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Contribution of Epithelial-to-Mesenchymal Transition and Cancer Stem Cells to Pancreatic Cancer Progression

Seth B. Krantz, MD^{1,*}, Mario A. Shields, MPhil², Surabhi Dangi-Garimella, PhD², Hidayatullah G. Munshi, MD^{2,3,4,*}, and David J. Bentrem, MD, MS, FACS^{1,3,4}

¹Department of Surgery, Northwestern University Feinberg School of Medicine / Chicago, IL 60611

²Department of Medicine, Northwestern University Feinberg School of Medicine / Chicago, IL 60611

³Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine / Chicago, IL 60611

⁴Jesse Brown VA Medical Center / Chicago, IL 60612

Abstract

Pancreatic adenocarcinoma remains among the most lethal of human malignancies. Overall 5-year survival is less than 5%, and only 20% of patients presenting with localized disease amenable to surgical resection. Even in patients who undergo resection, long-term survival remains extremely poor. A major contributor to the aggressiveness of multiple cancers and pancreatic cancer in particular is the process of epithelial-to-mesenchymal transition (EMT). This review highlights the growing evidence of EMT in pancreatic cancer progression, focusing on the contribution of EMT to the development of cancer stem cells and on interaction of EMT with other pathways central to cancer progression such as Hedgehog signaling, the K-ras oncogene, and transforming growth factor- β (TGF- β). We will also discuss EMT-targeting agents currently in development and in clinical trials that may help to reduce the morbidity and mortality associated with pancreatic cancer.

Keywords

pancreatic cancer; epithelial-mesenchymal transition; microRNA; stem cells; drug resistance

EMT and Cancer Progression

Epithelial-to-mesenchymal transition (EMT) is a process controlled by a family of transcription factors that leads epithelial cells to undergo a phenotypic shift from cells with tight cell-cell junctions, clear basal and apical polarity, and sheet-like growth architecture into spindle-like, fusiform, motile cells that express distinct mesenchymal markers such as vimentin, fibronectin, and N-cadherin (1–3). While EMT is an embryologic process

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*To whom correspondence should be addressed: Seth B. Krantz, MD or Hidayatullah G. Munshi, MD, Northwestern University Feinberg School of Medicine, Lurie Building, Room 3-117, 303 E Superior Street, Chicago Illinois 60611, p (312) 503-2301, f (312) 503-0386, s-krantz@fsm.northwestern.edu or h-munshi@northwestern.edu.

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essential to normal development, it also allows epithelial cancer cells to take on invasive properties and form distant metastases (1, 3–5). The process in tumors, while utilizing the same transcription factors and responsive to the same pathways as embryogenesis, is qualitatively different, given the large degree of genetic abnormality and instability inherent within the cancer cells (3). Numerous studies have shown that cells harboring a mesenchymal phenotype demonstrate biology associated with cancer progression. This includes increased cell invasion, angiogenesis, chemotherapy resistance, increased tumorigenicity, and the formation of side populations of cancer stem cells (6–12). Metastatic foci in several cancers show evidence of EMT and elevated stem cell markers, which correlate with worse clinical outcomes and response to standard chemotherapy (13, 14). This has been seen in multiple epithelial cancers including breast, prostate, colorectal, lung, ovarian, head and neck, and pancreatic cancers (10–22).

EMT is a dynamic process; however, by their nature, the *in vivo* studies from human cancer specimens that show a link between EMT and prognosis are static snapshots of tumors. It has been argued that what is thought to be EMT *in vivo*, simply reflects a shift in cell population resulting from a combination of epithelial apoptosis and mesenchymal proliferation, rather than a dynamic transition (23). More recently, transgenic mouse models of breast cancer and intestinal fibrosis have clearly demonstrated the role of dynamic EMT occurring in these settings (24, 25) and provide strong evidence for EMT as an active biological process within human cancers.

Pancreatic Cancer

In 2010, there were an estimated 43,000 new cases along with over 36,000 deaths from pancreatic cancer in the United States, making it the 4th leading cause of cancer death (26). Pancreatic ductal adenocarcinoma (PDAC) remains an extremely lethal disease, with a median survival of only 6 months and a dismal 5 year overall survival of less than 5% (27). Only 20% of patients present with disease amenable to resection, and even in these patients 5-year overall survival is still only 10%–20% (28–30). While surgical morbidity and mortality have improved significantly, long-term outcomes have remained relatively unchanged over the past 30 years (30, 31). The reasons for the extreme difficulty in treating PDAC are both anatomic and biologic. Given the retroperitoneal location of the pancreas the disease frequently remains relatively asymptomatic in its early stages. Yet even when patients present with localized disease amenable to resection the biology of the disease is such that it is resistant to standard chemotherapies. Histologically, the tumors are encased within a dense fibrotic stroma, known as the desmoplastic reaction, which has been shown to limit chemotherapy delivery, increase the innate chemo-resistance of tumor cells, and promote a more aggressive cancer cell biology (6, 8, 11, 30–33). Given this, an active area of investigation focuses on how malignant ductal cells transform and then invade through this stroma with such efficiency and the cross-talk between the fibrous integument and the epithelial elements. A better understanding of the underlying biology and the interactions and signaling processes within cancer cells is needed in order to make meaningful progress in the treatment of this uniquely lethal disease.

EMT and PDAC

EMT, with its contribution to invasion, metastasis, chemo-resistance and the propagation of cancer stem cells, plays an especially important role in PDAC. EMT has been demonstrated in resected PDAC specimens and is a prominent feature of both *in vivo* and *in vitro* models of the disease (13, 34–37). Mesenchymal cells, identified by increased fibronectin and vimentin staining, along with decreased E-cadherin staining, show a positive correlation

with high grade tumors (34). These changes have been linked to prognosis, with more mesenchymal tumors having worse survival and an increased number of metastases (13, 34).

EMT is controlled by a group of zinc finger transcription factors such as the Snail family (Snail and Slug), Zeb1, and Twist (1, 4, 5). In pancreatic cancer, Snail and Zeb1 are the most studied and are correlated with tumor grade and survival in vivo and with invasion and chemoresistance in several in vitro models (35–38). Over 80% of resected PDAC specimens have moderate to strong Snail expression, which was significantly more than either Slug or Twist. Snail expression, along with Zeb1, has been correlated with decreased E-cadherin levels and worse tumor grade and a poorer prognosis (35–38). Modulation of EMT pathways in vitro, Zeb1 in particular, leads to a reversal of EMT in pancreatic cancer cells and a restoration of chemosensitivity in previously resistant, mesenchymal cell lines (37, 39, 40).

EMT occurs in response to several distinct pathways, most notably and important to cancer include WNT, Notch, several receptor tyrosine kinase pathways, and transforming growth factor- β (TGF- β) (3). TGF- β plays an important and heterogeneous role in pancreatic cancer and is an essential driver of EMT (1, 32, 41–43). TGF- β ligands cause dimerization of the membrane bound T β RI and T β RII receptors, which leads to signal propagation through Smad dependent pathways (43, 44). Activated Smad-2 or Smad-3 localize to the nucleus with Smad-4 to serve as a transcriptional regulator (45). Mutations in TGF- β receptors and in Smad signaling are contributors to PDAC progression (44, 46). Over 50% of PDAC tumors have loss of Smad4; mouse models that replicate this show increased growth of pancreatic lesions owing to loss of TGF- β growth inhibition (46–48). While this suggests a tumor suppressive role for TGF- β , these tumors remain well differentiated. TGF- β responsive tumors (those with intact Smad signaling), conversely, show poor differentiation owing to an increase in EMT (48). Collagen, the primary component of the desmoplastic reaction, has been shown to increase Snail expression in pancreatic cancer cells through a TGF- β dependent process. This in turn, leads to an increase in membrane type 1-matrix metalloproteinase (MT1-MMP, a.k.a. MMP-14) expression and increased cell invasion (6). Matrix metalloproteinases, specifically MT1-MMP, represent novel therapeutic targets; silencing upstream TGF- β mediated EMT could decrease the progression of pancreatic cancer owed to MT1-MMP expression, though this remains to be verified (49). Additionally, tumors showing TGF- β driven EMT also show loss of oncogene dependent growth (50). Ablation of the oncogenic mutant K-ras leads to increased apoptosis in several cancer cell lines. Cells that underwent TGF- β induced EMT lose this K-ras dependence, which could be restored by targeting Zeb1 expression (50). This has important implications for future drug development, as molecules targeted against these growth pathways may be less effective in cells that have undergone EMT.

The WNT and Notch pathways are also important contributors to pancreatic cancer progression and regulators of both EMT (51, 52). Notch ligand Jagged1 downregulates E-cadherin via increased Slug, while Zeb1 expression increases Notch signaling (51, 53). Notch is also associated with gemcitabine resistance in pancreatic cancer cells (54). Blocking γ -secretase activity, essential for Notch mediated signaling, slowed tumor progression in mice (55). The WNT pathway has a strong association with cancer stem cells and the development of the stem cell-like phenotype (56). It can induce EMT directly, or through cross-talk with other pathways, including TGF- β , across several cancers, including PDAC (7, 57).

EMT, Cancer Stem Cells and PDAC

Populations of cells have been identified within a variety of cancers that possess properties such as self-renewal, tumor initiation, and differentiation. These cancer stem cells, originally identified in hematopoietic malignancies, have now been identified in numerous solid tumors and are associated with disease recurrence, metastases, and chemo-resistance (9, 14, 15, 58–61). It is becoming increasingly evident that non-stem cell populations within tumors can transform into stem cells (9–11). In breast cancer forced expression of a mutant K-ras in MCF-10 mammary cells leads to the development of a highly enriched population of CD44+/CD24– (90% of K-ras expressing cells vs. 1% of control cells) cells, which are the markers for breast cancer stem cells (11, 59). This transformation was associated with an increase in EMT markers, was potentiated by exogenous TGF- β treatment, and led to an increase in stem cell like behavior and drug-resistance (9–11, 62, 63).

Stem cell populations have also been identified in pancreatic cancer and their impact on disease progression continues to be elucidated (13, 22, 64, 65). Initial work utilized resected human PDAC specimens to generate xenograft models in nude mice. Within these tumor specimens, a small subpopulation of less than 1% of the total cell population possessed stem cell like properties. These cells, CD44+/CD24+/ESA+(epithelial specific antigen) showed tumorigenicity, the ability to differentiate into a heterogeneous tumor cell population, and maintenance of a self-renewing stem cell population (64). The triple positive cells were 100-fold more tumorigenic than unsorted cells. CD44 has long been associated with pancreatic cancer progression. Different processing of CD44, known as splice variants, are associated with various cancer phenotypes (66–69). Splice variant V6 is increased in metastatic pancreatic cancer relative to the primary tumor, while other splice variants have recently been linked to EMT in breast cancer (66, 69).

Recently, using aldehyde dehydrogenase (ALDH) activity as a more specific marker of cancer stem cells, it was shown that ALDH-high cells comprise an even more select subpopulation of cells in human pancreatic cancers that are tumorigenic and capable of producing tumors at very low numbers (13). These ALDH-high cells have evidence of EMT and are increased within metastatic pancreatic cancer lesions. Patients with ALDH-positive tumors had increased metastases and worse survival (13).

In studying signaling pathways that may be utilized or differentially regulated by these cancer stem cells, both the Hedgehog signaling pathway and EMT associated gene expression were found to play a role. In the CD44+/CD24+/ESA+ cells, Hedgehog signaling was upregulated ten-fold compared to unsorted cells and to non-stem cell cancer cells, and when comparing ALDH-high to ALDH-low tumors, those with increased ALDH-high cells had increased Slug expression and decreased E-cadherin (13, 64).

Hedgehog signaling, EMT and cancer stem cells

Hedgehog signaling is an embryologic pathway that has been strongly implicated progression of several gastrointestinal malignancies and pancreatic cancer in particular (70, 71). The secreted ligand Sonic Hedgehog is increased in not only pancreatic cancer but precursor PanIN lesions as well (70). Forced hedgehog expression in mouse pancreas leads to the development of PanIN-like lesions that also possess mutant K-ras, and inhibiting Hedgehog signaling in vitro promotes apoptosis and limits proliferation (70). Within the surrounding stroma, Hedgehog signaling has been shown to be important for tumor growth, and targeting Hedgehog signaling can improve the delivery of gemcitabine in pancreatic cancer in vivo (33, 72). Hedgehog signaling is also involved in effecting EMT in a variety of other pathologic processes and malignancies (7, 18, 57, 73–78). In the development of hepatic fibrosis, quiescent hepatic stellate cells undergo EMT to become activated

myofibroblasts in response to hedgehog signaling (73, 76). Whether hepatocytes, and not merely ductal cells in culture, or quiescent stellate cells, can undergo EMT and produce fibrosis is more controversial. In genetic labeling studies, mouse hepatocytes did not appear to be the source of type-I collagen producing cells (79, 80). However, while hepatocytes may themselves not undergo full EMT to contribute to fibrosis, they have been shown to be involved, upregulating their own expression of Snail and contributing the overall degree of liver fibrosis (81). In the kidney, tubular epithelial cells have been shown to undergo EMT and produce renal fibrosis (82). In non-small cell lung cancer, Hedgehog signaling mediates TGF- β induced EMT, and targeting Hedgehog pathways restored an epithelial phenotype and decreased invasion and tumorigenicity (77). Cross-talk between Hedgehog signaling and EMT pathways is also implicated in tumor progression in colon, esophageal, gastric, hepatocellular and pancreatic cancer (18, 22, 74, 75, 83).

The interaction between Hedgehog and EMT pathways leads to tumor progression through increased invasion, proliferation, and the induction and promotion of cancer stem cells (7, 18, 22, 84). In one set of experiments, pancreatic cancer cells selected based on slow cycling time, a feature of stem cell populations, demonstrated the traditional stem cell properties of tumorigenicity, the ability to differentiate, and self-renewal (84). Gene expression analysis of this sub-population demonstrated increased expression of Sonic Hedgehog and EMT associated genes. Furthermore, the cells had a mesenchymal morphology and showed increased invasion. Additional evidence of the link between EMT, Hedgehog Signaling, stem cells comes from work that sought to selectively target the Hedgehog pathway in a mouse model of pancreatic cancer (22). Hedgehog signaling was blocked in pancreatic cancer cells using the compound Cyclopamine, which led to the restoration of E-cadherin and reduction in cancer cell invasion. Treatment of a nude mouse model of pancreatic cancer led to a reduction of metastases and had a synergistic effect with gemcitabine (22). The authors also looked at the effect on stem cell populations and found that targeting Hedgehog signaling led to a reduction in the percentage of cells expressing the stem cell marker ALDH (22).

MicroRNA, EMT and Cancer Stem Cells

MicroRNAs (miRNAs), small, non-coding RNAs that affect a wide range of cell functions, are increasingly recognized to play a large role in many cancers (85–88). miRNAs primarily affect cell function by increasing or decreasing the stability of mRNA (85, 87). In cancer, they serve as both tumor promoters and suppressors and have been implicated in proliferation, apoptosis, and invasion (85–89). They have been shown to affect tumor progression through modulating both EMT and stem cell pathways (20, 89–93) (Figure 1). Work based on screening of over sixty cancer cell lines showed that the miR200 family of miRNAs are repressors of Zeb1 and Zeb2, thereby increasing E-cadherin expression and the epithelial phenotype, while conversely Zeb1 inhibits miR200 (20, 94). Additional work has shown that forced expression of the entire miR200 family (miR-200a, b, c, miR-141 and miR-429) and miR205 can completely block TGF- β induced EMT (95).

In pancreatic cancer, specific miRNA expression in vivo has been correlated with patient outcomes and has been shown to contribute to tumor cell invasion and metastatic ability (89, 96). Tissue microarrays of pancreatic cancer and multiple cell line analysis demonstrate increased miR200 expression is associated with increased E-cadherin expression and that patients with higher miR200 have improved survival over patients with low miR200 expression (89, 97). In contrast, miR21 expression, which is overexpressed in pancreatic cancer compared with normal pancreas, correlates with a poorer survival in patients with node-negative disease (27.7 months versus 15.2 months for patients with weak vs. strong miR21 expression) (96).

MicroRNAs also show an effect at the interplay between EMT and cancer stem cells, as several miRNAs effecting both EMT and stemness. In breast cancer, stem cell populations showed downregulation of the miR200 family, while increased miR200 expression abrogated stem cell colony growth and tumor formation (92). In pancreatic cancer specifically, EMT, miRNA and stemness correlated with gemcitabine resistance (90). The well differentiated epithelial pancreatic cancer line BxPC3 was treated with gemcitabine to select out a gemcitabine resistant cell population. This chemo-resistant line was significantly more mesenchymal in phenotype than the parental cells, with significantly increased Zeb1 expression, decreased E-cadherin and miR203, and an enhanced colony forming ability (90). Separate analysis of multiple pancreatic cancer cell lines showed that cell lines that are gemcitabine resistant were more mesenchymal and had decreased miR200 and *let-7* expression compared with sensitive cell lines (93). Treatment with natural compounds 3,3'-diindolylmethane or Isoflavone, or forced expression of miR200 or *let-7*, led to a decrease in EMT markers Slug and vimentin, along with restoring gemcitabine sensitivity in previously resistant cells (93). Interestingly, *let-7* is also repressed by type I collagen through a TGF- β and MT1-MMP dependent process (98). In the same cell line, collagen promoted Snail expression through TGF- β and subsequent MT1-MMP mediated invasion (6), further highlighting the interplay between the tumor microenvironment, TGF- β , miRNA, and EMT.

Targeting EMT and Future Directions

Given its role in invasion, chemo-resistance, and the development of cancer stem cells, EMT and its pathways are intriguing targets for new therapies to combat pancreatic cancer. Specific targets include modulation of microRNAs involved in EMT, such as miR-200, directly targeting EMT transcription factors, or inhibiting related pathways like Hedgehog. The difficulty in developing strategies targeting EMT has been primarily related to technical difficulties in translating the methods developed in vitro into vehicles suitable for clinical application (99, 100). An alternative to siRNA and cellular transfection is finding chemical and biologic compounds that can affect these pathways. Examples with in vitro efficacy include CDF, a Circumin analogue, which increased miR-200 in pancreatic cancer to restore gemcitabine sensitivity; Silibinin, a natural flavonoid, directly inhibited Zeb1 in prostate cancer, reversing EMT; even the oral antidiabetic metformin has been shown to specifically target stem-cell populations by attenuating EMT (39, 101, 102). A separate compound, Salinomycin, discovered as part of a drug screen designed to find compounds effective against EMT, not only was specifically cytotoxic to cells that had undergone EMT, but also reduced the population of cancer stem cells within breast cancer cells (58).

Several clinical trials targeting EMT and related pathways in pancreatic are underway, but they remain early as either phase I or II trials. They include targeting TGF- β , Hedgehog inhibitors, and two trials looking to target Notch signal (Table 1). As these pathways are all involved with stemness and tumor survival, it is hoped that targeting them may sensitize tumors to other chemo and radiation therapy. As such, they may play a particularly valuable role in the neoadjuvant setting, trials of which are underway. The results of existing and future trials will be instrumental in determining whether targeting EMT and of stromal targeting more broadly, can be translated into meaningful improvement in outcomes for patients suffering from cancer.

Conclusion

EMT plays a central role in cancer progression. Being able to simultaneously affect invasion, chemo-resistance, and cancer stem cells makes EMT an immensely attractive target for developing new treatments. In pancreatic cancer especially, with its unique lethality and its multicellular dense fibrous stroma, a product of mesenchymal fibroblasts

and EMT, halting this process is an extremely promising treatment avenue. Various existing compounds have been identified that can modulate several different aspects of EMT and its pathways, some of which are already being translated into clinical treatments, trials of which are currently underway. While the benefits are still uncertain, increasing our understanding of EMT, and its contribution to multiple components of tumor progression, will hopefully help identify additional targets and new therapies, and ultimately improve outcomes for patients with pancreatic cancer.

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Figure 1.

Schematic representation of the bidirectional feedback and regulation between EMT associated genes and the miR200 family of microRNAs. The miR200 family plays a central role in EMT and cancer progression, regressing the EMT associated gene Zeb1, TGF- β induced EMT, and the development of cancer stem cells. miR200 expression is in turn downregulated by Zeb1.

Table 1

Active Clinical Trials Targeting EMT and related Pathways in Pancreatic Cancer

Pathway Targeted	Phase	Disease	Notable Information
Cancer Stem Cells(OMP21M18)	I	Unresectable Pancreatic Cancer	Monoclonal antibody against stem cells http://clinicaltrials.gov/ct2/show/NCT01189929
Hedgehog (GDC-0449)	II	Metastatic Pancreatic Cancer	Combination with or without Gemcitabine http://clinicaltrials.gov/ct2/show/NCT01064622
Hedgehog (GDC-0449)	II	Metastatic Pancreatic Cancer	Combination with Gemcitabine, Nab-Paxitacel, GDC-0499, http://clinicaltrials.gov/ct2/show/NCT01088815
Hedgehog (GDC-0449)	II	Resectable Pancreatic Cancer – Neoadjuvant Therapy	http://clinicaltrials.gov/ct2/show/NCT01096732
Hedgehog (IPI-926)	I/II	Untreated Metastatic Pancreatic Cancer	http://clinicaltrials.gov/ct2/show/NCT01130142
Hedgehog (GDC-0449)	I	Metastatic Pancreatic Cancer	Combination with Erlotinib and with or without Gemcitabine http://clinicaltrials.gov/ct2/show/NCT00878163
Hedgehog (GDC-0449)	0	Pancreatic Cancer – Cancer Stem cells	http://clinicaltrials.gov/ct2/show/NCT01195415
Notch (MK0752)	I/II	Unresectable Pancreatic Cancer	Combination Therapy with Gemcitabine http://clinicaltrials.gov/ct2/show/NCT01098344
Notch (RO4929097)	II	Previously Treated Metastatic Pancreatic Cancer	Outcomes – Survival and correlation with Stem Cell Markers http://clinicaltrials.gov/ct2/show/NCT01232829
Notch (RO4929097)	I	Multiple Solid Tumors, including Pancreatic Cancer	Combination therapy with cediranib http://clinicaltrials.gov/ct2/show/NCT01131234
Notch (RO4929097)	I	Pancreatic Cancer – Neoadjuvant therapy	Outcomes – Notch Inhibition and Cancer Stem Cell population changes http://clinicaltrials.gov/ct2/show/NCT01192763
Notch (RO4929097)	I	Locally Advanced Pancreatic Cancer	Combination with Gemcitabine http://clinicaltrials.gov/ct2/show/NCT01145456
WNT (PRI-724)	I	Advanced Solid Tumors (Unresectable Pancreatic Cancer)	http://clinicaltrials.gov/ct2/show/NCT01302405