injection of antagomir, regulates genes essential for cholesterol biosynthesis, leading to a decline in concentration of miR-122 and reducing circulating levels of cholesterol [240]. Similarly, cardiac fibrosis and hypertrophy was significantly reduced after targeting miR-21 and miR-133 respectively [241, 242]. Further, chimpanzees chronically infected with Hepatitis C were protected against viremia and liver pathology after targeting miR-122 with the LNA-antimiR [243]. The major drawbacks of anti-miR oligonucleotides are off target effects and affect endogenous RNA species other than the intended target miRNAs. However, these off target effects can be minimized by conjugating antagomir with ligands for target organ/specific cell surface receptors. Besides antagomirs, several studies have successfully employed small molecule (low molecular weight compounds) inhibitors to target miRNAs such as miR-21, miR-122 and miR-27a [232, 244, 245] *in vitro*. Recent study has shown that morpholinos are capable of antagonizing miRNA by binding to the miRNA response element. They are totally immune to nucleases and at appropriate concentrations can only interfere with translation by binding to the 5' end of mRNA near the start codon. This approach is useful when a miRNA has both pro-cancer and anti-cancer targets simultaneously, because at appropriate lengths it can protect key anti-cancer miRNA targets while not interfering with the downregulation of the pro-cancer targets. In order to reach effective concentrations of synthetic oligos (*in vivo*) at the target cells, it is beneficial to conjugate them to cholesterol or arginine rich peptides to increase cellular uptake and diffusion out of endosomes [246]. A preclinical study involving administration of anti-miR-221 modified with cholesterol significantly enhanced liver tissue distribution, inhibiting tumor growth leading to increased survival compared to unmodified anti-miR-221 in a mouse model of hepatocellular carcinoma [247]. Similarly, substantial inhibition of tumor metastasis was achieved after therapeutic targeting of miR-10b with cholesterol-modified anti-miR-10b in a mouse mammary tumor model [248]. Another alternative method of antagonizing miRNA is the miRNA sponge, produced by a transgene. miRNA sponges have 4–10 miRNA binding sites with small spacer nucleotides in-between [249]. Efficient miRNA sponges are designed with imperfect complementarity; this hinders sponge degradation and results in longer miRNA sequestration. Sponges are typically complementary only to the seed region allowing targeting of an entire family of miRNAs. Silencing the miRNAs in miR-17-92 cluster by employing miRNA sponges harboring multiple binding sites for these family members demonstrates the proof of principle for this approach [250]. In addition, additional methods are being investigated, such as, small molecules that interfere with dicer function [232].

miRNA mimics are identical, or near identical to the guide strand of endogenous miRNA. Double stranded oligos are 100–1000 fold more effective than single stranded ones at mimicking miRNA, as they are present as duplexes of the guide and the passenger strand [251]. Although the passenger strand is usually modified with cholesterol at the 3' end to increase cellular uptake [252], miRNA mimics are still susceptible to nuclease degradation, can be targeted by the innate immune system, and also affect non target tissue. In light of the above, miRNA mimics have limitations with regard to therapeutic applications. To further enhance therapeutic delivery, lipid based delivery systems have been developed. Liposomes of smaller diameter (<100nm) allow for a high drug to lipid ratio. Liposomes have been

successfully used to deliver miR-34a mimic intravenously in a mouse model of non-small cell lung carcinoma resulting in reduction of tumor growth, while not having significant renal or hepatic toxicity or triggering an immune response [253]. In order to develop precise targeting of anti-cancer miRNAs, tumor specific promoters can be utilized to specifically express the reintroduced miRNA in the tumor cells [254]. An example of this is the T-VISA-miR-34a construct that utilizes the human telomerase reverse transcriptase promoter, which is activated only in cancerous tissue, to express miR-34a [254]. As with other gene therapies, adeno-associated viruses can be used to enhance miRNA construct delivery [255]. Adeno-associated viruses have different serotypes that have differing affinity for various organs aiding in the targeting of miRNA, and has already proven effective in various murine cancer models [255].

Pancreatic cancer

Some preclinical studies have shown that targeting mature miRNAs or their precursors by using synthetic or chemically modified antisense oligonucleotides, and/or overexpression of some miRNAs in PC results in decreased tumor burden [207]. The oncomir miR-21 is over expressed in the early stages of PDAC [256], and it has been shown that PC cells are addicted to miR-21 [257]. Lentiviral based targeting of miR-21 by its antagonists resulted in increased angiogenesis, strong inhibition of PDAC derived cell lines proliferation both *in vitro* and *in vivo* as well. Further, combinational treatment involving miR-21 antagonists and gemcitabine (125 mg/kg) leads to significant regression of tumor growth *in vivo*. Therefore, delivery of miR-21 antagonists may be a useful therapeutic intervention in several cancers including PC because of its up regulation in multiple cancer types [258]. The phase-I clinical trial (NCT01274455) involving 24 PDA patients with advanced disease treated with intra tumoral injection of CYL-02 (Gene therapy product) using non-viral vector mediated delivery such as endoscopic ultrasound followed by gemcitabine treatment showed the feasibility and safety of intra tumor injection. A recent study has shown that co-delivery of a nanosystem comprising of anti-miR oligonucleotide and human serum albumin-1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine:cholesterol resulted in effective silencing of individually overexpressed miRNAs (miR-21, miR-221, miR-222, and miR-10) in PC cells leading to upregulation of their target genes. Further, treatment of PC cells with the above nanosystem containing anti-miR-21 and chemotherapeutic drug sunitinib has produced a strong synergistic antitumor effect, therefore, the above combination therapy may have potential therapeutic value in PDACs [259]. Arora *et al* developed a poly (D, L-lactide-co-glycolide) (PLGA)-based nanoformulation of miR-150 (miR-150-NF) having high encapsulation efficiency (~78%) and sustained release capacity. When PC cells were treated with miR-150-NF, it resulted in effective intracellular concentration of miR-150 mimics leading to down regulation of its target gene (MUC4), which resulted in the inhibition of cell growth, clonogenicity, motility, and invasion [260]. Further, intra-peritoneal injection of LNA-based miR-21 antagonist resulted in down regulation of oncomir miR-21 leading to decreased splenomegaly in mice with systemic lupus erythematosus [261]. In contrast, over expression of oncogenic miR-21 and miR-221 enhanced the malignant phenotype of PC cells [187]. However, inhibition of miR-21 and miR-221 with the use of antisense oligonucleotides resulted in decreased proliferation and increased apoptosis of PC cells by increasing expression of PTEN, RECK, and p27 [187]. In

combination with gemcitabine, use of antisense oligonucleotides also synergistically killed PC cells [187]. In addition to oligonucleotides, targeting miR-10a expression by using retinoic acid receptor (RAR) antagonist repressed PC metastasis [209].

Over expression of miR-143 [208] and the viral mediated delivery of miR-143 or miR-145 resulted in decreased PC metastasis and reduced tumor formation in PC cells [57]. Restoration of tumor suppressor miRNAs like let-7, miR-96, and miR-150 in PC cells led to decreased tumorigenesis and cell proliferation [60]. Furthermore, restoration of let-7 led to KRAS downregulation and decreased mitogen-activated protein kinase activation [60]. The use of oncogenic miR-34 mimics or its virus mediated infection led to apoptosis, cell cycle arrest, decreased clonogenicity, invasion, and increased chemo/radiation sensitivity of PC cells by targeting Bcl-2 and Notch1/2 [210]. Several studies have shown that Let-7 and miR-34 are significantly down regulated in various solid tumors as well as in cancer stem cells. Administration of let-7 and miR-34 resulted in changes in cancer stem cell phenotypic properties. Therefore, therapeutic delivery of let-7 and miR-34 may result in decreased tumor growth as well as number of viable cancer stem cells [262, 263]. Further, restoration of let-7 either in the form of a let-7 mimic or a viral mediated therapeutic delivery resulted in significant reduction of tumor growth xenograft model for human non-small cell lung cancer as well as in the Kras^{G12D} transgenic mouse model [264]. Similarly, restoration of Let-7b by intra-nasal inhalation of adenovirus carrying Let-7b in a spontaneous Kras model for lung cancer led to a significant decrease in tumor growth compared to mice injected with only the vector [265], whereas, intra-tumor injection of Let-7 carrying plasmid into xenograft tumors produced by orthotopic implantation of Capan1 cells did not show any change in tumor growth/proliferation compared to control mice [60]. Growth of prostate and lung cancer was significantly inhibited after systemic delivery of a miR-34 mimic in lung and prostate cancer mouse models [266, 267]. Several studies have also revealed that administration of tumor suppressor miRNAs mimics in mouse models revealed no toxicity in normal tissues, and therefore are well endured [255, 265, 268]. Further, delivery of miR-34a mimics in combination with lipid-containing formulation also did not elicit any non-specific immune response [267]. All of these studies provide a rationale to use miRNAs for therapeutic intervention of lethal PC.

Not only in PC, several miRNAs including miR-208/499/195 are currently under development as therapeutic agents [269] in the area of cardiovascular disease. miR-208 is a heart muscle specific miRNA and it stimulates hypertrophy of cardiomyocyte, fibrosis and β -MHC expression under stress and hypoxia conditions. The miR-208 knockout mice were shown to be highly resilient to cardiomyocyte hypertrophy and fibrosis due to stress or hypoxia, therefore, targeting of miR-208 may significantly ameliorate the chronic heart disease [270]. Recently, miRagen Therapeutics in collaboration with Danish Santaris Pharma developed a major LNA-based anti-miR-208 therapeutic (MGN-9103 antimir) to target miR-208 expression for treatment of chronic heart failure. In addition, they also developed two more LNA-based antagomirs to target miR-15, miR-195 (MGN-1374 antimir) and miR-451 (MGN-4893 antimir) for the treatment of myocardial infarction [271, 272] and polycythemia vera [273] respectively. With another miRNA miR-195, which is overexpressed in cardiac hypertrophy, the transgenic mice over expressing this miRNA

resulted in cardiac failure and death of the animal [270], therefore anti-miR-195 can be used to treat cardiac disease.

Santaris Pharma has recently developed an LNA-modified antagomir Miravirsen (or SPC3649) against miR-122 that is important for induction of viral transcription of Hepatitis C virus (HCV) for Hepatitis C treatment [243, 274]. Treatment of chimpanzees having chronic HCV with SPC3649 has shown decreased viral RNA in serum without any adverse effects [243]. Currently, Miravirsen is under phase IIa clinical trials, and has provided a significant protection to patients with HCV [275]. Regulus Therapeutics (San Diego USA) has generated several antagomirs (miR-10b, miR-21, miR-103/107, miR-182, miR-380-5p) to target miRNAs over expressed in different conditions (Glioblastoma, hepatocellular carcinoma, atherosclerosis, kidney fibrosis, and HCV infection) but none of the above therapeutics has reached clinical phase. Later on they employed anti-miR-21/anti-miR-380-5p/anti-miR-182 to prevent metastasis and invasion of glioma [276], neuroblastoma [277] and melanoma [278] respectively. Mirna Therapeutics has developed miRNA mimics to restore the expression of specific miRNAs, which are lost during the course of tumorigenesis. They developed MRX34, the first miR-34a mimic compound that entered into Phase I clinical trials (NCT01829971). Administration of MRX34 (restoration of miR-34) produced potent anti-tumor effects in several mice cancer models [266]. Further, pre-clinical testing of the combination of MRX34 and liposomes in mouse models of hepatocellular carcinoma have shown very promising outcomes with minimal toxicity (NCT01829971) [279].

10. Conclusion and future perspectives

Although the involvement of miRNAs in disease pathogenesis is still emerging, deregulated miRNA expression in PC suggests their critical roles in its development and progression. Many studies have shown their involvement in a wide variety of biological processes including cell survival, proliferation, invasion/metastasis, apoptosis, and drug resistance of PC [55, 83, 91, 113, 158, 174, 181, 280]. Furthermore, differential expression profiles of circulating miRNAs among PC patients and healthy individuals render them potential biomarkers for diagnosis and prognosis. Besides the fact that a single miRNA targets multiple genes, several miRNAs frequently share the same target transcript, which makes miRNA actions more additive resulting in large changes in disease state (Table 1). In light of the above, it is apparent that cancer cells frequently deregulate miRNA expression to shortcut the selection process during the evolution of benign to malignant state. In this process these tumor cells may develop addiction to either aberrantly over expressed oncogenic or downregulated tumor suppressor miRNAs. Therefore, it seems logical that restoring tumor suppressor miRNAs at the pre-cancer levels [173, 188] or use of miRNA inhibitors may hold promise for therapeutic intervention against cancers. Tumor promoting effects have been directly countered in experimental models primarily in two ways; (a) by nanoparticle/virally delivered miRNA, used to restore tumor suppressive miRNAs [173] and (b) by antisense blocking oligos against oncogenic miRNAs. Pancreatic cancer is composed of dense stroma, also called the desmoplastic reaction that result in poor penetration of therapeutic agents resulting in reduced efficacy due to lower available drug concentration in the center of the tumor. Use of higher amounts of miRNAs is associated with off target

effects that results in higher toxicity and thus miRNA based therapy is a major challenge for PC therapy. For considering miRNAs as therapeutic targets in PC, several challenges need to be addressed. First specific delivery of the miRNA to PC tumors *in vivo* presents a significant challenge. In order to address this, nano-particles carrying the miRNAs for delivery can be coated with ligands or antibody fragments specific to up-regulated cell surface receptors on the cancer cells to increase tumor specific miRNA delivery that will enhance efficacy and reduce targeting to normal cells. Furthermore, antisense molecules do not readily cross cell membranes and this therefore limits their use. However, recently intravenous injection of morpholino-oligos against miRNAs have shown some promise [281]. These morpholinos are absorbed by endocytosis and can diffuse through endosomes with the aid of either a octaguanidinium dendrimer or arginine-rich cell-penetrating peptides (CPP) linked to their 3' or 5' ends [281]. Furthermore, target specific delivery of these morpholino-oligos may be enhanced by either altering the CPP structure or by attaching a ligand specific to an upregulated receptor on the surface of PC cells.

Most PC patients have multiple mutations that either inactivate tumor suppressors like p16, p53 or activate oncogenes, most importantly KRAS. Therefore, for better management of PC, inhibition of miRNA miR-491-5p, -34a, -214, -125b, -29, -504, -122, -33, -1285, -380-5p, -25 -133 and miR-30d to increase p53 expression; miR-24 and miR-10b to increase p16 expression; miR-130a/301a/454 to increase Smad4 expression; miR-106 to increase p21; miR-221/222 for p27 upregulation and miR-132 and miR-212 expression to increase Rb1 expression seems reasonable to combat this deadly disease. In addition, reintroduction of miRNA that suppress oncogenes and inhibit cell cycle progression like miR-217, miR-96, Let-7, miR-126, miR-143/145 to decrease KRAS expression; miR-192, -194, -215, -15a/16-1, -34a/b/c, 145, -200a/b/c, -141, -429 and miR-107 to down regulate c-Myc and cyclins expression can have significant effect on inhibiting PC growth (Table 1). miRNAs that target KRAS expression are not specific to the mutant form and therefore use of a single miRNA may not have sufficient downregulation of mutant KRAS to be effectual *in vivo*. It may however be of interest to utilize a cocktail of several miRNAs to downregulate mutated KRAS expression in PC. However, use of a cocktail of miRNAs at high doses to counteract the effects of oncogenic KRAS may have undesirable effects on healthy adult cells. To this end, improvements in delivery techniques and targeted delivery of miRNA cocktails may provide a larger window before toxicity becomes a problem. In addition, it will be worthless to use antisense-oligos against miRNAs targeting mutated p16 and/or p53 in a subset of PC patients. It may even be counterproductive in the cases where p53 has gain of function mutations. Therefore, it will be of interest to genotype PC tumors to identify aberrantly expressed miRNAs, mutations of key tumor suppressors and oncogenes for effective decision making with regard to which miRNAs should be blocked or reintroduced.

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Abbreviations

AKT	protein kinase B
ALDOA	Aldolase A, fructose-bisphosphate
Axl	AXL Receptor tyrosine kinase
BCL2	B-cell CLL/lymphoma 2
c-abl oncogene 1	non-receptor tyrosine kinase
CA19.9	Carbohydrate antigen 19.9
CCND1	Cyclin D1
CDC25B	Cell division cycle 25B, ABL-1
CDKN1C	Cyclin-dependent kinase inhibitor 1C
CDC7	Cell division cycle 7
CDK4	Cyclin-Dependent Kinase 4
CDK6	Cyclin-Dependent Kinase 6
CLL	Chronic Lymphocytic Leukemia
c-Met	Met proto-oncogene
c-MYC	v-Myc avian myelocytomatosis viral oncogene homolog
CUL5	Cullin 5
DNMT1	DNA methyltransferase
E2F1	E2F transcription factor 1
EMT	Epithelial-mesenchymal transition
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FGF7	Fibroblast growth factor 7
GEM models	Genetically engineered mouse models
GLI1	GLI family zinc finger 1
GTP	Guanosine Triphosphate
HDM4	Mouse Double Minute 4, Human Homolog Of P53-Binding Protein
HIF-1α/β	Hypoxia inducible factor-1 α/β
HPDE	Human pancreatic ductal epithelial cells
INK4A (p16)	Cyclin-dependent kinase inhibitor 2A
Jag1	Jagged 1
Jak2	Janus kinase 2
Kras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LATS2	large tumor suppressor kinase 2
LDH	Lactate dehydrogenase
LOH	Loss of heterozygosity
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)
Maml1/2	mastermind-like co-activators 1/2
MEK	Mitogen –activated protein kinase
miRNA	microRNA
MDM2	Mouse Double Minute 2
Nanog	Nanog homeobox
n-MYC	v-Myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
NSCLC	Non-small cell lung cancer
PanIN	Pancreatic Intraepithelial Neoplasm
P15	Cyclin-dependent kinase inhibitor 2B
p21	Cyclin-dependent kinase inhibitor 1A
PC	Pancreatic cancer
PDAC	Pancreatic Ductal Adenocarcinoma
PDCD4	Programmed cell death 4
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
PP2A	Serine/threonine protein phosphatase 2A
PUMA	P53 Up-Regulated Modulator Of Apoptosis
RAF	Raf/mil family of serine/threonine protein kinases
RREB1	RAS-responsive element-binding protein
Shh	Sonic Hedgehog
Spry2	Sprouty homolog 2
FNAs	fine needle aspirations
STAT3	Signal transducer and activator of transcription 3
SIRT1	Sirtuin 1
SMAD4	SMAD family member 4

SNAIL	Snail family zinc finger 1
TGF-β	Transforming growth factor beta
TGFβRI	Transforming growth factor beta Receptor I
TGFβRII	Transforming growth factor beta Receptor II
TPI1	Triosephosphate isomerase 1
TP53	Tumor suppressor p53
Wnt	Wnt oncogene analog
WNT3A	Wingless-type MMTV integration site family, member 3A
ZEB1	Zinc finger E-box binding homeobox 1
ZNF652	Zinc finger protein 652
3'UTR	3' Untranslated region

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Figure 1. Therapeutic potential of miRNA-mediated KRAS down-regulation in pancreatic cancer

More than 90% of pancreatic cancer (PC) patients have mutations (G12D/G12V) in codon 12 of KRAS that results in its constitutive activation and hyper activation of its downstream signaling pathways accompanied by increased proliferation, motility and survival of PC cells. KRAS targeting miRNAs like miR-217, -96, -126, -143/145 and Let-7 are significantly down regulated in PC patients that results in increased KRAS expression. Restoration of these miRNAs expression may result in abrogation of KRAS mediated signaling pathways such as (A) RAF/MEK/ERK that will result in decreased PC cell proliferation, (B) PI3K/AKT pathway abrogation will decrease PC cell survival, (C) Inhibition of NF-κB and ETV4 will result in decreased cell invasion and metastasis by up regulating E-Cadherin expression and (D) Down regulation of Tiam1 and Ral-GEF will effect cell polarity, vesicular trafficking, cell cycle and transcriptional dynamics in pancreatic cancer (miRNAs in color in green indicates downregulated in PC). RAF: Raf/mil family of serine/threonine protein kinases; MEK: Mitogenactivated protein kinase; ERK: Extracellular signal-regulated kinases; ETS: E-twenty-six family transcription factors; ELK1: ELK1, member of ETS oncogene family; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP3: Phosphatidylinositol (3,4,5)-trisphosphate; AKT: v-akt murine thymoma viral oncogene homolog; MDM2: Mouse double minute 2 homolog; ETV4: Human ETS translocation variant 4; NF-κB: Nuclear Factor Kappa B; MMP9: Matrix Metalloproteinase 9; SNAIL: Snail family zinc finger 1; δ-EF1: translational elongation factor 1 delta; TIAM1: T-cell lymphoma invasion and metastasis 1; Ral-GEFs: Ral guanine nucleotide exchange factors.

Figure 2. miRNA mediated signaling of p53 in pancreatic cancer

DNA damage and stress signals like starvation, hypoxia, oncogene hyperactivation etc. by upregulating p53 expression transcriptionally modulate several miRNAs (miR-107, -34a/b/c, miR-34) that induce cell cycle arrest, apoptosis cellular senescence and inhibits hypoxia. p53 mediated overexpression of miR-15a/16-1 clusture inhibits proliferation of pancreatic cancer cells by downregualting WNT3A and FGF7 proteins. By upregulating miR-145 expression, p53 down-regulates c-Myc expression thereby decreases cell survival *in vitro* and impedes tumor growth *in vivo*. Similarly, p53 by regulating miR-200a/b/c, miR-141 and miR-429 inhibits ZEB1 and ZEB2 transcription factors that favor mesenchymal to epithelial transition thereby impeding the tumor growth. However, mutated p53 negatively regulates miR-155, miR-205 (p63 dependent manner) and miR-130b, (p63 independent) that targets ZEB1 and ZNF652 expression thereby enhance the invasion, migration and metastatic potential of pancreatic cancer cells. Missense mutations in p53 also interferes miRNAs (miR-145 and miR-16-1) maturation resulting in enhanced cell proliferation, invasion and migration. Certain miRNAs like miR-491-5p, -34a, -122, miR-30d, miR-214, -125b, -29, -192, -194, -215, -25, -1285, -380-5p regulate p53 expression by binding to its 3'UTR. Through feedback mechanism, CpG promoter hypermethylation of miR-34a/b/c could also influence p53 transcription thereby hindering p53 mediated biological functions. Abbreviations: P53: Tumor protein p53; BCL2: B cell leukemia/lymphoma 2; c-MYC: v-Myc avian myelocytomatosis viral oncogene homolog; CDK4/6: Cyclin-Dependent Kinase 4/6; SNAIL: Snail Family Zinc Finger 1; c-MET: Met Proto-Oncogene; AXL: AXL Receptor Tyrosine Kinase; SIRT-1: Sirtuin1; ZEB1/2: Zinc Finger E-Box Binding Homeobox 1/2; LDH: Lactate Dehydrogenase; ZNF652: Zinc Finger Protein 652; WNT3A:

Wingless-Type MMTV Integration Site Family, Member 3A; FGF7: Fibroblast Growth Factor 7.

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Figure 3. MiRNAs involved in TGF- β /SMAD and SHH signaling and their targeting approaches for pancreatic cancer pathogenesis

(A) Binding of TGF- β to its cognate receptor leads to phosphorylation and therefore activation of SMAD signaling. (1) Phosphorylated SMADs (SMAD2 and SMAD3) form a complex with co-mediator SMAD4 and translocates to the nucleus for active transcription of the target genes. (2) Many of the miRNAs are involved in the positive and negative regulation of TGF- β /SMAD signaling cascade. miRNA130a/301a/454 abrogate TGF- β /SMAD mediated signaling by blocking SMAD4 activation. (3) TGF- β /SMAD signaling is also involved in miRNA biogenesis e.g miR-21. (4) Certain other miRNAs like miR-155 and miR-224 can also be influenced by TGF- β /SMAD mediated signaling cascade. (5) Pharmacological inhibition of SMAD4 protein by small molecule inhibitor SB-431542 leads to modulation of miRNAs downstream of TGF- β /SMAD signaling. (B) In the presence of SHH ligand, the PTCH1-SMO association gets remitted, resulting in enhanced downstream transcriptional activation of GLI family of proteins leading to target gene expression. (1) Use of small molecule inhibitor, Vismodegib (GDC-0449) inhibits hedgehog signaling pathway thereby interfering with regulation of miR-200b and let-7c, that are critical regulators of EMT and drug resistance (2) Whereas indirect inhibition of SHH activation by synthetic miRNA (GLI-1-miR-3548) targets GLI family of transcription factors that resulted in inhibition of pancreatic cancer cell division and proliferation. (3) Cyclopamine also appears to interrupt SHH signaling through regulating miRNAs (let7, miR-34, miR-107, miR-125, miR-128, miR-130, miR-132 and miR-141) responsible for stem cell self-renewal process.

Table 1

Therapeutically important miRNAs in pancreatic cancer pathogenesis

S. NO	Target gene	% Target gene mutations in PC	Regulation of target genes by miRNAs	Reference
1	Kras	90–95%		[53]
			miR-96,	[55]
			miR-126,	[56]
			miR-143/145	[57]
			miR-217	[58]
			Let-7	[59]
2	P53	50–75%		[65]
p53 mediated transcription of various miRNAs			miR-34a, miR-34b/c	[82–87]
			miR-130b, miR-155 and miR-205	[92–94]
			miR-15a/16-1	[15]
			miR-145	[96]
			miR-107	[109]
			miR-200a/b/c, miR-141 miR-429	[98, 99]
			miR-192, miR-194 miR-215	[102]
			miR-16-1, miR-143, miR-145	[104]
miRNAs regulates p53 gene expression by binding to its 3' UTR region			miR-491-5p	[106]
			miR-34a	[89, 108]
			miR-214	[111]
			miR-125b	[114]
			miR-29	[116]
			miR-504	[117]
			miR-122	[118]
			miR-33, miR-1285, miR-380-5p, miR-25, miR-133 and miR-30d	[119]
			miR-192/194/215 and miR-605	[121, 122]
4	INK4A/p16	90–95%		[282]
miRNAs that regulate p16 expression			miR-24	[283]
			miR-10b	[284]
			miR-128a	[285]
			miR-20a	[286]
			miR-148a and miR-152	[287]
miRNAs regulated by p16			miR-410 and miR-650	[288]
			miR-141 and miR-146b-5p	[289]
5	TGF- β /Smad			
TGF- β /Smad4 signaling mediated miRNA induction and maturation			miR-21	[144]
			miR-155	[145]

S. NO	Target gene	% Target gene mutations in PC	Regulation of target genes by miRNAs	Reference
			miR-143	[146]
6	SMAD4/DPC4	inactivated in 55% PC cases		[151]
miRNAs directly targeting SMAD4			miR-199a	[147]
			130a/301a/454	[148]
			miR-146a	[157]
			miR-483-3p	[158]
			miR-421	[159]
			miR-146b-5p	[161]
			miR-182-5p	[19]
7	SHH	Up regulation of Shh in 70% of PDAC specimens		
miRNAs targeting SHH			miR-196 and miR-452	[8, 166]
8	Gli 1		miR-3548	[168]