5. Epithelial-mesenchymal transition and pancreatic cancer progression

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) continues to be one of the most lethal human malignancies, with median survival of less than one year and overall 5-year survival of less than 5%. There is increasing evidence for contribution of epithelial-mesenchymal transition (EMT) to pancreatic cancer metastasis and to treatment resistance. In this chapter we will review the role of EMT in pancreatic cancer progression, focusing particularly on the transcription factors and microRNAs involved in EMT. We will examine how EMT is involved in the generation and maintenance of stem cells, and detail the role of EMT in modulating resistance of PDAC cells to drug therapies. Finally, we will identify putative EMT-targeting agents that may help to reduce the morbidity and mortality associated with pancreatic cancer.

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1. Introduction

Despite the use of aggressive combination therapy in highly selected patients (1), pancreatic ductal adenocarcinoma (PDAC) continues to be one of the most lethal human malignancies and remains a daunting challenge for patients, clinicians, and researchers alike. There are approximately 43,000 new cases each year in the United States, with over 36,000 deaths, making it the fourth leading cause of cancer death (2). Median survival is less than one year and overall 5-year survival is less than 5% (3, 4). Additionally, over 80% of patients present with advanced disease not amenable to surgical resection, and even for those who do undergo surgery, treatment remains difficult with a 5-year survival of only 20% (5-7). Several factors are thought to contribute to the aggressive nature of pancreatic cancer. Anatomically, the location of the pancreas means patients are often asymptomatic until the disease is advanced, when they present with jaundice from obstruction of the bile ducts, or pain from invasion of the surrounding nerves. Histologically, PDAC is associated with a dense fibrotic reaction, known as the desmoplastic reaction, which is thought to contribute to disease progression and chemoresistance (8, 9). Despite improvements in surgical technique, enhanced imaging, and new chemotherapeutic agents, outcomes for patients remain extremely poor, and a better understanding of the cellular and biochemical factors that contribute to this terrible disease is essential if we are to make any significant improvements in the treatment of PDAC.

2. Epithelial-mesenchymal transition and PDAC

Epithelial-mesenchymal transition (EMT) is a developmental process that allows cells that are a part of a rigid architecture to escape and spread to distant sites (10-12). As cells undergo EMT, they lose their epithelial features including loss of their sheet-like architecture, loss of polarity, and down regulation of E-cadherin (Fig. 1). The cells also develop a mesenchymal phenotype, taking on a spindle-like, fusiform morphology, become motile, and start expressing mesenchymal markers, e.g. N-cadherin, fibronectin, vimentin (11, 13). Although the features of EMT were initially characterized in vitro, studies from patients with a variety of cancers have provided evidence for EMT in vivo (14-17). In human pancreatic tumor samples, fibronectin and vimentin are increased in high-grade tumors and within poorly differentiated areas of low-grade tumors, with a corresponding decrease in E-cadherin expression. Significantly, these patients have worse survival than those patients whose tumors demonstrate less evidence of EMT. In a study based on a rapid autopsy program for patients with pancreatic cancer,
Figure 1. EMT regulation in pancreatic cancer. Some of the main drivers of EMT in pancreatic cancer are transcription factors Snail, Slug and Zeb1, which are in turn regulated by cytokines (NF-α) and growth factors (TGF-β) as well as microRNAs. These signaling molecules transform non-invasive and chemo-sensitive cells into motile and invasive chemo-resistant cells that have stem-cell-like properties.

75% of the primary tumors with mesenchymal features developed metastatic lesions to liver and lung (14).

The key regulators of EMT include Snail, Slug, Zeb1, and Twist, which are zinc finger transcription factors that repress genes responsible for the epithelial phenotype (10-12). In resected PDAC specimens nearly 80% have moderate to strong Snail expression while only 50% show similar Slug expression, with very few having strong Twist expression (15). Snail expression is inversely correlated with E-cadherin expression, with decreased E-cadherin expression associated with higher tumor grade. Similar results were seen in pancreatic cell lines, with poorly differentiated lines showing higher levels of Snail and lower levels of E-cadherin compared with moderately differentiated cell lines (15). Zeb1 expression in pathologic specimens also correlates with advanced tumor grade and worse outcomes (16-18). In one study, tissue microarray analysis of pancreatic cancer showed an inverse relationship between Zeb1 and E-cadherin expression (17). Furthermore, silencing of Zeb1 in pancreatic cancer cell lines leads to the upregulation of E-cadherin and restoration of an epithelial phenotype (17). Interestingly, Zeb1 is primarily responsible for the acquisition of an EMT phenotype, along with increased migration and invasion in response to NF-κB signaling in pancreatic cancer cells (18).

Transforming growth factor-β (TGF-β), one of the primary drivers of EMT (9, 11), can increase expression of Snail, Slug and Zeb1 in a variety of cancers. Recently, we published that pancreatic cancer cells on encountering type I collagen induce Snail expression through increased TGF-β signaling.
Collagen-induced Snail expression was abrogated using siRNA against TGF-β type I receptor or against Smad4. TGF-β2 expression of Snail in endothelial cells and subsequent endothelial-mesenchymal transition was also shown to involve Smad signaling. However, in contrast to ERK1/2 regulation of TGF-β2-induced Snail expression in endothelial cells, we have found that collagen-induced Snail expression does not involve ERK1/2 signaling and is primarily mediated by Smad signaling. Although TGF-β can promote EMT, it is important to note that TGF-β has both tumor suppressive and tumor promoting affects on pancreatic cancer (19-21). Loss of Smad4 early in tumor development leads to loss of TGF-β growth inhibition and unchecked tumor growth in mouse models of pancreatic cancer. These tumors, however, are generally well differentiated (22, 23). Tumors with intact Smad4 signaling, meanwhile, are associated with an increase in EMT and subsequently are poorly differentiated (23). Furthermore, these advanced tumors that have undergone EMT show increased tumor proliferation and migration in response to TGF-β (23).

Consistent with TGF-β driven EMT leading to tumor proliferation in advanced tumors, EMT is also associated with cancers becoming oncogene independent. Induction of EMT with TFG-β causes previously K-ras dependent cells to become K-ras independent; conversely, K-ras independent cells forced to undergo mesenchymal to epithelial transformation (24) by targeting Zeb1 with shRNA subsequently become K-ras dependent (25). As cells undergo EMT, tumors that once may have responded to interruption of oncogenic signaling pathways may become unresponsive (25), which has important implications for drugs specifically designed to target these growth pathways, such as epidermal growth factor receptor (EGFR) inhibitors.

Inflammation plays a significant role in pancreatic cancer (26, 27), and inflammatory signaling through NF-κB has been shown to increase both EMT and cancer cell invasion. Snail activity is increased via stabilization at the protein level in response to TNF-α driven NF-κB signaling (28). Additionally, knockdown of Snail in this system abrogates TNF-α driven cancer cell migration and invasion (28). A similar interaction between NF-κB and EMT is seen in pancreatic cancer cells following TNF-α treatment. Transfection with a dominant negative form of IκBα abrogates the effect of TNF-α (18). Interestingly, TGF-β-induced EMT is also dependent on NF-κB signaling (18).

Chronic pancreatitis, which is associated with ongoing inflammation and fibrosis (29), has been identified as a risk factor for pancreatic cancer in humans and contributes to PDAC progression in mouse models of pancreatic cancer (30, 31). In addition, acute pancreatitis can accelerate the progression of precursor pancreatic intraepithelial neoplastic (PanIN) lesions to PDAC in
Epithelial-mesenchymal transition and pancreatic cancer progression

99

mutant K-ras-driven mouse models of pancreatic cancer (32, 33). Interestingly, although expression of embryonic mutant K-ras has been shown to be sufficient for tumor initiation in various mouse models of pancreatic cancer, expression of K-ras in adult mouse pancreas does not result in any obvious phenotypic changes (31). Induction of chronic pancreatitis is essential for PDAC development in these adult mice (31). Stat3, one of the key mediators of inflammatory signaling (34), was recently shown to be required for initiation and progression of PDAC following cerulein-induced pancreatitis in mutant K-ras mice (32, 33). Increased Stat3 signaling was shown as a result of increased IL-6 expression by both cancer cells and inflammatory cells (32, 33). Importantly, Stat3 signaling is aberrantly activated in human PDAC tumor samples and controls proliferation and invasion of human pancreatic cancer cells (32, 33). Human PDAC patients have increased circulating IL-6 levels and human PDAC tumor specimens stain for increased IL-6 expression (32, 33). Interestingly, Snail can also modulate inflammatory signaling in vivo through upregulation of chemokines and cytokines (35-37). Snail overexpression in keratinocytes increases production of cytokines IL-6, IL-8 and the chemokine CXCL1 (35). Moreover, Snail overexpression in epidermal keratinocytes in a transgenic mouse model promotes cutaneous inflammation that is associated with increased IL-6 production by keratinocytes and increased Stat3 signaling (36).

3. Role of microRNAs in modulating EMT in pancreatic cancer

MicroRNAs are small single-stranded non-coding RNAs that have been reported in many cancers, including pancreatic cancer (38-41). They serve as either tumor promoters or suppressors depending on their downstream effects (38-41). MicroRNAs of the miR-200 family (miR-200a, b, c, miR-141 and miR-429) and miR-205 have been identified as key negative regulators of both EMT and the metastatic ability of cancer cells (42, 43). These microRNAs are downregulated in high grade and poorly differentiated tumors, while forced expression of miR-200 microRNAs has been shown to inhibit TGF-β1-induced EMT in MDCK cells. In lung cells, forced miR-200 expression abrogates the ability of the cells to become invasive and metastatic. The miR-200 family targets the key regulators of EMT including Zeb1 and Sip1 (also known as Zeb2), and as such leads to increased E-cadherin levels (42, 43).

Recent surveys of global microRNA expression patterns in pancreatic cancer cell lines have shown that 39 microRNAs, including the miR-200
family, are deregulated and have at least 2-fold differential expression in PDAC cell lines compared to control non-transformed pancreatic ductal cell lines (44). Expression of miR-200 family members correlates positively with E-cadherin expression and negatively with the miR-200 target Zeb1 (44). High levels of miR-200c expression strongly correlate with E-cadherin levels in resected human pancreatic tumor samples and are associated with significantly better survival rates compared with patients whose tumors have low levels of miR-200c expression (45). Interestingly, Zeb1 can also directly suppress transcription of miR-200 family members miR-141 and miR-200c (46), indicating a significant interplay between Zeb1 and miR-200 family microRNAs that contributes to the differentiation state of pancreatic cancer cells. Several microRNAs have been shown to be overexpressed in pancreatic cancer, one of which is miR-21. miR-21 was shown to be overexpressed in 79% of pancreatic cancers as opposed to 27% of chronic pancreatitis (75). Another study indicated that pancreatic tumors with elevated expression of miR-155, miR-203, miR-210 and miR-222 have a much higher risk of tumor-related death as compared to tumors with a lower expression (Greither, T., L. F. Grochola, et al. (2010). “Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int J Cancer 126(1): 73-80.

4. Contribution of EMT to stem cells in pancreatic cancer

There is increasing interest in the subpopulation of cells within tumors that have stem cell-like properties (47-49). These cells are frequently associated with metastatic foci and chemoresistance and are increasingly linked to an EMT phenotype (50-52). The resistance to chemotherapy of this subpopulation prevents eradication of cancer, and presents the looming threat of recurrence.

There is ever more evidence to suggest the existence of cancer stem cells in pancreatic cancer (53, 54). These cells are CD44-high/CD24-high and express epithelial specific antigen (ESA) (53). Although the CD44-high/CD24-high/ESA-high cells comprise a small population of any particular pancreatic tumor, these cells have the ability to self-renew and reproduce the original tumor heterogeneity. Moreover, gemcitabine-resistant cells isolated from established cancer cell lines were also found to be CD44-high (55). Pancreatic cancer stem cells can also be identified by high expression of CD133 and these cells are highly tumorigenic and resistant to standard therapy (56). 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 (14). These ALDH-high cells have reduced E-cadherin expression and increased Slug expression (14). Interestingly, overexpression of Snail in pancreatic cancer cells leads to increased ALDH expression. ALDH-high cells with a mesenchymal phenotype have also been found in metastatic lesions of patients with pancreatic cancer (14). MicroRNAs that are associated with EMT, such as the miR-200 family, also regulate stem cell behavior (57, 58). miR-200c cooperates with other microRNAs to suppress expression of stem cell factors, such as Bmi1, Sox2 and KLF4 in cancer cells and mouse embryonic stem cells (57, 58). Stem cells isolated from a number of different tumor types also show increased expression of miR-21 along with its upstream regulator AP-1, and these molecules were found to be responsible for chemoresistance of the tumors. A small molecular inhibitor of AP-1 and anti-miR-21 is able to sensitize the cells to topotecan and decrease colony formation (59). In glioma cells, ID4-mediated suppression of miR-9* results in induction of the stem cell protein SOX2, making the cells chemoresistant by upregulating the ABC family of transport proteins (60).

5. Importance of EMT in enhancing drug resistance in pancreatic cancer

Pancreatic cancer remains extremely lethal in large part due to the poor response to existing treatments (61, 62). EMT has been shown to be a significant contributor to chemo-resistance in several cancers, including in pancreatic cancer (17, 63-65). Induction of gemcitabine resistance in previously sensitive cell lines results in development of cells with an EMT phenotype that is associated with an increased migratory and invasive ability compared to gemcitabine sensitive cells (65). Moreover, gene expression profiling of chemoresistant cell lines has shown a strong association between expression of genes associated with EMT and chemotherapy resistance (17). Specifically, the EMT transcription factor Zeb1 is upregulated in resistant cell lines and correlates with decreased expression of E-cadherin. Silencing of Zeb1 with siRNA causes mesenchymal to epithelial transition (24) and restores chemosensitivity (17). Significantly, maintenance of chemoresistance in cell lines that have undergone EMT is dependent on Notch and NF-κB signaling (64). Inhibition of Notch-2 down regulates Zeb1, Snail and Slug expression, attenuates NF-κB signaling, and reduces the migratory and invasive capacity of the gemcitabine resistant cells. (64). Interestingly, the heparin binding growth factor Midkine, which is overexpressed in
chemoresistant PDAC, can interact with and activate Notch-2 to promote EMT (66).

EMT plays a role in modulating resistance not only to traditional chemotherapies, but to targeted biologic therapies as well. Cells that express either mutated E-cadherin, or have high levels of Snail, Zeb1, and vimentin, and thus a mesenchymal phenotype, show significantly decreased growth inhibition in response to treatment with the EGFR inhibitor erlotinib than cells with an epithelial phenotype (16). Interestingly, cells from the same patient have been shown to have differential response to drug treatment, with cells from the primary tumor being responsive while cells isolated from a liver metastases and demonstrating a mesenchymal phenotype being resistant to erlotinib (16).

Transgenic mouse models have established that pancreatic cancer cells may not be inherently chemoresistant (65). The pronounced fibrotic reaction, primarily generated by myofibroblast-like stellate cells (67-69), can limit the delivery of current chemotherapeutic agents to the cancer cells. While quiescent fibroblasts within the microenvironment are activated by TGF-β (70), a significant number of myofibroblasts have in fact been shown to arise from epithelial cells that have undergone EMT (71). In the adult kidney activation of Snail is sufficient to cause renal fibrosis (72), while Hedgehog signaling, which has been shown to contribute to EMT (73), was recently shown in pancreatic cancer to contribute to resistance to gemcitabine through modulation of the tumor microenvironment, specifically by affecting the stroma and type I collagen (8, 14, 64). Thus, EMT may modulate chemoresistance not only within cancer cells themselves, but also by modulating the tumor microenvironment through generation of desmoplastic reaction.

MicroRNAs have also been identified as mediators of chemo-resistance in various cancers. Although we showed that let-7 does not mediate gemcitabine resistance in pancreatic cancer either on 2D surfaces or in 3D collagen microenvironment, other microRNAs have been demonstrated to mediate chemo-resistance. For example, miR-21, which is overexpressed in PDAC tumors and predicts for poor outcome (74), contributes to gemcitabine resistance in part through modulation of PI3-kinase-Akt signaling (75, 76). miR-21 increases pro-survival PI3-kinase signaling through repression of PTEN, upregulates the pro-survival Bcl-2 protein and inhibits the pro-apoptotic Bax protein in PDAC cells to promote gemcitabine resistance. The effect of miR-21 on PI3-kinase-Akt signaling and Bcl2 is not unique to pancreatic cancer cells as it can also mediate doxorubicin resistance in bladder cancer cells (77). Recently, miR-15a and miR-214 were reported to be dysregulated in pancreatic cancer. miR-15a was identified as a suppressor
of pancreatic tumor growth while miR-214 was found to promote chemo-
resistance (78). It has also been hypothesized that microRNAs that contribute
to chemo-resistance of pancreatic cancer might be overexpressed in the stem
cell population of the cancer, thus helping the cells to resist apoptosis and aid
in cancer recurrence.

6. Targeting EMT in pancreatic cancer

Given the role of EMT in chemo-resistance and tumor progression
specifically targeting EMT could improve the survival rates of pancreatic
cancer patients. Although increasing expression of miR-200 family
microRNAs could restore the epithelial state and make the tumors more
sensitive to therapeutic agents, delivery of microRNAs have yet to be
translated to the in vivo environment due to a number of technical barriers
related to safety, delivery and efficacy (79, 80). Consequently, there is
increasing interest in using compounds that can modulate EMT-inducing
microRNAs or transcription factors. Curcumin analogue CDF can restore
miR-200 levels and sensitize pancreatic cancer cells to gemcitabine treatment
in vitro (81). The naturally occurring flavanoid Silibinin can downregulate
Zeb1 and Slug expression and thus attenuate EMT in prostate cancer cells
(82). The anti-diabetic drug metformin can also decrease expression of the
Zeb1, Twist1 and Slug in breast cancer cells. Metformin also decreases the
ability of breast cancer stem cells to form mammospheres through reduction
in the CD44-high/CD24-low population (83). Salinomycin, which was
discovered as part of a drug screen designed to find compounds effective
against EMT, can reduce the population of cancer stem cells.

Clinical trials targeting Hedgehog, Wnt and Notch signaling, known
EMT pathways that have been implicated in cancer stem cells and
chemoresistance (8, 14, 64), are also underway. For example, the Hedgehog
inhibitor GDC-0449 is being evaluated in combination with chemotherapy in
patients with metastatic pancreatic cancer (http://clinicaltrials.gov/ct2/show
/NCT01088815). GDC-0449 is also being evaluated to determine whether it
can specifically target cancer stem cells (http://clinicaltrials.gov/ct2/show/
NCT01195415). There are also a number of ongoing clinical trials using Notch
inhibitors in patients with locally advanced or metastatic pancreatic cancer. For
example, the Notch inhibitor RO4929097 is being evaluated as both neo-
adjuvant therapy (http://clinicaltrials.gov/ct2/show/NCT01192763) and in
patients with metastatic pancreatic cancer (http://clinicaltrials.gov/ct2/
show/NCT01232829). Finally, the Wnt inhibitor PRI-724 is being evaluated in
patients with advanced solid tumors, including in patients with unresectable
In summary, targeting EMT holds significant promise in treating pancreatic cancer patients. Targeting EMT could contribute to increased sensitivity to standard chemotherapy and to growth factor directed therapies, such as those against EGFR signaling. By attenuating fibrosis, it can also increase delivery of drugs to cancer cells. Targeting EMT can also reduce the population of cancer stem cells that are thought to contribute to metastatic disease and treatment resistance. As our understanding of the role and regulation of EMT in pancreatic cancer increases and as we identify how best to target EMT, we will be able to improve the outcomes of patients with pancreatic cancer.

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References


