Review Article

Stem Cell INVESTIGATION

microRNA regulation of human pancreatic cancer stem cells

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Abstract: microRNAs (miRNAs) are a group of small non-coding RNAs that function primarily in the post transcriptional regulation of gene expression in plants and animals. Deregulation of miRNA expression in cancer cells, including pancreatic cancer cells, is well documented, and the involvement of miRNAs in orchestrating tumor genesis and cancer progression has been recognized. This review focuses on recent reports demonstrating that miRNAs are involved in regulation of pancreatic cancer stem cells (CSCs). A number of miRNA species have been identified to be involved in regulating pancreatic CSCs, including miR-21, miR-34, miR-1246, miR-221, the miR-17-92 cluster, the miR-200 and let-7 families. Furthermore, the Notch-signaling pathway and epithelial-mesenchymal transition (EMT) process are associated with miRNA regulation of pancreatic CSCs. Given the significant contribution of CSCs to chemo-resistance and tumor progression, a better understanding of how miRNAs function in pancreatic CSCs could provide novel strategies for the development of therapeutics and diagnostics for this devastating disease.

Keywords: Pancreatic cancer; miRNAs; cancer stem cells (CSCs)

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Pancreatic cancer stem cells (CSCs)

CSCs are cancer cells that possess the ability to self-renew and persist in tumors as a specific cancer cell population that can contribute to relapse and metastasis by re-emerging or by initiating new tumors. The first conclusive evidence showing the existence of CSCs was provided in 1997 by Bonnet and Dick (1). In this study, a subpopulation of leukemia cells expressing CD34, but not CD38 ($CD34^+/CD38^-$), could initiate tumors in NOD/SCID mice that were histologically similar to the donor cells. Cancer stem-like cells were first identified in human cortical glial tumors and were highly similar to neural stem-like cells, but expressed astroglial and neuronal markers (2).

The first pancreatic CSC population was identified in 2007 using xenografts of human pancreatic adenocarcinomas grown in immunocompromised mice. In these mice, a highly tumorigenic subpopulation of pancreatic cancer cells expressing the cell surface markers CD44, CD24, and epithelial-specific antigen (ESA) were isolated (3). It was shown that the CD44(+)CD24(+)ESA(+) subpopulation of cells shared stem cell properties, such as self-renewal, pluripotency, and increased expression of developmental signaling pathways, such as the sonic hedgehog pathway (3). One of the most important aspects of CSC research is the identification of surface markers that can be used to define and isolate CSCs. The widely accepted common CSCs markers are CD44(+) and CD24(+) (4), which are also applicable to pancreatic CSCs. Other surface markers identified for pancreatic CSCs include ABCG2, ALDH1, CD133, c-Met, CXCR4, nestin and nodal-activin (5).

The clinical significance of CSCs has been well described. The concept that CSCs are responsible for the initiation of tumor metastases is supported by the association of CSCs with epithelial-mesenchymal transition (EMT) (6-9). In pancreatic cancer, hedgehog-

signaling was found to regulate EMT in cancer "stem-like" cells and promote tumor genesis and metastasis (10). A recent report further indicates that exosomes, a group of secreted membrane-bound vesicles, from pancreatic CSCs can reprogram neighboring non-CSCs toward EMT and function in metastatic niche preparation in distant tissues (11). In addition to their role in promoting tumor metastasis, pancreatic CSCs also contribute to chemo-resistance (12). Therefore, targeting CSCs may enhance drug sensitivity and inhibit tumor metastasis (12). Interestingly, CSCs in peripheral blood may also serve as tumor biomarkers for diagnosis or prognosis (13).

microRNAs (miRNAs) and pancreatic CSCs

miRNAs are short non-coding RNAs (19–22 nucleotides in length) that primarily function to repress target mRNA translation through complementary binding in the 3' untranslated region (3' UTR) of mRNAs (14,15). miRNAs are transcribed as primary transcripts (pri-miRNA) by RNA polymerase II. The pri-miRNAs are then processed in the nucleus by RNA enzymes into 70–100-nucleotide-long precursors (pre-miRNAs) (16,17). The pre-miRNAs are then translocated to the cytoplasm (18) and further processed by a complex composed of the RNase III enzyme Dicer and the trans-activating response RNA-binding protein (TRBP) (19,20), leading to the generation of the mature miRNA and the consequent degradation of the complementary strands (21,22). The mature miRNAs are loaded onto Argonaute proteins within the RNA-induced silencing complex (RISC) and function to guide the RISC to complementary sequences in the 3' UTR of specific target mRNAs (23-25). Previous studies have shown that some of the miRNAs highly expressed in cancer are oncogenic, such as miR-21, miR-155, miR-17-5p, miR-19, and miR-92, etc. (26-28); whereas miRNAs with reduced expression in cancer often act as tumor suppressive regulators, such as miR-34, miR-15, miR-16, and let-7, etc. in pancreatic cancer (29-31).

Many studies have shown that miRNAs play a critical role in the regulation of CSCs in malignant tumors, including pancreatic cancer, and are involved in the initiation, propagation, and regulation of EMT and the Notch-signaling pathway in CSCs (32-39). miRNAs that have been reported to regulate pancreatic CSCs include miR-21, miR-34, miR-1246, miR-221, miR-145, the miR-17-92 cluster, and the let-7, miR-200, and miR-30 families.

miRNAs that regulate pancreatic CSCs through the Notch-signaling pathway

The Notch-signaling pathway is associated with the regulation of cell development in mammary epithelial cells and has been implicated in cancer initiation and progression (40-43). It is deregulated in many kinds of CSCs, including pancreatic CSCs. Recent studies found that Notch-signaling regulates miRNA expression, and in return miRNAs modify Notch-signaling in pancreatic CSCs. For example, over-expression of Notch-1 in pancreatic CSCs led to an increase in expression of miR-21, and a decrease in expression of miR-200b, miR-200c, let-7a, let-7b, and let-7c *in vitro* (37). Furthermore, metformin, an experimental anticancer drug, decreased the expression of Notch-1, thereby elevating expression of miRNAs such as let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c in a xenograft mouse model (38). With regard to miRNA regulation of Notch signaling, over expression of miR-34 in pancreatic cancer cells either by transfection of miR-34 mimics or infection with lentviral miR-34-MIF led to a significant reduction of cancer initiating cell population likely due to the down-regulation of Notch1/2 and Bcl-2 by this miRNA (34). Similarly, DCAMKL-1 (a putative pancreatic stem cell marker) knockdown resulted in down-regulation of Notch-1 expression in a miR-144-dependent mechanism (44). Garcinol, a known plant-derived antioxidant, could down-regulate Notch-1 signaling via up-regulation of miR-200c, thereby suppressing oncogenic properties of PANC-1 cancer stem-like cells (45). Notably, quercetin-induced let-7c was shown to decrease pancreatic cancer initiating cell growth by posttranscriptional activation of Numbl and indirect inhibition of Notch in a fertilized chick egg tumor xenotransplant model (46).

In addition to targeting Notch-signaling, let-7 and miR-34 can regulate pancreatic CSCs through other cellular mechanisms. The let-7 family, which is one of the first discovered miRNAs (47) and the first known human miRNA family (23), was shown to be deregulated in cancer-stem-like cells and display tumor suppressor activity (48). In pancreatic cancer, down regulation of let-7 was associated with increased chemotherapy resistance (49,50). In these cells, DCAMKL-1 acted as a master regulator of pancreatic tumor genesis through regulation of multiple tumor suppressor miRNAs including let-7a in a xenograft tumor model (51). Diflourinated-curcumin (CDF) was found to decrease formation of pancreatospheres (spheres generated from pancreatic cancer cell lines' sphere formation assay),

cell invasiveness, and CSC function in human pancreatic cancer cells by reducing expression of EZH2 and increasing expression of a panel of tumor-suppressive miRNAs, including let-7a-d (52). Treatment of pancreatic CSCs with metformin could decrease the expression of the CSC markers CD44, EpCAM, EZH2, Notch-1, Nanog and Oct4, and induce re-expression of miRNAs (let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c) that are typically lost in pancreatic cancer and especially in pancreatospheres (38). Compared to its parental BxPC-3 cells, BxPC-3-LN (highly lymphatic metastatic pancreatic cancer cells derived from BxP-3 cells) cells showed stem cell-like properties, including high lymphatic metastasis potential, self-renewal and chemoresistance (53). The BxPC-3-LN cells also expressed higher levels of migrating CSC surface markers (CD133 and CXCR4) and lower levels of let-7 compared to the parental BxPC-3 cells (53), further supporting the role of let-7 in regulating pancreatic CSCs.

miR-34a is a transcriptional target of p53 and is down-regulated in pancreatic cancer (54). For example, highly lymphatic metastatic pancreatic cancer cells possess stem cell-like properties and express low levels of miR-34 in a xenograft model (53). miR-34 was found to be involved in pancreatic CSCs self-renewal by modulation of its downstream targets, Bcl-2 and Notch, implying that miR-34 may play an important role in pancreatic CSCs self-renewal and/or cell-fate determination *in vivo* (34). The restoration of miR-34 expression by demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-dC) and HDAC inhibitor Vorinostat (SAHA) in CSCs could boost patient response to existing chemotherapies potentially by eliminating the CSC characteristics, which provided mechanistic insight into new therapeutic strategies against pancreatic cancer (54). Another example is that systemic intravenous delivery of miR-34 with nanovectors regulated pancreatic CSCs survival and inhibited growth of MiaPaCa-2 subcutaneous xenografts ($P<0.01$). This effect was more pronounced in the orthotropic (intrapancreatic) setting ($P<0.0005$) when compared to vehicle controls (55).

miRNAs that regulate EMT in pancreatic CSCs

EMT refers to a process in which epithelial cells lose their epithelial properties and gain mesenchymal cell characteristics. Cancer cells that undergo EMT acquire stem cell-like properties, thus giving rise to CSCs. The miR-200 family is involved in the regulation of EMT in pancreatic CSCs. Over-expression of miR-200c had an inhibitory effect on human pancreatic CSCs by deregulating EMT-related genes *in vitro* and *in vivo* (39). It significantly down-regulated expression of zinc-finger E-box binding homeobox 1 (ZEB1) and vimentin (markers of mesenchymal cells), and up-regulated expression of E-cadherin (marker of epithelial cells), and decreased colony formation, invasion, chemoresistance, and xenograft growth (39). The loss of miR-200a expression was associated with an EMT phenotype and stem-like cell features, characterized by the expression of the cell surface markers CD24, CD44 and ESA in pancreatic cancer cells *in vitro* (56). Knockdown of DCAMKL-1 up-regulated miR-200 expression, resulting in a decrease in the expression of VEGFR1, VEGFR2 and the EMT-related transcription factors ZEB1, ZEB2, SNAIL and SLUG in a xenograft model (51). It was also reported that knockdown of DCAMKL-1 induces miR-200a, along with the down-regulation of EMT-associated transcription factors ZEB1, ZEB2, Snail, Slug, and Twist in human pancreatic cancer cells using (44). miR-200 could antagonize EMT driven by mutant KRAS (57,58) and suppression of miR-200 expression by activated oncogenic KRAS promoted cell survival and EMT in KRAS-driven pancreatic cancer in cell lines. Likewise, the activation of Notch-1 signaling contributed to the acquisition of EMT phenotype, which was mediated through miR-200b and CSC self-renewal capacity, and could be attenuated by genistein treatment *in vitro* (37).

Hypoxia and the HIF pathways also contribute to the acquisition of EMT and maintenance of CSC functions (59). For example, hypoxia could induce mR-21 expression in pancreatic cancer cells via the HIF-1α pathway. miR-21 over-expression in these cells allowed them to escape apoptosis in a hypoxic microenvironment using a xenograft model (59,60). In addition, over-expression of Notch-1 increased expression of miR-21 which led to the acquisition of EMT phenotype in pancreatic cancer cells *in vitro* (37).

In addition to regulating EMT in pancreatic CSCs, the miR-200 family and miR-21 are also involved in regulating pancreatic CSCs through other mechanisms. The miR-200 family, which consists of miR-200a, miR-200b, miR-200c, miR-141, and miR-429, is involved in cancer metastasis *in vitro*, as evidenced by a transwell migration assay (61). It was reported that miR-200c overexpression decreases colony formation, invasion and chemoresistance of pancreatic CSCs (62). As described above, CDF, a novel analogue of the turmeric spice component curcumin, decreased pancreatic cancer

cell survival, clonogenicity, formation of pancreatospheres, and cell invasion. These effects were associated with decreased expression of EZH2 and increased expression of a panel of tumor-suppressive miRNAs, including miR-200b and miR-200c *in vitro* and *in vivo* (52). In a xenograft mouse model of human pancreatic cancer, CDF treatment significantly inhibited tumor growth, and was associated with increased miR-200 expression (63). miR-21 was highly expressed in pancreatic cancer cell lines, tissues and the plasma of pancreatic cancer patients (64,65) and could promote pancreatic CSCs growth via regulation of FoxO1. FoxO1-negative cells are considered to have CSCs properties in pancreatic cancer and null expression of FoxO1 was associated with a high expression of miR-21 and rapid cell growth in cell lines, primary tumor tissues and a mouse model (66). Moreover, miR-21 expression was related to chemotherapy resistance in pancreatic cancer (49). Inhibition of miR-21 in pancreatic CSCs suppressed tumor genesis, metastasis, and chemotherapy resistance in cell lines (67). CDF treatment of pancreatic cancer *in vivo* significantly inhibited tumor growth, which was associated with decreased miR-21 expression in tumor remnants (63).

Other miRNAs that are related to pancreatic CSCs

miR-221 expression was described in pancreatic cancer holoclone-forming cells (a colony-forming stem cell that has a higher growth potential than a meroclone because it does not contain differentiated cells) (68). Inhibiting miR-221 in tumor-initiating stem-like cells could modulate tumor genesis, metastasis, and chemotherapy resistance in pancreatic cancer. The administration of antagomir-221 significantly reduced the stem-like cancer cell fraction, decreased stem-like cancer cell differentiation, thereby reducing chemoresistance to gemcitabine and 5-Fluorouracil in pancreatic cancer cells *in vitro* and *in vivo* (67). The miR-17-92 cluster could inhibit tumorigenicity, but enhanced chemoresistance in pancreatic CSCs via the TGF-β1 pathway (49). DNMT1 [DNA (cytosine-5)-methyltransferase] inhibition was reported to reprogram pancreatic CSCs in part via reactivation of the miR-17-92 cluster in primary tissue cultures and *in vivo* (69). miR-145 is a tumor suppressor miRNA that could regulate expression of critical pluripotency factors and oncogenes, such as OCT4, SOX2, NANOG, KLF4, KRAS and RREB1, resulting in repressed metastatic potential in pancreatic cancer cells in a xenograft model (51). miRNA-1246 expression was associated with CCNG2-mediated chemoresistance and stemness in pancreatic cancer in primary tissue cultures and an animal model (70). The miR-30 family could promote migratory and invasive characteristics in CD133(+) pancreatic cancer stem-like cells (71). miR-26a expression was reportedly lost in pancreatic cancer and especially in pancreatospheres (38). Finally, miR-99a, miR-100, miR-101, miR-125b, miR-192, miR-183 and miR-429 were differentially expressed in pancreatic CSCs compared to non stem-like pancreatic cancer cells (16,72,73).

Future perspectives

Differential expression of certain miRNA species in pancreatic CSCs and the involvement of these miRNAs in regulation of pancreatic CSCs has been recently documented. These discoveries provide new directions for the development of therapeutics and diagnostics against this malignancy. It remains inconclusive as to whether the miRNA species differentially expressed in pancreatic CSCs are also involved in regulating CSCs in other cancer types. Furthermore, detailed information on how these miRNAs, individually or in combination, regulate pancreatic CSCs thereby contributing to pancreatic cancer progression, merits further exploration. Current management strategies against pancreatic cancer, especially metastatic pancreatic cancer, are not effective. Pancreatic CSCs play a significant role in pancreatic cancer cell self-initiation, metastasis and chemo-resistance, therefore targeting pancreatic CSCs is a promising strategy that could lead to improved pancreatic cancer outcomes.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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